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Protein Therapeutics

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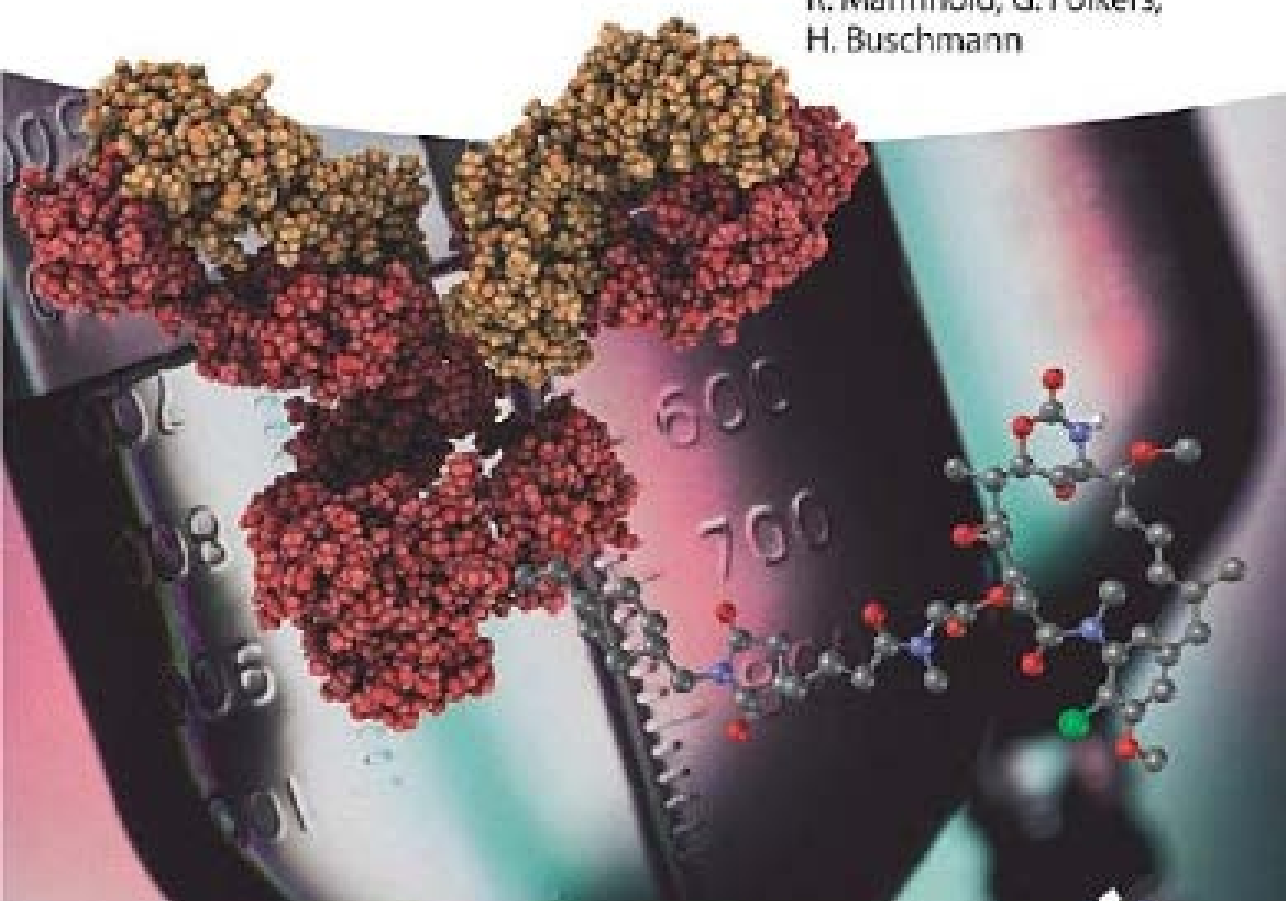

Methods and Principles in Medicinal Chemistry

Edited by Tristan Vaughan,
Jane Osbourn, and Bahija Jallal

Protein Therapeutics

Methods and Principles in Medicinal Chemistry

Previous Volumes of this Series:

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Protein Therapeutics

Volume 1

WILEY-VCH

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Preface

The number of marketed protein therapeutics [1–3] has increased enormously since the introduction of the first recombinant protein, human insulin, into the clinic several decades ago. Protein therapeutics play a very significant role in many various fields of medicine, and their use continues to steadily broaden. There are several key advantages of protein therapeutics over small-molecule drugs that contribute to this [1]:

- Proteins often exhibit highly specific and complex functions that cannot be mimicked by simple chemical compounds.
- The larger binding interface of a protein therapeutic enables them to be engineered with high affinity for their target.
- Due to their high level of specificity, there is often less potential for protein therapeutics to interfere with normal biological processes and cause off-target effects.
- Recombinant technology allows the production of proteins that provide a novel function or activity.
- Because the body naturally produces many of the proteins that are used as therapeutics, these agents are often well tolerated and are less likely to elicit immune responses.
- For diseases in which the product of a gene is absent or defective, protein therapeutics can provide an effective replacement treatment.
- The clinical development and approval time for protein therapeutics may be faster than for small-molecule drugs [4].
- Recombinant proteins can also be used in combination with both other large molecule, or indeed small-molecule, drugs to provide additive or synergistic benefit.

Many successful uses of protein therapeutics are documented in this volume. However some challenges still remain for the discovery and development of protein therapeutics: (i) The current route of administration is typically parenteral. Development of oral biologics remains largely an aspiration at this time. (ii) Although the production of recombinant proteins is becoming increasingly efficient and cost–effective, it remains relatively expensive compared to that of small molecules. (iii) The body may mount an immune response against the

therapeutic protein. In some cases, this immune response can neutralize the protein and reduce the efficacy of the potential drug.

Taken together, the early success of recombinant insulin production in the 1970s created an atmosphere of enthusiasm and hope, which was followed by an era of disappointment when the vaccine attempts, nonhumanized monoclonal antibodies, and cancer trials in the 1980s were largely unsuccessful. Despite these setbacks, significant progress has been made. With the large number of protein therapeutics both in current clinical use and in clinical trials for a range of disorders, one can confidently predict that protein therapeutics will have a further expanding role in future medicine and may – together with cell and gene therapy – dominate over classical therapeutic approaches based on small-molecule drugs.

Accordingly, this is an appropriate time to review our current knowledge and future perspectives of protein therapeutics as realized in this volume by experts in the field both from industry and academia. It is organized in six sections, first of which introduces the past and present development of protein therapeutics in the chapters on "Early Recombinant Protein Therapeutics" and "Evolution of Antibody Therapeutics." The second section is dedicated to antibodies as the ultimate scaffold for protein therapeutics and is covered in two chapters on "Human Antibody Structure and Function" as well as "Antibodies from Other Species." Discovery and engineering of protein therapeutics are described in the next section comprising detailed chapters on "Human Antibody Discovery Platforms," "Beyond Antibodies: Engineered Protein Scaffolds for Therapeutic Development," "Protein Engineering: Methods and Applications," "Bispecifics," and on "Antibody–Drug conjugates." Physiological and manufacturing considerations are given in the follow-up section including overviews on "Pharmacokinetics," "Safety Considerations," "Immunogenicity," "Expression Systems for Manufacture," and a chapter on "Stability, Formulation, and Delivery." The section on Clinical Applications discusses in detail "Protein Therapeutics in Autoimmune and Inflammatory Diseases, Oncology, Respiratory, and Infectious Diseases." Chapters on "Rescue Therapies" and "Biosimilars" supplement this section. Future horizons and new target class opportunities are the topics of the final section.

The series editors thank Tristan Vaughan, Jane Osbourn, and Bahija Jallal for organizing this volume and for identifying and working with such excellent authors. Last but not least we thank Frank Weinreich and Waltraud Wüst from Wiley-VCH for their valuable contributions to this project and to the entire book series.

May 2017

Raimund Mannhold, Düsseldorf
Gerd Folkers, Zürich
Helmut Buschmann, Aachen

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A Personal Foreword

To a diabetic patient, it is hard to imagine a world without biosynthetic insulin. For a new parent of a premature baby, Synagis provides potentially life-saving prevention of respiratory syncytial virus. And for someone suffering the devastating effects of rheumatoid arthritis, Humira, the world's first fully human antibody, introduced in 2002, is so effective that it has become the world's best-selling medicine. Today, biologics such as these account for nearly half of all new drug approvals across the globe and nearly 25% of overall sales. In fact, in 2015, 6 biologics were among the top 10 best-selling drugs worldwide. With more than 500 biopharmaceuticals on the market, biologics represent the fastest growing sector of this industry, targeting illnesses such as cancer, asthma, cardiovascular disease, infectious diseases, multiple sclerosis, hepatitis, inflammatory disease, and so many others.

As a student of physiology and biochemistry in Paris, I was struck by the possibility of modifying proteins to increase their potential to treat illness. My postdoctoral work in molecular biology and oncology at the Max-Planck Institute for Biochemistry in Munich allowed me to focus on the analysis of epidermal growth factor receptor (EGF-R and HER2) signaling and to investigate the antitumor properties of the novel secreted tumor-associated antigen 90K.

My later work building the translational sciences function at Sugen further heightened my interest. But the day I met a patient with renal cell carcinoma who was successfully being treated with Sutent, a multitargeted receptor tyrosine kinase (RTK) inhibitor, made me realize that my true calling was to work in the biopharmaceutical industry to discover new drugs to help patients. Today, as the head of MedImmune, the global biologics early research and development unit of AstraZeneca, I am even more excited by the possibilities of using protein therapeutics to not just treat or prevent disease but to provide a durable cure for so many illnesses affecting patients across the globe.

This book dissects the field of protein therapeutics, from its early struggles to its promising future, and provides a thorough look into this dynamic industry. It touches on exciting developments around immuno-therapies for oncology – advancements I never thought were possible in my lifetime. It delves into the considerable energy going into the development of antibody drug conjugates and their potential for new therapies. Other topics include human and nonhuman

antibodies; technological advances in protein therapeutics, including human antibody discovery platforms, nonantibody scaffolds and antibody mimetics; protein engineering and physiological and manufacturing considerations around pharmacokinetics, immunogenicity, safety, and manufacturing; and clinical applications for protein therapeutics.

Protein Therapeutics concludes with a view into innovation of the future, including the potential to target protein therapeutics across the blood–brain barrier for the treatment of diseases ranging from brain tumors to Alzheimer's disease and how protein therapeutics could be delivered intracellularly to gain a better understanding of protein interactions and, for example, modify the RAS/MAPK pathway that could be potentially transformative for the treatment of leukemia and other cancers.

With contributions from recognized academic and industry experts, including Professor Pierre De Meyts, the leading academician in the field of insulin research and one of the fathers of protein therapeutics, and Herren Wu, MedImmune's chief technology officer who offers just a glimpse at potential future innovations, *Protein Therapeutics* will be a valuable addition to a field that is profoundly changing the way we treat disease.

Gaithersburg, MD
December 2016

Bahija Jallal

Acknowledgments

We would like to express our gratitude to Karen Stanger for her proactive stewardship over early stages of this project and to Fay Larman who helped us see it through to completion. We are, of course, most indebted to all the authors for taking time out from their daily activities to make so many excellent and insightful contributions to this handbook, as well as to all the reviewers for providing valuable critique and comment. Finally, we would like to especially thank all of you, the scientists, who have contributed so much to this most exciting, dynamic, and motivational field. There is nothing more rewarding than having the opportunity to meet a patient who is benefitting from a protein therapeutic that you have had the good fortune to have helped discover and develop.

Part I
Introduction to Protein Therapeutics: Past and Present

1
Early Recombinant Protein Therapeutics

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1.1
Introduction

The successful purification of pancreatic insulin by Frederick Banting, Charles Best, and James Collip in the laboratory of John McLeod at the University of Toronto in the summer of 1921 [1–3], as reviewed in the magistral book of Bliss [4], ushered in the era of protein therapeutics. Banting and McLeod received the Nobel Prize in Physiology or Medicine in 1923. The discovery of insulin was truly a miracle for patients with Type 1 diabetes, for whom the only alternative to a quick death from ketoacidosis was the slow death by starvation on the low-calorie diet prescribed by Allen of the Rockefeller Institute [5–7]. Insulin went into immediate industrial production (from bovine or porcine pancreata) from the Connaught laboratories of the University of Toronto and, under license from the University of Toronto by Eli Lilly and Co. in the United States, by the Danish companies Nordisk Insulin Laboratorium and Novo (who merged in 1989 as Novo Nordisk), and by the German company Hoechst (now Sanofi), all of which remain the major players in the insulin business today.

Insulin also turned out to be a blessing for scientists interested in protein structure. It was the first protein to be sequenced [8, 9], earning Fred Sanger his first Nobel Prize in 1958. It was the first protein to be assembled by total peptide synthesis [10–14]. It was the first peptide hormone to have its minute blood levels measured by radioimmunoassay [15], earning Rosalyn Yalow the Nobel Prize in Physiology or Medicine in 1977. The structure of insulin was solved by X-ray crystallography in 1969 by Nobel Laureate Dorothy Hodgkin and her team in Oxford [16], providing a rationale for detailed structure–activity relationships studies [17] and, later, for protein engineering of insulin and insulin analogs.

Protein Therapeutics, First Edition. Edited by Tristan Vaughan, Jane Osbourn, and Bahija Jallal.

For about 60 years after the discovery of insulin, major therapeutic advances were in formulation to improve pharmacokinetic and pharmacodynamic (PK/PD) properties and provide longer acting insulins, and in purification (reviewed in Ref. [18]). The introduction of the first insulin injection pen in 1985 (Novopen) made insulin treatment somewhat more bearable for diabetic patients.

In 1982, insulin once again was at the forefront of a therapeutic revolution by becoming the first DNA recombinant protein therapeutics on the market (human insulin, Humulin by Eli Lilly), kickstarting the biotechnology era. By 2008, 130 protein therapeutics (of which 95 were produced by genetic engineering) had been approved by the US Food and Drug Administration (FDA) [19], and in 2016, 206 [20]. These comprise hormones, interferons, interleukins, hematopoietic growth factors, tumor necrosis factors, blood clotting factors, thrombolytic drugs, enzymes, monoclonal antibodies, and vaccines.

In this introductory chapter, I will attempt to retrace the pioneering early steps in the development of recombinant protein therapeutics.

1.2 The Birth of Genetic Engineering

The term "molecular biology" is said to have been coined in 1938 by Warren Weaver, head of the Division of Natural Sciences at the Rockefeller Foundation. While the field developed since the 1930s, it got a major impetus in 1953 with the discovery of the double-helical structure of DNA by Watson and Crick [21, 22], who deduced from it the mechanism of genetic self-duplication. For this achievement, Watson and Crick were awarded the Nobel Prize in Physiology or Medicine in 1962.

While studying the structure, function, and replication of the genes of simian virus 40 (SV40), Paul Berg of Stanford University had the idea in the early 1970s that viral DNA could be used to transduce inserted nonviral genes into mammalian cells. He and his colleagues succeeded in developing a general way to join two DNAs together *in vitro,* a process called DNA recombination. This involved synthesizing cohesive ends onto the end of DNA segments and covalently joining them *in vitro* using DNA ligase. They succeeded in inserting both lambda phage genes and the galactose operon of *Escherichia coli* into circular SV40 DNA [23]. Recombinant DNA technology was born. Paul Berg received the Nobel Prize in Chemistry in 1980 for this work, shared with Walter Gilbert and Fred Sanger (his second Nobel Prize) for their work on sequencing DNA methods. Interestingly, this landmark paper does not report successful expression of the recombinant DNA into mammalian cells (in part due to biohazard concerns), but Berg and colleagues later developed suitable vectors for this purpose (Figure 1.1) [24].

Berg's very basic goal was to insert bacterial, viral, and simple eukaryotic genes into mammalian cells, in order to understand better how human genes are organized and function. What triggered the rise and explosive growth of the biotechnological industry was a different postulate: how to transduce mammalian genes encoding proteins of therapeutic importance into bacteria or simple eukaryotes (and eventually mammalian cells). The key experiments toward this end were done

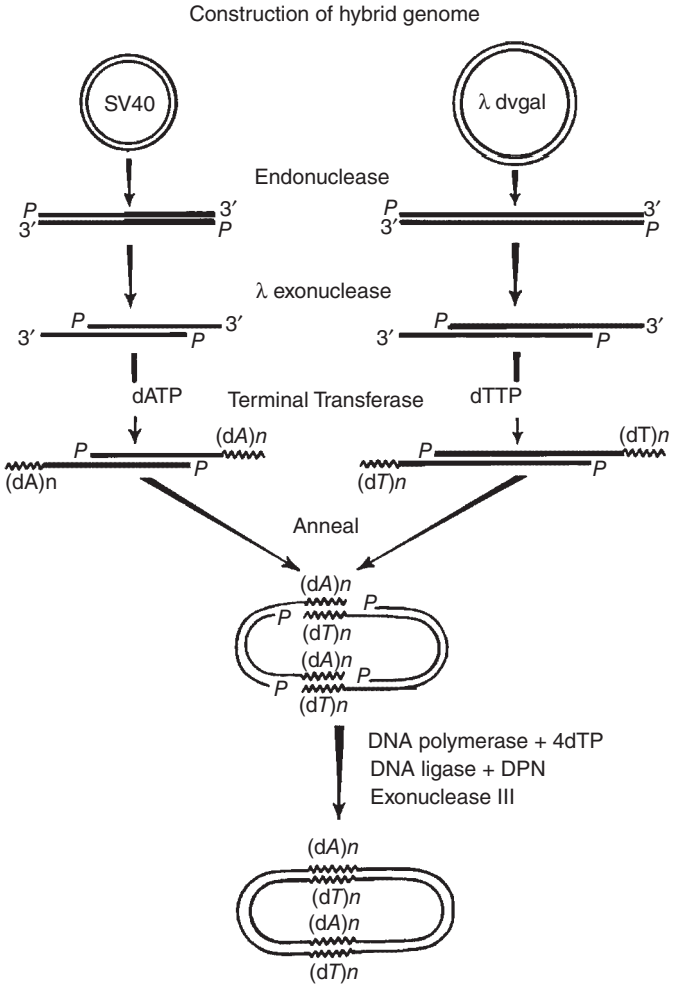

Figure 1.1 Paul Berg's construction of hybrid genome. (From his speech: The Nobel Prize in Chemistry 1980. Nobelprize.org. Nobel Media AB 2014. Web. 12 Jan 2017. http://www.nobelprize.org/nobel_prizes/chemistry/laureates/1980/ [24].)

by a team of scientists that included Stanlry N. Cohen from Stanford University, as well as Herbert Boyer and Howard Goodman from the University of California, San Francisco (UCSF), who were to become major players in the nascent biotechnology industry [25, 26].

They transferred and expressed frog ribosomal DNA into bacterial cells using a constructed plasmid vector, pSC101. This plasmid contained a single site for the restriction enzyme EcoRI and a gene for tetracycline resistance. EcoRI was used to cut the frog DNA into small fragments, which were combined with the plasmid that had also been cleaved with EcoRI. The aligned sticky ends of the DNA fragments were joined using DNA ligase. The plasmids were then transferred into *E. coli* and plated onto a growth medium containing tetracycline. The

cells that incorporated the plasmid carrying the tetracycline resistance gene grew and formed colonies of bacteria, some of which carried the frog ribosomal RNA gene. The colonies were shown to express frog ribosomal DNA.

This led to the very first biotechnology patent granted to Cohen and Boyer in 1980, to the Wolf Prize in Medicine in 1981 and the National Medal of Science in 1988 to Cohen, and to the Lasker Award in 1980 and National Medal of Science in 1990 to Boyer.

Berg Boyer and Cohen with other leading molecular biologists raised concern in 1974 about the potential biohazards of recombinant DNA [27], and organized the influential Asilomar Conference in February 1975, which resulted in stringent National Institutes of Health (NIH) guidelines for recombinant DNA work in June 1976; however, these were later relaxed when the technology became more familiar.

Herbert Boyer made a bold move in April 1976 by founding the biotechnology company Genentech in South San Francisco with a young venture capitalist Robert A. Swanson. Genentech to this day (acquired by Roche, Switzerland, in 2009) remains one of the most successful biotech companies. Since 1976, more than 2000 such companies have been founded, and major pharmaceutical companies have subsequently strived to become more biotechnologically oriented.

1.3 Recombinant Human Insulin

1.3.1 The Race to Clone the Human Insulin Gene and to Produce Human Recombinant Insulin

The race to produce the first recombinant protein therapeutics has been vividly described in the excellent book of Hall [28]. By 1976, Eli Lilly and Co. became concerned that the supply of animal pancreata would become insufficient at some point in the future to meet the needs of diabetic patients worldwide. This was supported by a report of the National Diabetes Advisory Board published 2 years later [29]. Lilly was aware of the promise of genetic engineering and had established an in-house group of scientists to evaluate its possibilities.

Forty years ago, in May 1976, Lilly convened one of its periodic academia–industry symposia (the 16th since 1922), focused this time on the theme of making insulin by genetic engineering. Leading molecular biologists and insulin researchers from all over the United States were invited: Donald F. Steiner (the discoverer of proinsulin [30]) and Shu Jin Chan from the University of Chicago, Grady Saunders and Peter Lomedico from University of Texas, William J. Rutter, Raymond Pictet, and Howard M. Goodman from UCSF, and William (Bill) Chick, Argiris Efstratiadis, and Walter Gilbert (who would later become a co-winner of the 1980 Nobel Prize in Chemistry) from Harvard University.

Bill Chick presented his results with establishing an insulin-producing cell line from an irradiated rat insulinoma, which became an obvious tool for attempting to isolate the rat insulin gene (rat and mice have two, in fact). The race was on [28].

The following year, Axel Ullrich and colleagues in the UCSF team led by Bill Rutter and Howard Goodman reported the construction of recombinant bacterial plasmids containing complementary DNAs (cDNAs) for the coding sequence or rat preproinsulin I and the A sequence or rat preproinsulin II [31]. In 1980, Graeme Bell in the Rutter–Goodman team determined the sequence of the human insulin gene [32]. The same year, Talmadge and colleagues in the Gilbert team showed that bacteria (*E. coli*) can process mature rat preproinsulin to proinsulin [33].

However, in the end the race to produce recombinant human insulin was won by a team that scooped all others by choosing a radically different approach from those trying to use cloned genes: they chose to use completely synthetic DNA.

Herbert Boyer missed the Lilly May 1976 meeting because he was busy founding Genentech in April, and forming an alliance with a team at the City of Hope National Medical Center in Duarte, California, in order to produce recombinant human insulin, and ultimately sell it [28].

The City of Hope team included Arthur (Art) D. Riggs, a geneticist and molecular biologist whose major interests were how genes are turned on and off, X-chromosome inactivation, and DNA methylation, basically founding the now popular field of epigenetics. Riggs had recruited in 1975 a highly talented Japanese organic chemist Keiichi Itakura (then in Ottawa), who was at the forefront of the difficult art of making synthetic DNA.

Riggs and Itakura had realized the potential power of combining chemical DNA synthesis technology and recombinant DNA technology, and Herbert Boyer had independently come to the same conclusion. The project got strong support from Rachmiel Levine, the Medical Director of the City of Hope, considered by many to be the father of modern diabetes research. Riggs and Itakura chose to first try the synthetic approach on a simpler peptide hormone, somatostatin, with only 14 amino acids. The project was rejected for funding by the NIH as unrealistic, but with Boyer's collaboration and Genentech's support, the effort to make somatostatin in *E. coli* was completed successfully in 1977 [34]. This represented the first synthesis of a functional polypeptide product from a gene of chemically synthesized origin. Then, in 1978, the City of Hope–Genentech team reported the successful chemical synthesis of separated genes for the A chain and B chain of human insulin [35], and in 1979 their successful expression in *E. coli* [36]. Synthetic genes for human insulin A and B chains were cloned separately in plasmid pBR322. The cloned synthetic genes were then fused to an *E. coli* β-galactosidase gene to provide efficient transcription and translation and a stable precursor protein. The insulin peptides were cleaved from β-galactosidase, detected by radioimmunoassay, and purified. Complete purification of the A chain and partial purification of the B chain were achieved. These products were mixed, reduced, and reoxidized. The presence of insulin was detected by radioimmunoassay.

This triumph launched the biotechnology industry. It also endeared Genentech with investors [28]. Some of the key players behind this historical milestone are shown in Figure 1.2. In this figure are David Goeddel, one of the first scientists hired by Genentech and who later became Genentech's research director, and Roberto Crea, who soon after the insulin project set up the DNA chemistry lab at Genentech.

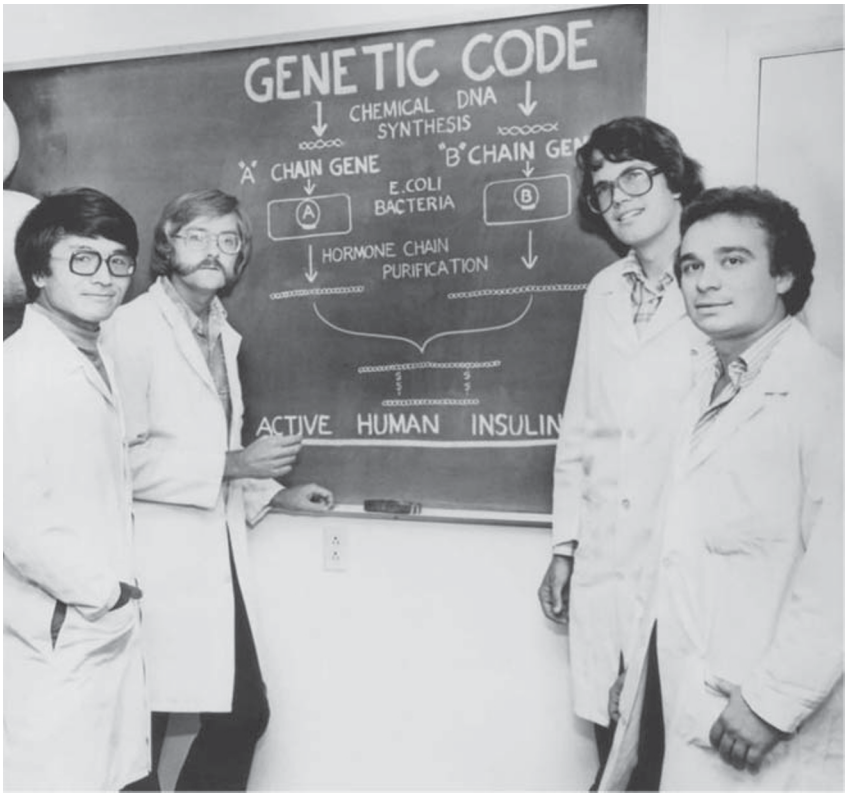

Figure 1.2 From left to right: Keiichi Itakura, Art Riggs, David Goeddel, and Roberto Crea. (Picture courtesy of City of Hope, used with permission of the scientists pictured.)

The process (Figure 1.3) was licensed by Genentech to Eli Lilly and Co. [38]. Crystals of the recombinant insulin are shown in Figure 1.4. In 1981, my group showed that the recombinant insulin had the same affinity for the insulin receptor as native insulin [39]. On October 29, 1982, recombinant human insulin was approved by the FDA and went on the market under the name Humulin. The two-chain recombination process was later replaced by the expression in *E. coli* of a gene for human proinsulin, which after expression and purification was then enzymatically converted to insulin [40]. More details about the precise processes can be found elsewhere [18, 41].

Retrospectively, aside from preventing an eventual shortage of animal insulins, it is not clear whether recombinant human insulin presented a significant therapeutic advantage over the highly purified mono-component porcine insulins that Novo had on the market since 1973 [42]. However, it paved the way for the entire biotechnology industry and cleared the hurdles for FDA approval of a recombinant protein therapeutic. Moreover, it also opened up the possibility to prepare recombinant insulin analogs with improved PK/PD properties [43].

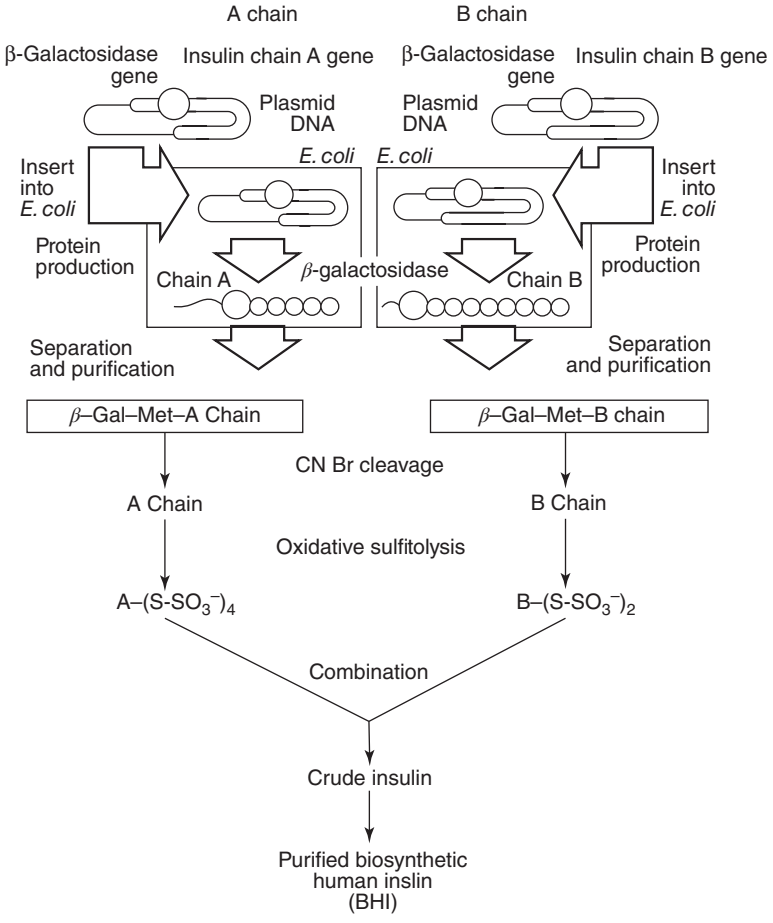

Figure 1.3 Production of insulin from separate genes encoding the A and B chains of human insulin. (From Ref. [37], used with permission. © E.P. Kroeff.)

1.3.2
Novo's Counter Strategy: Semisynthetic Human Insulin

The research management at the Danish company Novo, one of the main competitors to Eli Lilly, headed at the time by Jørgen Schlichtkrull, the father of highly purified mono-component porcine insulins [42], had been cautious of the whole DNA recombinant approach. Some of their chemists even referred to the bacterial insulin as "sewer insulin"! The prospect of a shortage of animal pancreata, which had motivated Lilly in the first place, did not appear to be an issue in a country that has 4.5 times more pigs than humans. However, when it became clear that Lilly was going soon to be on the market with a recombinant human insulin, it was too late to jump on the genetic engineering bandwagon. A quick strategy was needed to counter the risk of losing European market share to Humulin.

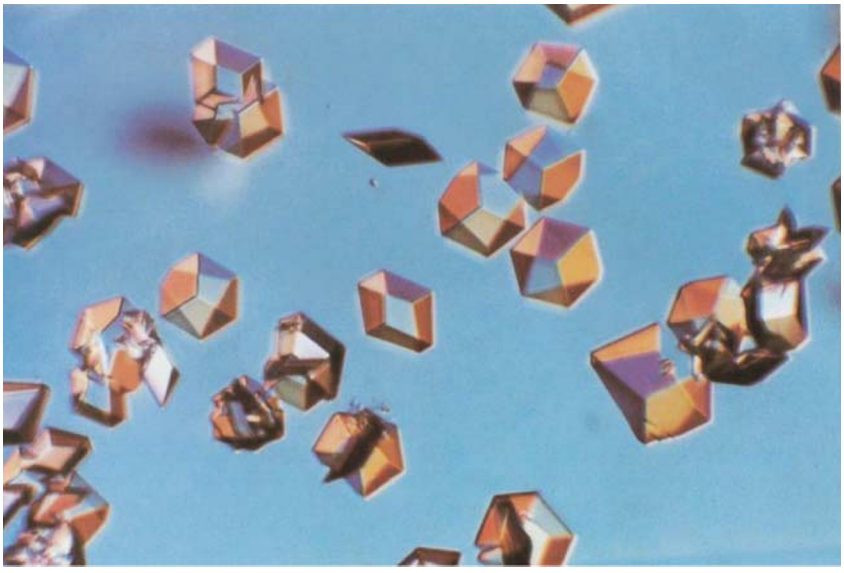

Figure 1.4 Zinc crystals of recombinant human insulin produced from the separate chains recombination process. (From Ref. [38], used with permission © Raven Press 1981.)

It had been known for some time that peptide bond formation can be enzymatically catalyzed by the reverse reaction of proteases (for review see Ref. [44]). Ken Inouye in Japan pioneered the enzymatic semi-synthesis of insulin analogs from desoctapeptide insulin [45]. Dietrich Brandenburg and Axel Wollmer were pioneers in insulin chemistry at the German Wool Research Institute in Aachen, where the total insulin synthesis had been previously accomplished by director Zahn in 1965 [11]. In 1979, Brandenburg and Wollmer organized the second International Insulin Symposium in Aachen, which was attended by the top insulin researchers in the world [46]. Jan Markussen, the leading insulin chemist from Novo was there. So was I.

Hans Gattner from Aachen presented how to make human insulin from porcine insulin by enzymatic semi-synthesis [47]. The only amino acid difference between the two is a Thr at the C-terminal position 30 of the B chain in human insulin replacing an Ala in porcine insulin. The semi-synthesis consisted in removing the Ala with carboxypeptidase from porcine insulin (extracted from pancreas) and using trypsin to replace with a Thr in a two-step process. A similar approach was published independently in 1979 by Morihara *et al.* [48]. Neither scientist patented their method.

Upon return to Denmark, Markussen applied for a patent to make a one-step trypsin transpeptidation of porcine insulin to human insulin [49]. The Aachen group published their similar one-step procedure in 1981 [50]. This process was easily scalable for industrial production, and Novo had its semisynthetic human insulin (Novolin) approved in Europe and on the market in early 1982, beating Eli

Lilly to the finish line by several months for their human insulin. The progress in insulin semi-synthesis is reviewed in Ref. [51].

Retrospectively, one could argue that this whole process of humanizing porcine insulin was a rather futile exercise, aside from the marketing strategy. The marketing of the time was focused on "Why use an insulin that is not your own"? In fact, the B30 amino acid has no influence on the biological activity of insulin, and modern insulin analogs prepared today in yeast lack entirely this B30 residue (the single-chain precursor can be cleaved at Lys B29 by the processing enzyme). Indeed, modern insulin analogs could therefore be considered both porcine and human.

1.3.3 Yeast Recombinant Insulin

In order to fill the expertise gap in DNA recombinant methods, Lars Thim, a young Novo protein chemist, and Niels P. Fiil, a seasoned microbiologist from the University of Copenhagen who had joined Novo in 1980, made contact in 1982 with Zymogenetics, a young biotech company based in Seattle, founded in 1981 by two scientists from the University of Washington, Earl W. Davie and Benjamin D. Hall, and by the late Michael Smith from the University of British Columbia, who became a Nobel Laureate in Chemistry in 1995. Zymogenetics was acquired by Novo in 1988, became independent again in 2000, and was acquired by Bristol Myers Squibb in 2010.

In collaboration with Zymogenetics scientists, Thim established a method for the secretion and processing of dibasic insulin precursors in the yeast *Saccharomyces cerevisiae* [52]. Proinsulin in the pancreas is processed to insulin by a trypsin/carboxypeptidase B-like enzyme system [53]. *S. cerevisiae* possesses a similar enzymatic system (*KEX2*) [54] within the secretory compartments to process the pheromone mating factor α1 (MFα1) gene product into a peptide with 13 amino acid residues [55]. The construct used for yeast expression consisted of a double-stranded human preproinsulin cDNA cloned from human pancreas, fused to MFα1, and inserted within the triose phosphate isomerase gene. Together, these fragments provide sequences that ensure a high rate of transcription for the insulin precursor-encoding genes and also provide presequences that direct the insulin precursors into the yeast secretory pathway and lead to the secretion of the expression products into the growth medium [52]. The entire expression unit was inserted into the plasmid CPOT [52] (Figure 1.5).

The purified secretion product was converted to insulin by trypsin and carboxypeptidase B [52].

Novo and Nordisk merged in 1989 as Novo Nordisk, ending 66 years of competition. The Novo Nordisk yeast DNA recombinant insulin was approved and on the market in 1991 under the name Novolin.

And what about Hoechst (today Sanofi), the other major player in the insulin field? They had also missed the nascent genetic engineering revolution. In order to catch up, they made a deal in 1981 with Howard M. Goodman who had by then

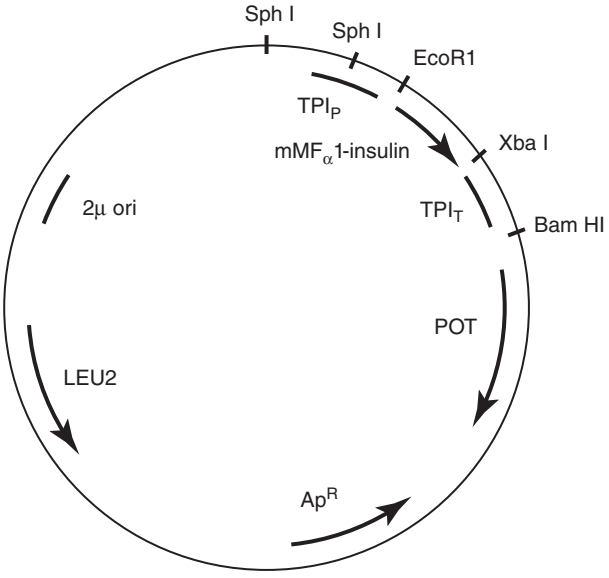

Figure 1.5 Yeast expression plasmid for dibasic insulin precursors. (From Ref. [52], used with permission © PNAS 1986.)

moved to Massachusetts General Hospital in Boston (part of Harvard Medical School), giving him a US$ 70 million unrestricted research grant over 10 years, with the understanding that he would train some Hoechst scientists and that Hoechst would get licensing rights from discoveries. However, there was a level of general uncertainty within German public opinion around genetically modified organisms, and coupled with efficient opposition from Green parties, Hoechst's *E. coli*-based DNA recombinant insulin (Insuman) was significantly delayed and did not start production and achieve approval until 1998 [56].

1.4 Recombinant Human Growth Hormone

Up to 1985, children with growth hormone deficiency (dwarfism or small for gestational age) were treated with the growth hormone (somatropin) extracted from the pituitary glands of human cadavers. This led to over 160 cases of the prion-caused Creutzfeldt–Jakob disease, which has a mean incubation time of 12 years but may not be obvious until 30 years after contamination. This gave some urgency to ensure the availability of DNA recombinant growth hormone. Growth hormone is a much larger peptide than insulin (191 compared to 51 amino acids), so it was not possible to manufacture it via the complete DNA synthesis route that worked so well for insulin. However, unlike insulin, it is a linear peptide that does not require complex processing of a pro form to a two-chain form, so it did prove amenable to direct expression in *E. coli.*

Growth hormone became a focus of research in Howard Goodman's lab at UCSF in the late 1970s, where Axel Ullrich was still struggling to express a cloned

insulin gene in bacteria [28]. A German post-doctoral scientist, Peter Seeburg, together with John Shine from Australia (and Joseph Martial from Belgium, and John D. Baxter from the United States from a separate department), determined the sequence of the rat growth hormone gene in 1977 and demonstrated that it could be expressed in bacteria [57, 58]. The cDNA sequence of the human growth hormone RNA was subsequently determined [59], and the gene (GH1) was shown to be part of a cluster of five genes [60]. In August 1978, Howard Goodman reported the successful expression of the human growth hormone gene in *E. coli* at a Benson Symposium in Copenhagen, with Peter Seeburg, Joseph Martial, John Shine, Axel Ullrich, and John D. Baxter as coauthors [59].

In 1978, Peter Seeburg and Axel Ullrich moved to Genentech after a difference of opinion with Goodman [28]. In October 1979, a Genentech team led by David Goeddel and Peter Seeburg, and in collaboration with Itakura and colleagues at the City of Hope, reported in *Nature* the successful expression in *E. coli* of a hybrid native and synthetic DNA sequence coding for human growth hormone ([61], Figure 1.6). The path to the recombinant human growth hormone has been reviewed by both Seeburg and by the UCSF team [63, 64].

The recombinant growth hormone was approved by the FDA in 1985 as Protropin, and later as Nutropin. The research was funded by the Swedish company

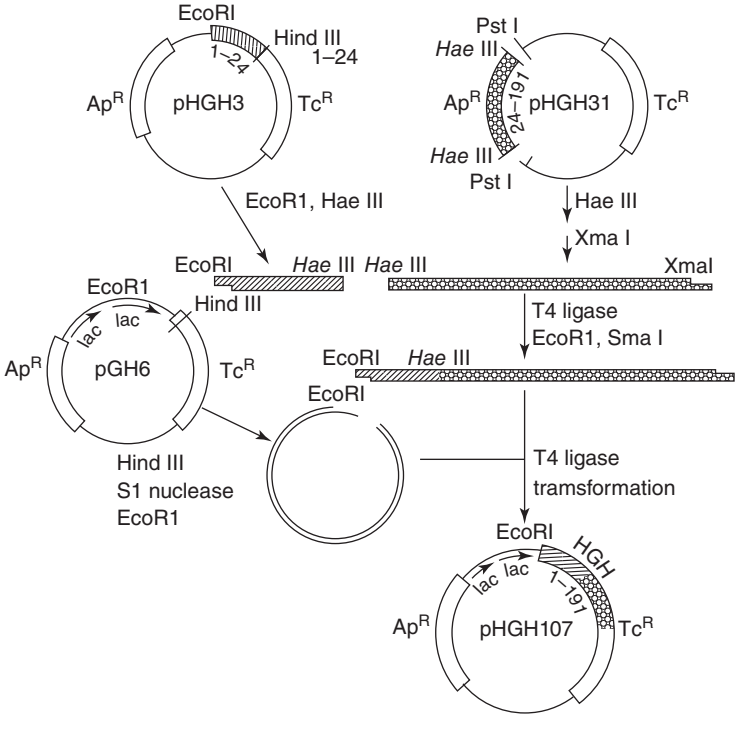

Figure 1.6 Assembly of the plasmid containing the human growth hormone gene. For detailed explanation, see Ref. [61]. (From Ref. [62], used with permission, © G. Miozzari and Walters Kluwer.)

AB Kabi, and both Genentech and Kabi (later acquired by Pharmacia followed by multiple mergers) received co-marketing rights.

Human growth hormone became the first major product directly marketed by Genentech. This success story became somewhat overshadowed when it became apparent that Peter Seeburg had taken to Genentech aliquots from his human growth hormone clones from UCSF without permission from the lab chief or the University, and may have used them for the process to make the recombinant growth hormone [28]. By then Seeburg was Director of Molecular Neuroscience at the Max Planck Institute in Germany. He was accused of making up one of the plasmids described in the 1979 *Nature* paper to hide the supposed theft, which he acknowledged (calling it a "technical inaccuracy") in the trial for patent infringement that ensued between UCSF and Genentech [65]. David Goeddel and the other coauthors have strongly denied Seeburg's allegations that the experiments were not conducted exactly as described in the *Nature* paper (see Ref. [66] for both Goeddel's and Seeburg's comments).

In any case, in 1999 Genentech settled for a $200 million payment to UCSF, of which $85 million went to Seeburg, Shine, Goodman, Baxter and Martial [65].

In 1985, Nordisk Insulin Laboratorium in Denmark (who had been selling human pituitary-extracted growth hormone since 1973 under the name Nanonorm) came up with its own version of bacterial recombinant growth hormone, under the name Norditropin, approved in Europe and Japan in 1988 and by the FDA in 1995 (by then Nordisk had merged with Novo).

1.5 Recombinant Human Interferons

In parallel to the race to produce recombinant human insulin with the involvement of Genentech as narrated above, another race at the end of the 1970s was in place for the production of recombinant human interferon, with heavy involvement of another biotech start-up, Biogen, and some of the same key players as in the insulin and growth hormone sagas. The narrative that follows is based on the lively and candid account of Weissmann [67] as well as other sources.

Interferon, a substance that protected cells from viral infection, was discovered and named in 1957 [68] by Alick Isaacs and Jean Lindenmann at the National Institute of Medical Research in London. This was the first of a class of molecules known as cytokines.

Interferons are proteins with antiviral activity that are produced from cells in response to a variety of stimuli [69]. They turned out to be a highly complex class of multifunctional proteins encoded by over 20 different genes in animals including humans (for contemporary reviews see Refs [70,71]).

In the summer of 1977 at a Gordon Conference, Charles Weissmann, then director of the Institute for Molecular Biology at the University of Zurich, and Peter Lenguyel, Professor of Molecular Biophysics and Biochemistry at Yale University [72, 73], decided to collaborate to clone mouse interferon, for which Lenguyel had a sensitive assay [67, 73]. Weissman and Lenguyel were both born in Hungary 2

years apart and became friends while working together in Nobel Laureate Severo Ochoa's lab at New York University [73]. Cloning interferon was a huge challenge because at the time no sequence information was available, and it was not appreciated that it consisted of multiple proteins. Moreover, this very potent substance was produced in very tiny amounts by induced cells, such as leukocytes or fibroblasts.

In 1977, and again in 1978, Charles Weissmann was approached by two venture capitalists from Inco, a company that had invested in the early days of Genentech [67], with a proposal to create a similar biotech venture in Europe, to be called Biogen. In 1978, they organized the "First European Microbiology Conference" in Geneva, with a roster of prominent European molecular biologists as prospective members of Biogen's Scientific Board, plus the future Nobel Laureate Walter Gilbert of Harvard University (already mentioned in the insulin saga) and Phillip Allen Sharp, then Director of the Massachusetts Institute of Technology (MIT) Center for Cancer Research (who would share in 1993 the Nobel Prize in Physiology or Medicine with Richard J. Roberts for the discovery of mRNA splicing). Biogen was thus founded.

Weissmann was commissioned by the Scientific Board to clone human IFN cDNA, a move promoted by the prospect of interferon being not only an antiviral agent but also potentially an antitumor agent [67, 71]. Weissmann made a partnership with Kari Cantell, a virologist from the Central Public Health Laboratory in Helsinki who was able to produce about a gram of crude IFN per year from leukocytes from 90 000 blood donors from the Finnish Red Cross [67, 74].

In 1979, Walter Gilbert became the CEO of Biogen, and the Board attracted support from Schering Plough, with an investment that allowed the creation of a Biogen laboratory in Geneva. At Christmas the same year, the Weissmann team had established conclusive evidence that they had cloned and expressed human leukocyte interferon (later known as IFNα) in *E. coli.* Weissmann announced the results in a seminar at MIT on January 15, 1980, and then at a highly publicized press conference [75], which raised questions about the then rather untested relationships between university labs and commercial companies [67]. The article was published in *Nature* on March 27, 1980 [76]. Six months later, David Goeddel and colleagues at Genentech, in collaboration with the group of Stanley Pestka at the Roche Institute of Molecular Biology (a leading interferon researcher), also reported the production of leukocyte human interferon in *E. coli,* but this time in sufficient amounts to demonstrate *in vivo* antiviral activity in monkeys [77]. In September 1980, Tadatsugu Taniguchi, a former postdoc of Weissmann now back in Japan, reported the cloning and expression of the human fibroblast interferon gene (later known as IFNβ) in *E. coli* [78]. He is now a member of the National Academy of Sciences, USA. In 1982, Goeddel and colleagues at Genentech reported the cloning and expression of IFNγ in *E. coli* [79].

From then on, the research, both academic and commercial, on interferons progressed at a fast pace (see Refs [67, 70, 71]).

Recombinant human IFN α2b interferon from Schering-Plough (licensed from Biogen) and from Hoffmann-La Roche (licensed from Genentech) was approved

by the US FDA in 1986 for the treatment of hairy cell leukemia. The further evolution of Biogen has been well documented [67]. The company went public in 1983 and merged with the San Diego-based IDEC pharmaceuticals. It went through major reorganizations, moving to Cambridge, MA, USA, and divesting the Geneva, Zurich, and Ghent labs. Walter Gilbert was replaced as CEO in December 1984 and Charles Weissmann resigned in 1988. Biogen is today a multi-billion dollar company. A dozen different interferon preparations from several companies have now been approved by the FDA for various indications including cancers, multiple sclerosis, and chronic hepatitis C infection.

1.6 Recombinant Human Erythropoietin

Erythropoietin (EPO) is a glycoprotein cytokine/hormone/growth factor principally produced in the kidney and controls red blood cell production in the bone marrow. For a good overview, see the excellent review of Jelkmann from the University of Lübeck [79]. EPO was the first of the hematopoietic growth factors – a group that includes thrombopoietin, granulocyte-colony stimulating factor (GCSF), and granulocyte-macrophage colony-stimulating factor (GMCSF) – to be characterized.

Its existence was first suggested 110 years ago by Paul Carnot, Professor of Medicine at the Sorbonne in Paris, France, and his coworker Clotilde Deflandre [80, 81]. Although the experimental data are now believed to be an artifact [79], the hypothesis they generated regarding the existence of a circulating hemopoietic substance proved to be correct and influential. This hemopoietic substance was named EPO by Bonsdorff and Jalavisto at the Institute of Physiology of the University of Helsinki in 1948 [82]. It took another 30 years to finally be able to isolate and purify it. This was done by the group of Eugene Goldwasser in the Department of Biochemistry at the University of Chicago [83], starting from 2550 l of urine from Japanese patients with aplastic anemia collected by Takaji Miyake from Kumamoto University, generating 8 mg of protein. Goldwasser (who died in 2010 at age 88) has written a nice personal account of the struggle to purify, clone, and express EPO [84]. He and the University of Chicago did not patent EPO, and neither made a cent from what became a blockbuster drug [85].

Enter Amgen. Initially called Applied Molecular Genetics, the biotech company Amgen (in Thousand Oaks, California) was founded on April 8, 1980, by the venture capitalist William K. Bowers and associates, and went public in 1983. The first CEO was one of the co-founders, George B. Rathman, a scientist–businessman. They partnered with Goldwasser's group and obtained the purified EPO. Amgen scientists Fu-Kuen Lin from Taiwan and colleagues managed to clone the EPO gene and express it in Chinese hamster ovary (CHO) cells [86]. Amgen had naturally patented EPO. The article came out in the *Proceedings of the National Academy of Sciences* (PNAS) in October 1985 [86].

However, the Amgen/Goldwasser teams were scooped with respect to publication by Takaji Miyake (now at Wright University in Dayton, Ohio), who had made

an alliance with a competing biotech company, Genetics Institute Inc. in Boston, founded in 1980 by two leading molecular biologists from Harvard, Thomas Maniatis and Marc Ptashne. Miyake provided Genetics Institute with the purified EPO he had made with Goldwasser, and ended up as last author on the paper that came out in *Nature* on February 28, 1985 [87], reporting the cloning and expression of human EPO in COS cells, 9 months ahead of the Amgen article. The project was supported by Chugai Pharmaceuticals in Japan. But Amgen had patented first. This resulted in protracted patent litigation, ultimately won by Amgen. Amgen's EPO was approved by the FDA on June 1, 1989, and produced under the name Epogen. Shortly afterward, Amgen (then associated with Japan's Kirin Breweries) cloned and produced GCSF [88], which was approved by the FDA in February 1991 as Neupogen. The Genetics Institute was subsequently (1992–1996) absorbed by Wyeth (then American Home Products).

1.7
Recombinant Tissue – Type Plasminogen Activator

Tissue-type plasminogen activator (t-PA) is a serine protease normally present on endothelial cells, which is therapeutically used for thrombolysis, the pharmacological dissolution of a blood clot by the IV infusion of plasminogen activators that activate the fibrinolytic system (Figure 1.7, see Refs [89, 90] for reviews). Thrombolysis by t-PA has become the staple of the treatment of acute myocardial infarction [90].

t-PA was originally purified and characterized in 1981 by Désiré Collen from the Katholieke Universiteit Leuven in Belgium from human melanoma cells in culture [91]. t-PA was cloned and successfully expressed, in collaboration between Collen and Diane Pennica and colleagues at Genentech, in both *E. coli* [92] and mammalian CHO cells [93]. This project stimulated another important

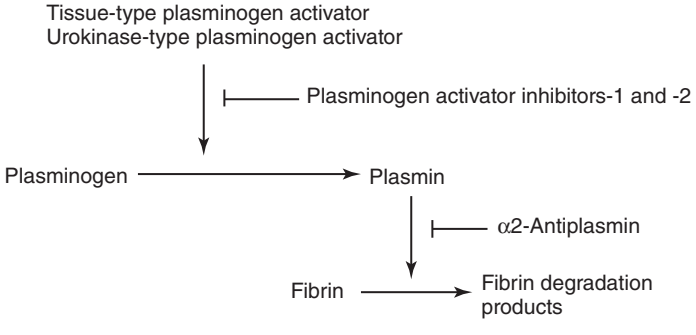

Figure 1.7 The fibrinolytic system. The proenzyme plasminogen is activated to the active enzyme plasmin by tissue-type or urokinase-type plasminogen activator. Plasmin degrades fibrin into soluble fibrin degradation products. Inhibition of the fibrinolytic system may occur through plasminogen activators, by plasminogen activator inhibitors, or more directly via plasmin, mainly by α2-antiplasmin. (Adapted from Ref. [90].)

biotechnological development, that of large-scale tissue-culture fermentation and purification procedures [94], critical for recombinant proteins that require specific biological processes such as glycosylation, which cannot be obtained in unicellular organisms like *E. coli* or yeast.

Genentech's CHO-produced t-PA (Activase or alteplase), the first of the early recombinant protein therapeutics not produced in unicellular organisms, was approved by the FDA in 1987 for acute myocardial infarction and later for acute ischemic stroke and pulmonary embolism.

Désiré Collen received several major scientific awards for his work on t-PA, including the Belgian Francqui Prize in 1984, the Louis-Jeantet Prize for Medicine in 1985, and the Interbrew-Baillet Latour Prize in 2005.

1.8 Recombinant Hepatitis B Virus (HBV) Vaccine

The very first FDA-approved recombinant vaccine for human use was developed by Chiron Corporation, a US West Coast biotech company (based in Emeryville, CA) founded in 1981 by three academic scientists from the University of California, William (Bill) J. Rutter (chairman), already mentioned above in the insulin saga, Edward Penhoet, and Pablo DT Valenzuela. It went public in 1983. They were active in the areas of biopharmaceuticals, vaccines, and blood tests. Based on their research [94–96], they managed to express hepatitis B surface and core antigens in *E. coli* [96]. This became the basis of a vaccine licensed to Merck and Co. and approved by the FDA in July 1986 as Recombivax.

Chiron was acquired by Novartis AG from Switzerland in April 2006.

1.9 Postscript

In this introductory chapter, I have not attempted to provide an encyclopedic description of the processes involved in producing the first DNA recombinant therapeutic proteins, nor of their biological properties and all therapeutic applications that developed after the initial FDA approval. I have focused on the period 1972–1985, which saw the transition from basic molecular biology to industrial biotechnology, trying to give a feeling for the challenges of the time, especially in terms of the sometimes delicate relationships between academic and industry-sponsored research, and the fierce competition that developed between the leading groups involved, which was in fact a major contributor to success. This melting pot in the end broke down barriers between academia and industry, making it much easier to transit between the two. I should also make it clear that I have in no way attempted to give a complete picture of the scientific accomplishments of the scientific protagonists involved in this story, most of whom went on to brilliant careers in both academia and biotech, and became some of the most influential scientists of the last decades.

Acknowledgments

I would like to dedicate this essay to the memory of Ronald E. Chance of Eli Lilly and Co., who sadly passed away in 2015. Ron was the driving force (with Bruce Frank and colleagues) in bringing recombinant human insulin to the market, and transforming the perspectives of the whole pharmaceutical industry in the process. I fondly remember our collaboration in the early days preceding the launch of Humulin, and many years of friendly interactions afterward.

I also would like to acknowledge many enlightening interactions over the years with many of the scientists cited in this chapter: in particular, Arthur Riggs, Keiichi Itakura, and Rachmiel Levine during the 5 years I spent at City of Hope, and Jan Markussen and colleagues during my 20 years at the Hagedorn Research Institute at Novo Nordisk in Denmark. Axel Ullrich kindly served on the advisory board of the Hagedorn Research Institute for several years. I also had a nice collaboration with Jim Wells at Genentech on growth hormone. I am grateful to Arthur Riggs, Keiichi Itakura, and Yoko Fujita-Yamaguchi for a critical reading of the manuscript.

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2
Evolution of Antibody Therapeutics

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2.1
Overview of Antibody Therapeutics Development

The history of antibody therapy spans over 120 years, combining technological breakthroughs with marked evolutions in the concepts and understanding of their pharmacology and their therapeutic potentialities. This complex story is recapitulated in Figure 2.1, which shows two successive and interconnected paths. The ultimate source of both paths was the experimental discovery of serotherapy in 1890 [1], which led to the development of polyclonal antibody therapies, and then a second path diverged after the discovery of a method to produce monoclonal antibodies (mAbs) in 1975 [2]. The wide range of technologies and concepts arising from the discovery of serotherapy have enriched the divergent path, which led to the development of mAb therapies and an even broader range of technologies, concepts, and applications. However, discoveries related to polyclonal antibodies also inform the development of mAbs, since some old approaches are still very relevant in modern therapy.

Following the divergent path, mAbs evolved from basic science laboratory tools into therapeutics with a total global market of nearly $75 billion. As of the end of 2016, over 500 novel antibody-based therapeutics were in clinical development, and nearly 70 had been granted marketing approvals (Table 2.1). This extraordinary outcome started with Köhler and Milstein's landmark paper, published as a letter to *Nature* in 1975, which described a long-sought method to make cells that would continuously secrete antibodies of predefined specificity, that is, mAbs [2]. Niels Jerne, Georges Köhler, and Cesar Milstein were awarded the Nobel Prize in Physiology or Medicine in 1984 for their theories concerning the specificity in development and control of the immune system and the discovery of the principle for production of mAbs. Because of the versatility of the method and the resulting mAbs, as described in the following, this achievement was followed by a

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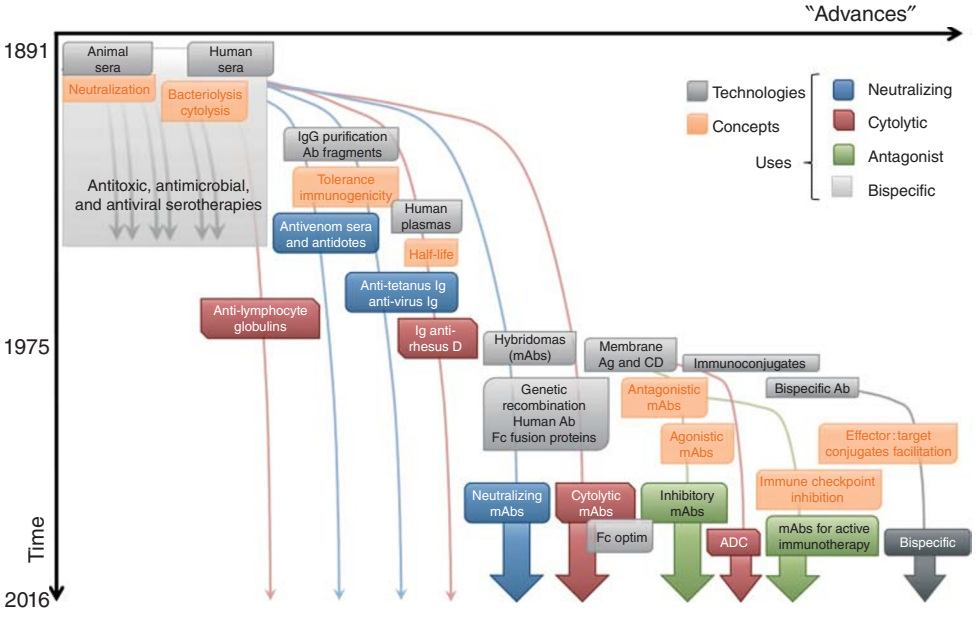

Figure 2.1 Chronological, technological, and conceptual evolution of antibody therapeutics from 1891 to 2016. Rather than presenting the evolution linearly and chronologically only (*Y*-axis, from top to bottom), a second dimension (*X*-axis) has been added to better highlight the technological evolutions (gray boxes), which led to new concepts and therapeutic principles (pink boxes) and a large panel of therapeutic uses, which are categorized into neutralizing Abs (blue boxes), cytolytic Abs (red-brown boxes), antagonist Abs (green boxes), and bispecific Abs (dark gray box). The *Y*-axis starts in 1891, year of the first clinical use of serotherapy. The trajectory of the thin arrows indicates how today's applications originate from older therapeutic concepts, having gradually benefited from new technological inputs. The thick arrows indicate the importance of the deployment of the applications.

succession of notable events in the development of therapeutic mAbs, including first approvals of antibody–drug conjugates (ADCs), radioimmunotherapeutics, bispecific antibodies, and glycoengineered antibodies, culminating with a record-breaking number of antibody marketing approvals in 2015.

In 1986, patients began to directly benefit from the advances in mAb development when muromonab-CD3 (Orthoclone OKT3®) became the first therapeutic mAb of any kind to be granted a marketing approval. Muromonab-CD3, a murine IgG2a mAb that targets the TcR–CD3 complex on the surface of circulating human T cells, functions as an immunosuppressant. Its approval for use as an anti-rejection agent for renal transplantation was a landmark for the fields of mAb therapeutics and clinical transplantation of solid organs. Over the course of the next decade, advances in recombinant protein production and antibody engineering techniques delivered antibodies that reduced the amount of murine sequence in mAbs, that is, chimeric and humanized mAbs, and thus

Table 2.1 Monoclonal antibody therapeutics approved or in review in the European Union or the United States.

International non-proprietary name	Brand name	Target; format	Indication first approved or reviewed	First EU approval year	First US approval year
Atezolizumab	Tencentriq	PD-L1; humanized IgG1	Bladder cancer	In review	2016
Bezlotoxumab	Zinplava	*Clostridium difficile* enterotoxin B; human IgG1	Prevention of *Clostridium difficile* infection recurrence	In review	2016
Brodalumab	(Pending)	IL-17R; human IgG2	Immune-mediated disorders	In review	In review
Sarilumab	(Pending)	IL-6R; human IgG1	Rheumatoid arthritis	In review	In review
Olaratumab	Lartruva	PDGFRalpha; human IgG1	Soft tissue sarcoma	2016	2016
Reslizumab	Cinqair	IL-5; humanized IgG4	Asthma	2016	2016
Obiltoxaximab	Anthim	Protective antigen of *B. anthracis* exotoxin; chimeric IgG1	Prevention of inhalational anthrax	NA	2016
Ixekizumab	(Pending)	IL-17a; humanized IgG4	Psoriasis	2016	2016
Necitumumab	Portrazza	EGFR; human IgG1	Non-small cell lung cancer	2015	2015
Elotuzumab	Empliciti	SLAMF7; humanized IgG1	Multiple myeloma	2016	2015
Daratumumab	Darzalex	CD38; human IgG1	Multiple myeloma	2016	2015
Mepolizumab	Nucala	IL-5; humanized IgG1	Severe eosinophilic asthma	2015	2015
Idarucizumab	Praxbind	Dabigatran; humanized Fab	Reversal of dabigatran-induced anticoagulation	2015	2015
Alirocumab	Praluent	PCSK9; human IgG1	High cholesterol	2015	2015
Evolocumab	Repatha	PCSK9; human IgG2	High cholesterol	2015	2015
Dinutuximab	Unituxin	GD2; chimeric IgG1	Neuroblastoma	2015	2015

(*continued overleaf*)

Table 2.1 (*continued*)

International non-proprietary name	Brand name	Target; format	Indication first approved or reviewed	First EU approval year	First US approval year
Secukinumab	Cosentyx	IL-17a; human IgG1	Psoriasis	2015	2015
Nivolumab	Opdivo	PD1; human IgG4	Melanoma, non-small-cell lung cancer	2015	2014
Blinatumomab	Blincyto	CD19, CD3; murine bispecific tandem scFv	Acute lymphoblastic leukemia	2015	2014
Pembrolizumab	Keytruda	PD1; humanized IgG4	Melanoma	2015	2014
Ramucirumab	Cyramza	VEGFR2; human IgG1	Gastric cancer	2014	2014
Vedolizumab	Entyvio	α4β7 integrin; humanized IgG1	Ulcerative colitis, Crohn's disease	2014	2014
Siltuximab	Sylvant	IL-6; chimeric IgG1	Castleman disease	2014	2014
Obinutuzumab	Gazyva	CD20; humanized IgG1; glycoengineered	Chronic lymphocytic leukemia	2014	2013
Ado-trastuzumab emtansine	Kadcyla	HER2; humanized IgG1; immunoconjugate	Breast cancer	2013	2013
Raxibacumab	(Pending)	*B. anthracis* PA; human IgG1	Anthrax infection	NA	2012
Pertuzumab	Perjeta	HER2; humanized IgG1	Breast cancer	2013	2012
Brentuximab vedotin	Adcetris	CD30; chimeric IgG1; immunoconjugate	Hodgkin lymphoma, systemic anaplastic large cell lymphoma	2012	2011
Belimumab	Benlysta	BLyS; human IgG1	Systemic lupus erythematosus	2011	2011
Ipilimumab	Yervoy	CTLA-4; human IgG1	Metastatic melanoma	2011	2011
Denosumab	Prolia	RANK-L; human IgG2	Bone loss	2010	2010
Tocilizumab	RoActemra, Actemra	IL-6R; humanized IgG1	Rheumatoid arthritis	2009	2010

Ofatumumab	Arzerra	CD20; human IgG1	Chronic lymphocytic leukemia	2010	2009
Canakinumab	Ilaris	IL-1β; human IgG1	Muckle–Wells syndrome	2009	2009
Golimumab	Simponi	TNF; human IgG1	Rheumatoid and psoriatic arthritis, ankylosing spondylitis	2009	2009
Ustekinumab	Stelara	IL-12/23; human IgG1	Psoriasis	2009	2009
Certolizumab pegol	Cimzia	TNF; humanized Fab, pegylated	Crohn's disease	2009	2008
Catumaxomab	Removab	EPCAM/CD3; rat/mouse bispecific mAb	Malignant ascites	2009	NA
Eculizumab	Soliris	C5; humanized IgG2/4	Paroxysmal nocturnal hemoglobinuria	2007	2007
Ranibizumab	Lucentis	VEGF; humanized IgG1 Fab	Macular degeneration	2007	2006
Panitumumab	Vectibix	EGFR; human IgG2	Colorectal cancer	2007	2006
Natalizumab	Tysabri	α4 integrin; humanized IgG4	Multiple sclerosis	2006	2004
Bevacizumab	Avastin	VEGF; humanized IgG1	Colorectal cancer	2005	2004
Cetuximab	Erbitux	EGFR; chimeric IgG1	Colorectal cancer	2004	2004
Efalizumab	Raptiva	CD11a; humanized IgG1	Psoriasis	2004[a)]	2003[a)]
Omalizumab	Xolair	IgE; humanized IgG1	Asthma	2005	2003
Tositumomab-I131	Bexxar	CD20; murine IgG2a	Non-Hodgkin lymphoma	NA	2003[a)]
Ibritumomab tiuxetan	Zevalin	CD20; murine IgG1	Non-Hodgkin lymphoma	2004	2002
Adalimumab	Humira	TNF; human IgG1	Rheumatoid arthritis	2003	2002
Alemtuzumab	MabCampath, Campath-1H; Lemtrada	CD52; humanized IgG1	Chronic myeloid leukemia[a)]; multiple sclerosis	2001[a)]; 2013	2001[a)]; 2014

(*continued overleaf*)

Table 2.1 (*continued*)

International non-proprietary name	Brand name	Target; format	Indication first approved or reviewed	First EU approval year	First US approval year
Gemtuzumab ozogamicin	Mylotarg	CD33; humanized IgG4 conjugated to calicheamicin	Acute myeloid leukemia	NA	2000[a)]
Trastuzumab	Herceptin	HER2; humanized IgG1	Breast cancer	2000	1998
Infliximab	Remicade	TNF; chimeric IgG1	Crohn's disease	1999	1998
Palivizumab	Synagis	RSV; humanized IgG1	Prevention of respiratory syncytial virus infection	1999	1998
Basiliximab	Simulect	IL-2R; chimeric IgG1	Prevention of kidney transplant rejection	1998	1998
Daclizumab	Zenapax; Zinbryta	IL-2R; humanized IgG1	Prevention of kidney transplant rejection; multiple sclerosis	1999[a)]; 2016	1997[a)]; 2016
Rituximab	MabThera, Rituxan	CD20; chimeric IgG1	Non-Hodgkin's lymphoma	1998	1997
Abciximab	ReoPro	GPIIb/IIIa; chimeric IgG1 Fab	Prevention of blood clots in angioplasty	1995[b)]	1994
Muromonab-CD3	Orthoclone Okt3	CD3; murine IgG2a	Reversal of kidney transplant rejection	1986[b)]	1986[a)]

NA, not approved or in review in the EU; information on review status in US not available.
Abbreviations: EU, European Union; GD, glycolipid disialoganglioside; IL, interleukin; INN, international nonproprietary name; PCSK9, proprotein convertase subtilisin kexin Type 9; US, United States.
a) Withdrawn or marketing discontinued for the first approved indication.
b) Country-specific approval.
Source: Data as of December 31, 2016.

reduced issues associated with immunogenicity of the murine mAbs. The first recombinant chimeric antigen-binding fragment (Fab), abciximab (ReoPro®), was approved in 1994 for cardiovascular indications; this Fab binds to the platelet surface protein GPIIb/IIIa receptor and inhibits platelet aggregation. This achievement was followed in 1997 by the first approval of a full-length chimeric antibody, the anti-CD20 rituximab (Rituxan®, MabThera®), as well as the first approval of a humanized antibody, the anti-interleukin (IL)-2 receptor daclizumab (Zenapax®). The 1997 approval of rituximab is also notable because it was the first approval of an antibody therapeutic for cancer (non-Hodgkin's lymphomas). The first approval of an Fc fusion protein, etanercept (Enbrel®), was granted in 1998. Composed of the 75 kDa soluble extracellular domain (ECD) of tumor necrosis factor (TNF) receptor II fused to a human IgG1 Fc, etanercept is widely used as a treatment for immune-mediated disorders such as rheumatoid arthritis (RA).

The approvals of human antibodies were additional landmarks for the field of antibody therapeutics development that occurred in the 2000s. The development of human antibodies was a long-sought goal, and the substantial research in the field was validated with the first approval in 2002 of the anti-TNF-α adalimumab (Humira®) (for details, see Section 2.3.2).

During the 2000s, first marketing approvals were granted for an ADC, a radioimmunoconjugate therapeutic, and a pegylated antibody fragment. These three approvals were notable for the field of antibody therapeutics in general, but also specifically for the area of antibody conjugate development. In 2000, gemtuzumab ozogamicin (Mylotarg®) became the first ADC to be granted a marketing approval. Composed of a humanized anti-CD33 IgG4 mAb conjugated to a semisynthetic derivative of calicheamicin, a cytotoxic agent, gemtuzumab ozogamicin was granted an accelerated approval by the US Food and Drug Administration (FDA) as a single agent for the treatment of patients with acute myeloid leukemia experiencing their first leukemia relapse, based on response rates from three pilot studies initially reporting on 142 patients [3]. Although a lack of evidence to confirm clinical benefit and safety concerns ultimately led to a decision to withdraw the accelerated approval for the drug in 2010, the approval of gemtuzumab ozogamicin remains an important landmark for the ADC field.

Over two decades of research on radioimmunoconjugates [4] culminated in the first approval, in 2002, of a radiolabeled mAb therapeutic, ibritumomab tiuxetan (Zevalin®). This radioimmunoconjugate results from a stable thiourea covalent bond between the anti-CD20 murine IgG1 mAb ibritumomab and the linker-chelator tiuxetan, which provides a high-affinity, conformationally restricted chelation site for yttrium-90. Beta emission from the radioisotope induces damage in the target and neighboring cells. Ibritumomab tiuxetan is approved as part a therapeutic regimen indicated for the treatment of patients with non-Hodgkin's lymphomas. The first approval of a pegylated antibody fragment, certolizumab pegol (Cimzia®), was granted in 2008. Certolizumab pegol is an anti-TNF humanized Fab' conjugated to polyethylene glycol, and it was first approved in the United States for reducing the signs and symptoms

of Crohn's disease (CD) and maintaining clinical response in adult patients with moderate to severe active disease who have had an adequate response to conventional therapy, and subsequently approved in the European Union (EU) for RA, spondyloarthritis, and psoriatic arthritis.

The first approval of the bispecific antibody, catumaxomab (Removab®), was a further milestone. The numerous problems associated with the production of bispecific antibodies, including low yield of the desired antibody, and the difficulty of selecting two suitable targets hampered the clinical development of these molecules. The 2008 approval of catumaxomab in the EU was thus a notable event because of its unique (at the time) mechanism of action. The antibody can mediate the formation of a synapse between a T cell and a tumor cell, allowing upregulation of cell adhesion molecules, production of cytolytic proteins, and release of inflammatory cytokines, which results in redirected lysis of tumor cells. This is a function that naturally occurring antibodies cannot perform because they do not bind T cells. It must be noted, however, that catumaxomab is murine-derived (mouse/rat) and the method of production (quadroma) is not widely used. There is thus a continuing need for advances in the area of bispecific antibody development.

Advances in glycoengineering and protein engineering of antibodies over the past 40 years have enabled the generation of antibodies with properties that are enhanced compared to those of naturally occurring antibodies. In 2012, the first approval of a glycoengineered antibody, mogamulizumab (Poteligeo®), was granted in Japan. Mogamulizumab lacks core fucose because it is expressed in an FUT8 (α-1,6-fucosyltransferase) knock-out variant of Chinese hamster ovary (CHO) cells. This alternative glycosylation has been shown to significantly enhance antibody-dependent cell-mediated cytotoxicity (ADCC) because antibodies without a core fucose bind to the Fc gamma receptor IIIa of effector cells with higher affinity compared to those with a core fucose [5, 6]. Mogamulizumab targets the CC chemokine receptor 4, and it was approved for the treatment of patients with relapsed or refractory adult T-cell leukemia–lymphoma and peripheral T-cell lymphoma. In 2014, the first approval of a single-chain variable fragment (scFv), blinatumomab (Blincyto®), was granted by the FDA. This approval was a landmark generally for the protein engineering field, but also specifically for recombinant bispecific antibody development. Blinatumomab is also a bispecific antibody composed of two scFvs in tandem, one that binds CD19, which is expressed on cells of B-lineage origin, and the other that binds CD3, which is expressed on T cells. It thus has a similar mechanism of action as catumaxomab (i.e., engagement of T cells and tumor cells), but it is produced recombinantly. Blinatumomab was approved for the treatment of Philadelphia chromosome-negative relapsed or refractory B-cell precursor acute lymphoblastic leukemia. Because it does not have an Fc region, the half-life of blinatumomab in humans is very short (~2 h), and it must be administered to patients via continuous intravenous infusion.

Last but not least of the notable landmarks in antibody therapeutics development, the number of first approvals of antibody-based therapeutics in 2015 was the highest ever recorded to date. In total, FDA or the European Commission

approved 10 such new products (9 new antibody products and 1 new Fc fusion protein) in 2015. The landmark achievements described here are the results of decades of work in many different areas of both basic and applied research. The evolution of our understanding of how antibodies can be made to function as therapeutics is described in more detail in the following sections.

2.2 Polyclonal Antibodies – Laying the Foundation for mAb Therapy

Initially described by von Behring and Kitasato in 1890 [1], serotherapy was the origin of fantastic developments in many infectious diseases (Figure 2.1). The extent to which serotherapy revolutionized therapeutic practices by providing doctors with actual therapeutic agents against infections is hardly imaginable today. Sera from patients recovering from infections such as typhus or measles were sometimes used, but the vast majority of sera were collected from large animals, usually horses, that were deliberately immunized with antigens (e.g., toxins, bacteria, venoms). Serotherapy, therefore, usually consisted of infusing nonhuman proteins into patients, and was sometimes followed by adverse events associated with the immunization against these proteins (anaphylaxis, serum sickness). These drawbacks led to several improvements in the 1920s and 1930s, such as antibody purification (serum fractionation) and even elimination by pepsin cleavage of what was later called the Fc portion [7–9] (Figure 2.1). While antibiotics replaced most sera after 1945, some remaining indications of serotherapy benefited from progresses in human blood and plasma processing in the 1960s (Figure 2.1). Immunoglobulins were then purified after drawing blood from convalescent and cured blood donors (e.g., hepatitis B), from vaccinated donors (e.g., tetanus), or from deliberately immunized donors (e.g., rhesus D). These human immunoglobulin preparations, which are still used today, were shown to be better tolerated and to confer a longer protection (antibody half-life) than animal preparations [10], thus progressively replacing the latter. However, animal sera continued (and still continue) to be used, notably when it is impossible to immunize human donors (e.g., with reptile venom) (Figure 2.1).

2.3 Evolution of Monoclonal Antibody Therapeutics

The discovery of the hybridoma technology by Milstein and Köhler in 1975 [2] constituted another remarkable technological advance that led to notable improvements in the practice of medicine. A single B cell (or its progeny in an immortalized B cell clone) was demonstrated to produce a single antibody species, called an mAb. In contrast, all preparations extracted from organisms (animal sera, human immunoglobulins) were then called "polyclonal" antibodies because they are composed of a mixture of antibodies produced by many B-cell

clones. In comparison with polyclonal antibodies, mAbs have several advantages, notably a rather constant product over time (polyclonal preparations were highly variable from batch to batch, requiring very extensive controls), and a perfectly defined molecule recognizing a defined epitope on the target antigen, satisfying the definition of what should be a drug under the pharmacopoeia definition.

Murine (i.e., rodent-derived) mAbs were quickly viewed as an extension, and a possible reawakening, of ancient animal serotherapy, and hybridomas were then cultured on a larger scale, with initiation of clinical trials rapidly following. However, modern scourges were no more acute infections but chronic diseases (e.g., cancer, inflammatory diseases), requiring repeated infusions of antibodies, which carries the potential for a reaction to the antibody therapeutic by the patient's immune system. Such immunogenicity can adversely affect both the safety and efficacy of antibody therapies. The development of human anti-murine antibodies (HAMAs) with neutralizing properties was indeed viewed as a real obstacle to the clinical use of mAbs, despite some encouraging results. An exception was Orthoclone-OKT3®, an IgG2a mAb against human CD3, which was approved in 1986 for the prevention of acute allograft rejection, in replacement of equine anti-lymphocyte globulins (ALGs). This was actually a short-term treatment, not influenced by immunogenicity. Because of their immunogenicity, it was also imagined that mAbs could be at least good vectors for toxins, drugs, or radioisotopes (immunoconjugates and radioimmunoconjugates) (Figure 2.1), but this strategy eventually took time to became a clinical reality (see the following sections).

2.3.1 Chimeric and Humanized mAbs

Rendering mAbs more human (and therefore potentially less immunogenic) was the next achievement. Molecular biology (genetic engineering) was critical to this revolution, leading to the production of recombinant antibodies in the late 1980s in the form of chimeric [11] and humanized antibodies [12]. Whatever their degree of humanization, these recombinant antibodies were later shown in the clinic to be less immunogenic than the murine mAbs and, like previously observed with polyclonal antibodies, had a much better half-life (also contributed to the loss of immunogenicity), and were also more capable of interacting with human immune effectors through their Fc portion.

2.3.2 Fully Human Antibodies

In parallel with the efforts to make chimerized and humanized antibody therapeutics, substantial work was directed toward the production of fully human antibodies [13]. A human IgM therapeutic (nebacumab; Centoxin®) was in fact briefly approved in some countries in Europe in the early 1990s [14]; however, limitations of the available production methods curtailed the advancement of these therapeutics during the 1980s and 1990s, and only 16 human mAbs developed

by companies entered clinical study through 1996 [15]. In the two decades since then, technological advances in discovery methods, such as the development of phage display and transgenic mice, and manufacturing have enabled the clinical study of hundreds of human antibody therapeutics. As of the end of 2015, over 160 human antibodies were in clinical development and 17 human antibody products (Table 2.1) were on the market in the United States and EU, as well as other countries.

The first human IgG to gain marketing approval was adalimumab (Humira®). Adalimumab was first approved in 2002 for RA, and since then it has been approved for other indications such as psoriatic arthritis, ankylosing spondylitis, CD, plaque psoriasis, juvenile idiopathic arthritis, ulcerative colitis, non-radiographic axial spondyloarthropathy, and intestinal Behcet's disease [16]. Global sales for the product were ~US$11 billion in 2013, making it the top-selling therapeutic of any class on the market [17]. Adalimumab, which targets TNF-α, was the first, and most successful based on global sales, of two human anti-TNF-α mAbs developed as potentially better versions of previously approved biologic therapeutics. At the time of adalimumab's first approval in 2002, the anti-TNF Fc-fusion protein etanercept and the chimeric antibody infliximab were already marketed for RA. Golimumab (Simponi®), another human anti-TNF antibody, was approved in 2009 for three indications: moderately to severely active RA in adults, in combination with methotrexate; active psoriatic arthritis in adults, alone or in combination with methotrexate; and active ankylosing spondylitis in adults.

The anti-epidermal growth factor receptor (EGFR) panitumumab (Vectibix®) and the anti-CD20 ofatumumab (Arzerra®) represent additional examples of the development of the human mAbs that could potentially be better than previously approved mAbs of a different format. Panitumumab's first approval in 2006 for the treatment of EGFR-expressing metastatic colorectal cancer followed that of the chimeric anti-EGFR cetuximab in 2004 for the same disease. Ofatumumab's first approval in 2009 for chronic lymphocytic leukemia (CLL) followed that of the chimeric anti-CD20 rituximab.

The development of human mAbs that bind novel targets, that is, targets that are unique compared to those of antibody therapeutics marketed at the time the candidate mAb entered clinical study, has also proven to be a successful strategy. Ten of 11 human mAbs that were granted their first approval after 2009 target such antigens (Table 2.1); the one exception is the anti-EGFR necitumumab (Portrazza®), which was approved in 2015 for the treatment of metastatic, squamous, non-small-cell lung cancer. Nivolumab (Opdivo®) and daratumumab (Darzalex®) are notable examples of recently approved human antibodies that target antigens that were novel at the time of their development. Nivolumab was the first antibody targeting programmed death (PD)-1 to be approved. The product was granted a marketing approval in Japan in July 2014 for the treatment of patients with unresectable melanoma, and was subsequently approved as a treatment for melanoma in both the United States (December 2014) and the EU (June 2015). The biopharmaceutical industry has devoted

substantial resources to the development of inhibitors of PD-1 and other antigens that act as immune checkpoints because they may be efficacious as treatments for multiple types of cancer. Following its approval for melanoma, nivolumab received marketing approvals for the treatment of non-small-cell lung cancer and renal cell carcinoma, and it is being evaluated in Phase III clinical studies of patients with small-cell lung cancer, glioblastoma multiforme, head and neck cancer, gastric cancer, and esophageal cancer. On November 16, 2015, daratumumab became the first CD38-targeted antibody to be approved and also the first antibody approved for multiple myeloma. During its development and review, daratumumab was granted multiple FDA designations intended to assist and expedite the process, including breakthrough designation, priority review, and orphan drug designations, and it was approved under FDA's accelerated approval program.

2.4 Fc Fusion Proteins

The Fc domain of an antibody endows the molecule with a number of properties that are beneficial for therapeutics, including the ability to bind to Fc receptors [18]. In addition, the modular nature of antibodies allows the Fc domain to maintain its functions when separated from the antigen-binding portion. These aspects of the antibody molecule have been exploited through innovative protein engineering to produce Fc fusion proteins, which are recombinant proteins comprising an Fc domain linked to a targeting domain that is not derived from an antibody. Such Fc fusion proteins typically include a hinge region and the heavy chain constant domain (CH)2 and CH3 of either IgG1 or IgG4 [19]. Because the Fc domain engages with the salvage neonatal Fc receptor (FcRn), Fc fusion proteins have an extended circulating half-life, which allows longer intervals between doses. Fc-containing fusion proteins are thus pharmacologically very similar to mAbs, although this is not necessarily evident from their international nonproprietary names (INNs), which do not end with "–mAb."

An excellent example of the utility of the approach lies in the case of onercept, a soluble TNF-RI receptor with a short half-life (terminal elimination half-life of ~15 h [20]). It was fused with an IgG Fc portion to produce lenercept, which was evaluated in clinical studies for a variety of indications, including sepsis, multiple sclerosis, and RA, although it was not found to be sufficiently efficacious. The molecule evolved further when the TNF-RI ectodomain was replaced by the TNF-RII ectodomain, resulting to the well-known etanercept (Enbrel®), which in 1998 became the first Fc fusion protein to be approved for marketing.

As of the end of 2015, a total of 12 Fc fusion proteins had been granted marketing approvals in the United States or EU (Table 2.2). As with antibodies, most marketed Fc fusion proteins are produced in CHO cells [21]. The exceptions are eftrenonacog alfa (Alprolix®) and efmoroctocog alfa (Eloctate®/Elocta®), which are produced in human embryonic kidney cells [22, 23], and romiplostim

Table 2.2 Fc fusion protein therapeutics approved in the European Union or the United States.

International nonproprietary name	Brand name	Format	Indication(s) first approved	First EU approval year	First US approval year
Asfotase alfa	STRENSIQ	Two identical polypeptide chains consisting of the catalytic domain of human tissue nonspecific alkaline phosphatase, the human IgG1 Fc domain and a deca-aspartate peptide used as a bone targeting domain	Perinatal/infantile- and juvenile-onset hypophosphatasia	2015	2015
Efraloctocog alfa; efmoroctocog alfa	Eloctate (US); Elocta (EU)	B-domain deleted analog of human coagulation Factor VIII covalently linked to human IgG1 Fc domain	Control and prevention of bleeding episodes, perioperative management, routine prophylaxis to prevent or reduce the frequency of bleeding episodes in adults and children with hemophilia A (congenital Factor VIII deficiency)	2015	2014
Eftrenonacog alfa	Alprolix	Human coagulation Factor IX (Thr148 allelic form) covalently linked to human IgG1 Fc	Control and prevention of bleeding episodes, perioperative management, routine prophylaxis to prevent or reduce the frequency of bleeding episodes in adults and children with hemophilia B	2016	2014

(*continued overleaf*)

Table 2.2 (*continued*)

International nonproprietary name	Brand name	Format	Indication(s) first approved	First EU approval year	First US approval year
Dulaglutide	Trulicity	Two identical, disulfide-linked chains, each containing an N-terminal GLP-1 analog sequence covalently linked to the Fc portion of a modified human IgG4 heavy chain by a small peptide linker	Improvement of glycemic control in adults with Type 2 diabetes mellitus	2014	2014
Ziv-aflibercept	Zaltrap	ECDs of VEGF receptors 1 and 2 fused to human IgG1 Fc (formulated for intravenous injection)	Metastatic colorectal cancer that is resistant to or has progressed following an oxaliplatin-containing regimen	2013	2012
Aflibercept	Eylea	ECDs of VEGF receptors 1 and 2 fused to human IgG1 Fc (formulated for ocular injection)	Wet age-related macular degeneration	2012	2011
Belatacept	Nulojix	ECD of CTLA-4 disulfide-linked homodimer fused to human IgG1 Fc; differs from abatacept by two amino acid substitutions (L104E, A29Y) in the CTLA-4 region to increase potency	Prophylaxis of organ rejection in adult kidney transplant recipients	2011	2011

Rilonacept	Arcalyst	Two chains, each comprising the C-terminus of the IL-1R accessory protein ligand binding region fused to the N-terminus of the IL-1RI ECD, fused to human IgG1 Fc	Cryopyrin-associated periodic syndromes (CAPS), including familial cold autoinflammatory syndrome and Muckle–Wells syndrome in adults and children 12 and older	2009	2008
Romiplostim	Nplate	Two identical single-chain subunits, each consisting of human IgG1 Fc domain, covalently linked at the C-terminus to a peptide containing two thrombopoietin receptor-binding domains	Thrombocytopenia in patients with chronic immune (idiopathic) thrombocytopenic purpura who have had an insufficient response to corticosteroids, immunoglobulins, or splenectomy	2009	2008
Abatacept	Orencia	ECD of human CTLA-4 disulfide-linked homodimer fused to human IgG1 Fc	Reducing signs and symptoms, inducing major clinical response, slowing the progression of structural damage, and improving physical function in adult patients with moderately to severely active rheumatoid arthritis who have had an inadequate response to one or more disease-modifying anti-rheumatoid drugs	2007	2005

(*continued overleaf*)

Table 2.2 (*continued*)

International nonproprietary name	Brand name	Format	Indication(s) first approved	First EU approval year	First US approval year
Alefacept	Amevive	First ECD of LFA-3 fused to human IgG1 Fc	Moderate to severe chronic plaque psoriasis	NA	2003[a)]
Etanercept	Enbrel	75 kDa soluble ECD of TNF receptor II fused to human IgG1 Fc	Reduction in signs and symptoms of moderately to severely active rheumatoid arthritis in patients who have had an inadequate response to one or more disease-modifying antirheumatic drugs	2000	1998

Abbreviations: CTLA, cytotoxic T-lymphocyte-associated protein; EC, European Commission; ECD, extracellular domain; Fc, crystallizable fragment; Ig, immunoglobulin; IL, interleukin; LFA, lymphocyte-function-associated antigen; NA, not approved; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor.

a) Astellas Pharma US Inc. voluntarily discontinued the promotion, manufacturing, distribution, and sales of alefacept in the US in 2011 for business reasons.

Source: Data as of December 31, 2016.

(Nplate®), which is produced in *Escherichia coli* and is therefore not glycosylated [24]. Large-scale manufacturing is facilitated by the presence of the Fc, which allows the protein product to be purified via Protein G or Protein A affinity chromatography. As with antibodies, annual global sales for Fc protein products are quite variable. Etanercept's global sales ($8.3 billion in 2013) now rival that of the best selling antibody therapeutics, but only two other Fc fusion protein products, abatacept (Orencia®) and aflibercept (Eylea®), have global sales that exceed $1 billion [17].

Although most of the marketed Fc fusion proteins are novel, abatacept and belatacept (Nulojix®) bear obvious similarities to each other, and aflibercept and ziv-aflibercept (Zaltrap®) are actually the same molecule. Abatacept is composed of an ECD of human CTLA-4 disulfide-linked homodimer fused to a human IgG1 Fc that was mutated to limit Fc receptor binding. Belatacept differs from abatacept by two amino acid substitutions in CTLA-4 ECD (L104E, A29Y), and may be considered an improved version of abatacept. Both abatacept and belatacept bind to CD80 and CD86, which are expressed on the surface of antigen-presenting cells (APCs), and thus inhibit T cells by blocking interactions of the APC with receptors expressed on the T cells [25, 26]. Abatacept was first approved in 2005 for reducing signs and symptoms, inducing major clinical response, slowing the progression of structural damage, and improving physical function in adult patients with moderately to severely active RA. A supplemental approval for use of abatacept in the treatment of moderately to severely active polyarticular juvenile idiopathic arthritis in pediatric patients 6 years of age and older was granted by FDA in 2008. Global sales for abatacept were $1.4 billion in 2013 [17]. Suppression of T-cell activity is also relevant in transplant rejection; however, the immunosuppressive properties of abatacept were not sufficiently potent for this indication, and the product was not efficacious in primate transplant models [26]. Only two amino acid substitutions in the parent molecule were made to produce belatacept, which dissociates from its targets more slowly than abatacept and was shown to be more potent *in vitro* and in transplant models [26]. Belatacept was first approved in 2011 for prophylaxis of organ rejection in adult kidney transplant recipients, which has a relatively small global market ($26 million in 2013) [17].

The products aflibercept and ziv-aflibercept did not result from a process of molecular evolution, as was the case with abatacept and belatacept. In fact, aflibercept and ziv-aflibercept are the same molecule, but the products' formulation, route of delivery, approved indication, and manufacturer are all different. First approved in 2011 and marketed by Regeneron and Bayer, aflibercept is administered by intravitreal injection for the treatment of patients with neovascular, age-related macular degeneration. First approved in 2012, ziv-aflibercept is marketed by Sanofi, and it is administered by intravenous infusion in combination with chemotherapy to patients with metastatic colorectal cancer. Thus, to avoid confusion, the products were given different nonproprietary names in the United States as well as different brand names.

The 12 marketed Fc fusion protein products are a testament to the utility of these molecules as therapeutics. In the future, Fc fusion proteins are likely to evolve

in parallel with antibody therapeutics because advances in Fc protein and glycoengineering can be applied to both types of molecules [27, 28]. We thus may soon see a next generation of Fc fusion protein therapeutics with enhanced effector functions and extended half-life on the market.

2.5 Evolution of Concepts in Antibody Pharmacology

2.5.1 Neutralization

In parallel to these technological advances, concepts in antibody pharmacology also evolved, progressively multiplying the possibilities offered by antibody therapy and, hence, their indications. At the outset, protection conferred by serotherapy was attributed to specific "antitoxins" able to neutralize the immunizing agent (diphtheria or tetanus toxins), but serotherapy was soon demonstrated to be useful for neutralizing a plethora of poisons, from bacterial toxins to venoms and drugs (Figure 2.1). This was at the origin of the term "antibody": whatever the body (i.e., substance) used for immunization, the organism was able to produce a specific antidote. Neutralization of a "poison" still remains the basic property of many therapeutic agents today, whether polyclonal (human anti-tetanus immunoglobulins, equine anti-venoms $F(ab')_2$, ovine anti-digoxin Fab), or monoclonal (anti-anthrax toxin raxibacumab; anti-dabigatran idarucizumab (Praxbind®)). Whereas antibacterial endotoxin mAbs were developed to treat septic shocks [14], it appeared that septic shocks were all mediated by an endogenous factor, TNF-α, which proved be an alternative antibody target. Although no therapies were found for septic shocks, anti-TNF agents were found to be efficacious for RA [29] and later CD. This story clearly illustrates a conceptual shift in the thinking about targets, from exogenous to endogenous "poisons," that is, soluble factors involved in the pathophysiology. These soluble mediators of disease now constitute a large field of therapeutic targets, very prone to antibody neutralization. Among the numerous possible targets of this kind corresponding to commercialized antibodies, there are inflammatory cytokines, for example, TNF-α, IL-1β (canakinumab (Ilaris®) and rilonacept (Arcalyst®)), IL-5 (mepolizumab (Nucala®)), complement factor (C5; eculizumab (Soliris®)), pathogenic immunoglobulins (IgE; omalizumab (Xolair®)), or proprotein convertase subtilisin kexin Type 9 (PCSK9) (evolocumab (Repatha®) and alirocumab (Praluent®)), the latter being a factor that recycles cholesterol and whose neutralization is able to reduce cholesterol.

Extending the concept of neutralization to viruses, polyclonal antibodies were, or are, used as prophylaxes against viral diseases, including rabies, cytomegalovirus infection, and hepatitis B. The humanized mAb palivizumab (Synagis®) is used to prevent respiratory syncytial virus infection in infants. There are many mAbs in development to fight influenza, HIV, or viruses responsible

for hemorrhagic fevers, but obstacles lie in the variability of virus strains (epitope variation) and, sometimes, in the necessity to block more than one viral protein or epitope to be fully neutralizing, requiring mAb cocktails. Ironically, mAbs devolve to their polyclonal roots in this approach.

2.5.2 From Killing Bacteria to Killing Human Cells

From the beginning of serotherapy, bacterial cells were also considered possible targets (and not only their toxins), and specific sera were produced, showing efficacy against diseases such as plague, meningitis, pneumonia, gangrene, and typhoid fever. However, results were hardly reproducible. Indeed, these sera needed specificity (to recognize the bacteria and agglutinate them), but had to be bactericidal through complement activation, or opsonization and phagocytosis. Since then, antibodies have been recognized as bifunctional molecules, that is, as drugs having two categories of functions: (i) antigen binding (Fab functions), and (ii) activation of immune effectors (Fc functions). However, even today, targeting bacterial cells, as well as yeasts, molds, protozoa, or parasites, with mAbs remain extremely challenging, and many attempts at developing such mAbs have failed in the past.

Early attempts were also made to develop antibodies against other disease-causing cells, including cancer. Although anticancer antibodies were not found at that time, it was also observed that sera could kill human leukocytes [30]. This was the origin of the development of ALGs in the 1960s, which is inseparable from the development of allotransplantation [31]. These antibody therapeutics were prepared either in horses or rabbits, and were shown to have excellent cytolytic activity, preventing acute allograft rejection. During the same period, it appeared that anti-rhesus D immunoglobulins collected in immunized women prevented rhesus D alloimmunization and newborn hemolytic disease in rhesus D-negative pregnant women [32]. Such immunoglobulins are still in use today because of the difficulty of replacing them with mAbs having an equivalent activity.

ALGs are polyclonal and polyspecific because they are directed against many membrane antigens of human lymphocytes. Replacement of ALGs was the first envisioned application for mAbs because of their extreme and controlled specificity. OKT3 and Campath-1 were both selected for their ability to kill T lymphocytes *in vitro* in order to replace ALGs, and were secondarily shown to recognize CD3 and CD52, respectively. OKT3 was a murine IgG2a that was developed as such [33], and was eventually approved in 1985 under the name muromonab-CD3 for the prevention of acute allograft rejection. Ironically, OKT3 was subsequently abandoned because it was too immunosuppressive, and transplant surgeons went back to rabbit ALG, which is still in use! Campath-1 (Campath-1M) was a rat IgM and could never have been developed as such. It was subjected to substantial molecular engineering to refine its pharmacological activity: Campath-1G was a rat IgG produced after spontaneous class switching *in vitro*; Campath-1H was one of the first version of a humanized antibody, and was formatted as IgG1 and IgG4

[34]. The Campath-1H IgG1 version (alemtuzumab) was ultimately approved in 2001 for the treatment of CLL (MabCampath®), and more recently for multiple sclerosis (Lemtrada®).

Following along the same path, but aiming to be extremely specific for cancer cells, Ron Levy's group developed an anti-idiotype strategy to specifically kill B-cell hemopathies [35]. In 1986, IDEC Pharmaceuticals was created for commercial development of this strategy. Ironically, IDEC's success was rituximab, a cell-killing antibody developed in collaboration with Genentech/Roche that targets CD20, an antigen expressed on all B cells [36]. Rituximab is thus far from being tumor-specific, although it has been highly successful in oncohematology, transforming the therapeutic management of patients and the prognosis of many lymphomas. Rituximab was also the first antibody whose therapeutic response was associated with a polymorphism in the gene coding for FcγRIIIA, an Fc receptor expressed on cytotoxic immune effectors [37], revealing the importance of ADCC in the mechanism of action of cytolytic antibodies. This led to the development of mAbs engineered specifically to have enhanced ADCC activity, for example, the approved antibodies mogamulizumab (Poteligeo®) and obinutuzumab (Gaziva®, Gazivaro®).

2.5.3 Targeting Membrane Receptors

As already shown with the OKT3 and Campath versus ATG comparison, mAbs were able to resolve the complexity of membrane antigens, leading to the cluster of differentiation (CD) classification of leukocyte antigens [38], and, more generally, to the identification of many membrane antigens. Several of these membranes antigens were subsequently shown to be receptors, with some mAbs unable to interfere with ligand binding while others were able to block ligand binding. The latter were thus envisioned as antagonistic drugs, exactly as small-molecule drugs. An early attempt to use this approach involved the blockade of the recently discovered IL-2Rα, namely CD25, to prevent allograft rejection. This was initially done with a murine mAb, with some success [39], and eventually led to the development of basiliximab (Simulect®) by Novartis, and daclizumab (Zenapax®) by Roche. In the field of thrombosis, it was discovered that the platelet integrin GPIIb/IIIa was a fibrinogen receptor essential for clot formation and that mAbs were able to antagonize GPIIb/IIIa and to have an anti-aggregating effect. An mAb was chimerized and developed under an Fab format, mainly to have a short-term effect to prevent hemorrhages [40]. In 1994, the resulting product abciximab became the first approved recombinant antibody. Since then, many antagonistic antibodies against other cytokine receptors (IL-6R for tocilizumab (Actemra®/RoActemra®), integrins (LFA-1 for efalizumab (Raptiva®), VLA-4 for natalizumab (Tysabri®), α4β7 for vedolizumab (Entyvio®)), and growth factor receptors (HER-2 for trastuzumab (Herceptin®) and pertuzumab (Prejeta®), EGFR for cetuximab (Erbitux®), panitumumab (Vectibix®), and necitumumab (Portrazza®))) have been approved, and many more are in clinical development.

The most recent – and explosive – development of antagonistic mAbs is due to agents targeting inhibitory receptors on immune cells or their membrane ligands. By blocking inhibitory signals, these antibodies activate the immune system, reactivating many quiescent cells, some of them having antitumor properties. These immune check-point inhibitors, three of which are now approved (ipilimumab (Yervoy®), pembrolizumab (Keytruda®), nivolumab (Opdivo®)), appear very promising.

Some other mAbs were shown to be agonistic, that is, mimicking the ligand effect, mostly by bridging two receptors at the surface. Although some approved antibodies were secondarily shown to have discrete agonistic effects, there are currently no approved mAbs developed for such an activity. The most attractive of such mAbs are those targeting death receptors and inducing the death of their target cells.

2.6 Future Directions for Antibody Therapeutics Development

Antibody therapeutics have evolved from their early beginnings as proteins extracted from humans or animals into highly engineered molecules that can be modified in many ways to enhance functionality. Molecular biology techniques developed over many years have provided the opportunity to tailor recombinant biopharmaceuticals with desired pharmacological properties. This "cut-and-paste" function at the cDNA level and the many possibilities of mutations offer an endless source of inspiration for molecular designers. While canonical antibody therapeutics (i.e., full-length monospecific antibodies composed of amino acids and carbohydrates only) can certainly be improved via molecular engineering, future expansion of the biopharmaceutical industry's pipeline is likely to depend on successes in the development of antibodies with noncanonical structures, for example, ADCs, bispecific antibodies, and mechanisms of actions, for example, immunomodulatory mAbs.

It is well established that the potency of mAbs can be enhanced by conjugating highly cytotoxic drugs to them. Development of ADCs, however, involves aspects that do not need to be considered for canonical antibodies, for example, linker stability, drug/antibody ratio (DAR), drug distribution, and antigen internalization rate [41]. The stability of the drug linker must be assessed because stability in circulation is needed to ensure the potency and safety of the ADCs. The DAR must be optimized – high DARs lead to faster clearance and a shorter half-life, but low DARs reduce potency. The drug loading distribution, which is determined by the conjugation strategy, must also be assessed. Strategies that rely on reactive lysine or cysteine residues result in attachment of the drug at different locations on the antibody; this heterogeneity can be reduced by using site-specific conjugation [42]. For both canonical antibodies and ADCs, the specificity and affinity of the antibody toward the antigen, as well as the antigen expression levels, are important, but a suitable internalization rate is also critical to the successful

development of an ADC because ADCs must be internalized after binding to function properly. The design of an ADC is also more complex because of the substantial number of choices in linkers (e.g., cleavable, noncleavable) and drugs (e.g., monomethyl auristatins, maytansine derivatives, calicheamicin, pyrrolobenzodiazepine) now available. Despite the added molecular complexity, the recent expansion of the number of ADCs in the clinical pipeline has been remarkable. Over 50 ADCs are currently undergoing clinical evaluation, and 60% of these entered clinical study in just the past 3 years. ADCs now comprise ~20% of all mAbs in clinical studies of cancer patients, and two ADCs for cancers, brentuximab vedotin (Adcetris®) and ado-trastuzumab emtansine (Kadcyla®), are now marketed.

Bispecific antibodies exemplify how adjustable antibodies can be. The boundless creativity of antibody engineers and designers has now yielded over 60 bispecific antibody formats, which may be classified into five categories: (i) full-length bispecific IgG (14 formats); (ii) IgG with an added antigen-binding moiety (16 formats); (iii) bispecific antibody fragments (25 formats); (iv) bispecific fusion proteins (5 formats); and (v) bispecific antibody conjugates (3 formats) [43]. Like ADCs, bispecific antibodies face additional challenges in development compared to canonical antibodies. Those based on a full-length antibody are more complex, and may be more difficult to manufacture. Fragment-based bispecifics have short half-lives, but do have positive attributes, however, because they may have more rapid tissue distribution, and therefore better penetration, and they may be easier to manufacture and characterize because they have a less complex structure compared to a canonical antibody. Many of the bispecific antibodies currently in development are designed to engage effector cells, particularly T cells, and a tumor-associated antigen [44]. The recent approval of such a bispecific antibody (blinatumomab; Blincyto®) constitutes a remarkable proof of concept, opening new exciting ways of thinking antibody therapy.

The development of mAbs or Fc fusion proteins with immune-modulatory activity has great potential because of the large number of targets involved [45]. To date, mAb therapeutics that inhibit only two immune check-point targets, CTLA-4 and PD1, have reached the market, but many more are in clinical development. For example, antibodies that target important co-inhibitory receptors, such as lymphocyte activation gene 3, or important co-stimulatory receptors, such as CD137, CD27, OX40, glucocorticoid-induced TNFR-related protein, and CD40L, are in clinical development. The future of the development of antibody therapeutics thus appears very bright, as numerous products that benefit patients may result from the exploration of these pathways.

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Part II
Antibodies: The Ultimate Scaffold for Protein Therapeutics

3
Human Antibody Structure and Function

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3.1
Introduction

The antibody, also known as immunoglobulin (Ig), is one of the most well-studied proteins in terms of structure and function, and the knowledge of its structure–function relationships has led to the understanding of humoral immunity, innovative applications in biotechnology and medicine, and antibody scaffold developments for therapeutic applications. In this chapter, we provide an overview of the fundamental aspects of human antibody structure and function to provide sufficient background for therapeutic antibody design and engineering in a range of formats including scaffolds. We will discuss in detail antibody fragments, immunogenetic mechanisms involved in the generation of diversity, structural insights gained from antibody conformation, specifically antibody loops, and antibody interactions with antigens and fragment crystallizable (Fc) receptors. Understanding of these would facilitate structural and immunogenetic considerations for designing better antibody therapies. With the advent of new technologies, millions of antibody sequences and thousands of crystal structures for antibody fragments are currently available. A proper systems approach to manage and analyze these data using custom bioinformatics algorithms specific for human antibodies will lead to insights into the relationship between structure and functions for the generation of novel antibody-based therapeutics.

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3.2
General Sequence and Structural Features of Antibodies

Human antibodies have a basic structure comprising two identical pairs of light and heavy chains linked together by disulfide bonds, schematically shown in Figure 3.1. Each of the chains is divided into smaller units of about 110 amino acids called the Ig domains, and the N-terminal domains are highly variable in terms of the amino acid composition compared to the other domains. The light chain has a variable domain (V_L) at the N-terminal and a constant domain (C_L) at the C-terminal, while the heavy chain has a variable domain (V_H) at the N-terminal followed by three constant domains (C_{H1}, C_{H2}, and C_{H3}) at the C-terminal. Previously, comparative sequence analysis of many different antibody sequences had shown that only certain amino acid residues at specific positions in the variable domains are highly variable, referred to as hypervariable regions or complementarity-determining regions (CDRs) [1]. Each variable domain contains three CDR regions, CDR1–3, which vary in sequence and/or length among different antibodies. The CDR regions determine the specificity of particular antigen–antibody interaction and are alternatively arranged within the framework regions (FRs) 1–4 (FR1–4). Unlike CDRs, the FRs share amino acids extensively between other antibodies. Each antibody domain of light and heavy chains has an intra-disulfide bond at the conserved sites. Further, interchain disulfide bonds connect the four chains of the antibody together near the hinge regions. The penultimate constant domains of both heavy chains, C_{H2} in the IgG, have a conserved glycosylation site. The constant domains attached to glycans along with hinge regions play key roles in some cellular effector functions. Antibody sequence and structural features are discussed in detail in the following sections.

3.2.1
Antibody Numbering Schemes

To efficiently analyze and compare antibody sequences and structures, different antibody numbering schemes have been developed (Table 3.1). Kabat *et al.* first proposed an antibody numbering scheme based on the analysis of sequence data available at that time, solely considering the sequence variability of amino acid sequences of variable domains [1a]. However, insertions at CDR L1 and CDR H1 led to situations where structurally equivalent residues in those CDRs did not get the same number. Later, Chothia and Lesk improved the Kabat numbering scheme by considering the structural context of the insertions in CDR L1 and CDR H1 and provided a new numbering system. Further, a different method based on contact analysis of the antigen–antibody complex crystal structures was proposed, which also allowed the prediction of antigen-contacting residues with the defined CDRs. Although all these numbering schemes have common features as found in the relationship between the antibody sequence and structures, and enabled systematic comparison of antibody sequences, the definitions of CDRs still depend on the specific methods and datasets. Yet another numbering scheme, called AHo,

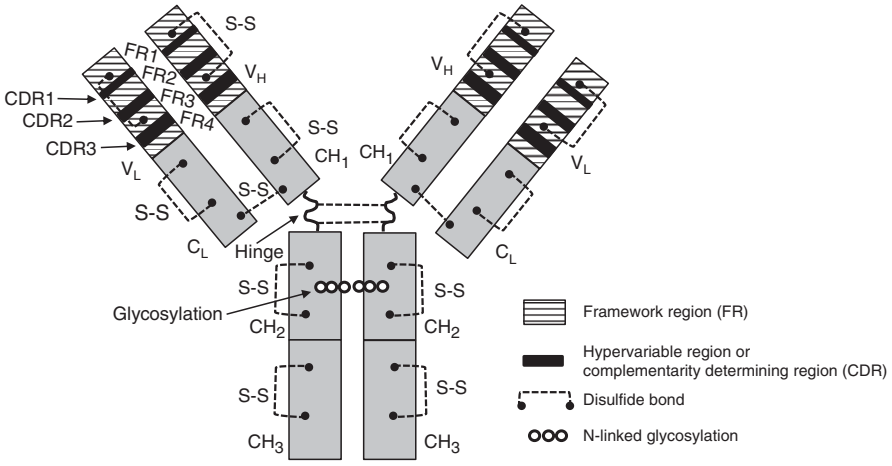

Figure 3.1 Schematic representation of a prototypic antibody IgG, which contains four polypeptide chains, two copies of each with two different chains, heavy (H) and light (L). The heavy chain has four domains: one variable (V_H) followed by three constant domains (C_{H1}, C_{H2}, and C_{H3}). The heavy chain also contains the hinge region. The light chain has one variable (V_L) and one constant domain (C_L). The N-terminal variable domains of both chains have hypervariable regions, also known as CDRs, interspersed with framework regions. Disulfide bonds connect the four chains. N-linked glycosylation site is found at the C_{H2} domain.

Table 3.1 Different numbering schemes for antibody variable domain sequence annotation.

Loop	Kabat	Chothia	Contact	AbNum	IMGT
L1	24–34	24–34	30–36	24–34	27–38
H1	31–35B	26–32	30–35	26–35	
L2	50–56	50–56	46–55	50–56	56–65
H2	50–65	52–56	47–58	50–58	
L3	89–97	89–97	89–96	89–97	105–117
H3	95–102	95–102	93–101	95–102	

was devised for all Ig variable and T-cell receptor variable domains. In the AHo numbering scheme, insertions and deletions were placed symmetrically on the turn and loop positions to accommodate them without distorting the surrounding structure. To facilitate the comparison of V-REGION sequences of B- and T-cell receptors from all species, a unique numbering has been implemented in the IMGT (ImMunoGeneTics) database (http://www.imgt.org). The IMGT numbering system provided by Lefranc and colleagues has many advantages, as conserved amino acids always have the same positions, and this unique numbering has allowed the redefinition of the limits of the FR and CDR regions.

3.2.2
Antibody Isotypes

Humans produce five major classes or isotypes of antibodies: IgG, IgD, IgE, IgM, and IgA, which are determined by the heavy chain isotypes μ, δ, γ, α, and ε, respectively. In contrast, there are only two types of light chain isotypes, κ and λ, but there are no functional differences between them. Individual B cells express either κ or λ but never both. Each heavy chain isotype can combine with either of the light chain isotypes. The five antibody isotypes have different structures, properties, and functions (Figure 3.2, Table 3.2). Figure 3.2 shows the schematic representation of the polypeptide chain structures of all five antibody isotypes. Soluble secreted IgG, IgD, and IgE are monomeric, while IgM is a pentamer and IgA is either a monomer or dimer. In all five isotypes, light chains contain one variable (V_L) and one constant domain (C_{L1}), while heavy chains contains up to four constant domains ($C_{H1}-C_{H3}$ for IgA, IgD, and IgA; $C_{H1}-C_{H4}$ for IgE and IgM). Each isotype generally contains two identical light and heavy chains, with an additional polypeptide chain J serving as a linker in pentameric IgM and dimeric IgA.

IgG is the predominant isotype with four subclasses (IgG1, IgG2, IgG3, and IgG4). Among them, IgG1 is the most prevalent, and IgG4 the least. Collectively, IgG makes up the greatest proportion of the antibody form in the serum, and

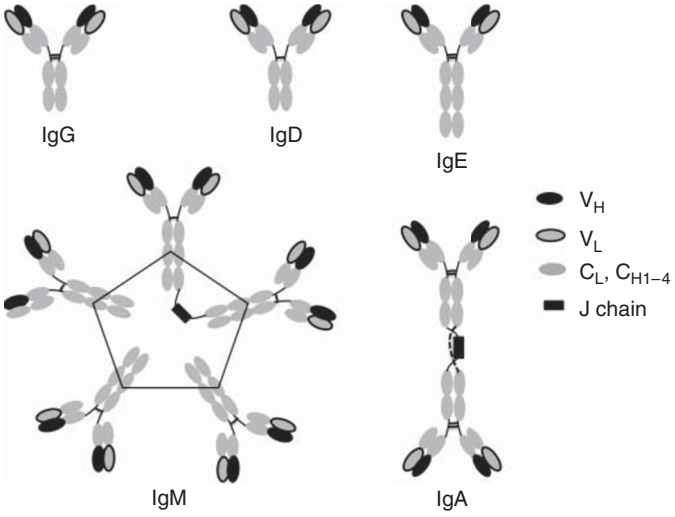

Figure 3.2 Five major isotypes of antibodies. All subtypes contain light and heavy chains and each with one variable domain (V_H) and different number of constant domains ($C_{H1}-C_{H4}$). Generally, two heavy (H) and two light (L) chains form a monomer Ig module as in IgG, IgD, and IgE. IgM is a pentamer with four constant domains, as in IgE, and an additional polypeptide, the J chain, connecting two monomer Ig units. The J chain is also found in IgA linking two units to form the IgA dimer. Carbohydrate moieties attached to N-glycosylation site of the penultimate constant domains are not shown in this figure.

Table 3.2 Properties and function of five different antibody isotypes.

Ig	Molecular weight (kDa)	Structure/ valency	Inter-heavy chain S–S bonds	Carbohydrate (%)	N-/O-glycosylation sites	Serum (%)	Serum half-life (days)	Biological responses/ functional properties
IgG1	150	2	2	2–3	1/0	45–53	21–24	Secondary response to pathogens, placental transfer, binding to macrophages, and other phagocytes by FcγR binding
IgG2	150	2	4	2–3	1/0	11–15	21–24	
IgG3	160	2	11	2–3	2/0	3–6	7–8	
IgG4	150	2	2	2–3	1/0	1–4	21–24	
IgD	175	2	1	9–14	3/7	0.2	2–8	Mature B-cell marker
IgE	190	2	1	12–13	7/0	0.004	1–5	Allergy reactions, binds to FcεR
IgM	950	10	1	10–12	5/0	10	5–10	Primary response, weak binding to pIgR
IgA1	150	2 or 4	2	7–11	2/8	11–14	5–7	Secretory antibodies, binding to pIgR
IgA2	150	2 or 4	2	7–11	4/0	1–4	4–6	

various IgG isotypes are able to elicit different effector functions and other biological activities including fragment crystallizable receptor (FcR) binding, complement activation and serum half-life, and, importantly, placental transfer (see Table 3.2). The membrane-bound IgD is found along with IgM on all mature, naïve B cells and has a relatively shorter half-life. IgE is found in the lowest serum concentration and has the shortest half-life. IgE binds to FcεRI with a high affinity, and cross-linking of IgE on mast cell surfaces by antigens leads to allergic reactions. IgM is the first antibody to be formed following antigen stimulation. IgM is highly active in agglutination and activating the classical pathway of complement. The IgM pentamer is connected by disulfide bonds through the C_{H4} domains, and two of the monomer units are connected by a 15 kDa polypeptide chain J. The J chain is also found in IgA. IgA is the dominant antibody isotype in mucosal secretions, breast milk, and gastrointestinal secretions. IgA has two subclasses, IgA1 and IgA2; IgA1 dominates in the serum, and IgA2 in mucosal secretion. Some important physical properties for antibody isotypes and subclasses, including the number of disulfide bonds, carbohydrate content, and glycosylation sites, along with their biological activities, are given in Table 3.2.

3.2.3 Antibody Fragments

A typical human IgG consists of two identical pairs of protein chains, each pair containing a light chain (~24 kDa) and a heavy chain (~55 kDa). The two pairs of light and heavy chains create three structural fragments containing two fragment antigen-binding (Fab) units and one Fc unit, which are linked by a flexible "hinge" region of heavy chains that can be readily cleaved into Fab and Fc fragments by proteases. Recent electron tomography analysis has shown 120 unique IgG1 antibody conformations at intermediate resolution (~1–3 nm), confirming the extraordinary conformational flexibility and dynamics for this class of antibody [2]. Because of the highly flexible nature of the whole IgG, only a very few X-ray structures of intact IgGs have been reported [3]. On the other hand, more than 1000 crystal structures of antibody fragments including Fabs, single-chain variable fragments (scFvs) and Fc, as well as several of them in complexes with antigens, are available in the Protein Data Bank (PDB) [4]. A ribbon diagram of the X-ray crystal structure of a complete human IgG1 [3c] is given in Figure 3.3a. As shown, all antibody domains have a characteristic Ig fold [5], which consists of a pair of β sheets connected by a disulfide bond and loop regions. The IgG tertiary structure shows that individual Ig domains exhibit strong associations through extensive van der Waals contacts between V_H and V_L, C_{L1} and C_{H1}, and C_{H3} and C_{H3} domains, while the relatively weak interactions between C_{H2} and C_{H2} domains occur mainly through carbohydrates. The two antibody fragments of the IgG, namely Fab and Fc, are produced by enzymatic cleavages. The Fab comprises one complete light chain (V_L and C_{L1}) linked by a disulfide bond to a fragment of the heavy chain (V_H and C_{H1}). The IgG Fc is a dimer consisting of C_{H2} and C_{H3} of both heavy chains which are connected by interchain disulfide bonds. The antibody fragments

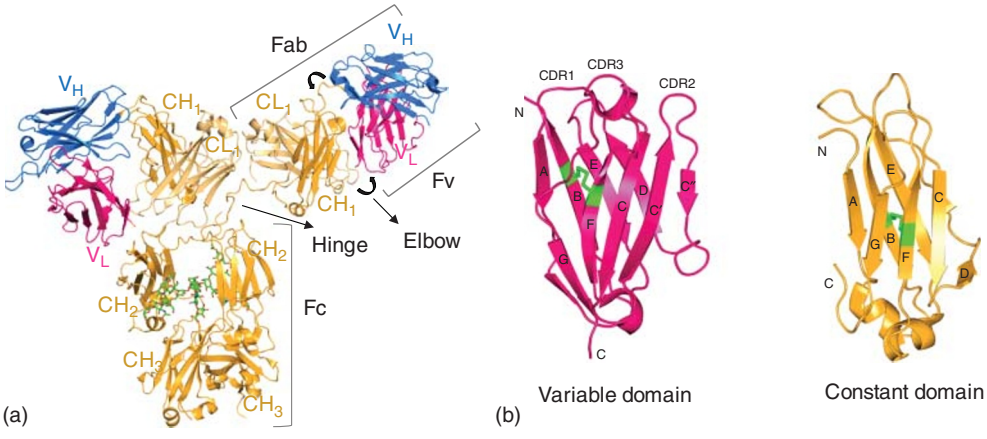

Figure 3.3 (a) Cartoon ribbon diagram of an intact human antibody IgG1. The variable domains of heavy (V_H) and light (V_L) chains at the N-terminal are shown blue and purple, respectively. The constant domains of light (C_{L1}) and heavy (C_{H1}, C_{H2}, and C_{H3}) chains at the C-terminal are shown in orange. IgG contains the two Fab units that are linked to Fc. The carbohydrates (in green sticks) are found between the C_{H2} domains of the IgG. The flexibility of IgG is achieved through the hinge regions that connect the Fab to the Fc and the elbow regions along the peptides linking V_H to C_{H1} and V_L to C_{L1}, as shown. (b) Structural comparison of variable and constant domains of IgG. Both domains share similar topology comprising of a pair of β sheets in which the β strands are labeled with A–G. The variable domain has CDR loops, whereas those loops in the constant domains are shorter and invariable. The CDR1 connects the β strands B and C, while CDR3 connects the β strands F and G. The variable domain has two additional β strands, C′ and C″, that supports CDR2. The conserved intradomain disulfide bond is shown in green.

produced by different biochemical methods such as proteolysis, denaturation, and recombination are described in Table 3.3. Detailed structural information of antibody fragments are given in the following sections.

3.2.3.1 *V* and *C* Domains

Variable (V) and constant (C) domains share a common Ig fold with characteristic sequence and structural properties. The IMGT databases and tools provide a highly standardized analysis of antibody V and C domains [6]. Figure 3.3b shows topological comparison between the V and C domain structures. At the sequence level, the V and C domains are quite dissimilar except for the conserved cysteine residues between the B and F β strands that connect the two β sheets (shown as green sticks in Figure 3.3b). The residues in the V domain are highly variable in the CDRs and less variable in the FRs. The residues throughout in the C domains are conserved. At the structural level, both V and C domains are very similar but with two major differences – the V domain has nine β strands, and the C domain has seven. Although the numbering scheme runs through A to G, the additional two β strands between C and D in the V domain are designated with C′ and C″. The other major difference is within the loop regions; the V domain has relatively

Table 3.3 Definitions of different antibody fragments and domains.

Abbreviations	Details of antibody fragments
Fab	The antigen binding fragment (~50 kDa) consists of the entire light chain (V_L and C_{L1}) and heavy chain's variable domain with its first constant domain ($V_H - C_{H1}$), linked through a disulfide bond
F(ab)′2	The divalent Fab fragment (110 kDa) is generated by pepsin digestion of whole IgG to remove most of the Fc region while keeping intact some of the hinge region. The fragment has two Fab portions linked together by disulfide bonds
Fab′	The monovalent Fab fragment is deduced by a mild digestion of from F(ab)′2 and it is similar to Fab but have some part of the hinge region
Fv	The variable fragment (~25 kDa) of Fab′ portion that consists of $V_H - V_L$ dimer
scFv	The single-chain variable fragment is a recombinant protein of the variable regions of heavy (V_H) and light (V_L) chains, which is generally connected with a short linker
Fb	The constant partition of the Fab ($C_{H1} - C_L$) dimer
Fc	The fragment crystallizable (50 kDa) is a dimer consisting of constant domains of the two heavy chains, except their first constant domain
Fd	The heavy chain portion of the Fab ($V_H - C_{H1}$) as formed from the denaturation of Fab
pFc′	The C_{H3} dimer unit
mFc	Monomeric Fc
mC_{H2}	Isolated, unglycosylated soluble C_{H2} domain
mC_{H3}	Monomeric C_{H3} domain

long loops, particularly the CDRs with variable sequences, while the C domain has shorter invariant loops. In the V domain, CDR1 connects the two β sheets through strands B and C, CDR2 connects the C′ and C″, and CDR3 is generally the longest loop that connects the last two strands F and G. A large portion of residues in the CDRs are surface-exposed and interact with antigens [7]. Most residues in FRs contribute to interdomain interactions, forming the hydrophobic core at the $V_H - V_L$ interface [8]. Sometimes, certain residues in the FRs can contribute to antigen binding by being directly involved in the antigen interactions, or indirectly by influencing the CDR conformations [9]. In the C domain, where residues in the loops that are structural homologs to the CDRs of the V domain do not have any known function but could involve in and affect the effector functions of antibodies and/or stabilize the overall structure of antibody molecule. Both the V and C domains have a conserved hydrophobic core structure, which is stabilized by a triad with two cysteine residues forming a disulfide bond and a neighboring tryptophan residue that are conserved in the core [10]. The other outer parts of the V and C domains are mainly covered with polar and charged residues at the surface. Interestingly, there are sequence and structural properties naturally found in β-sheet proteins including the V and C domains of antibodies that decrease or eliminate aggregation [11]. However, aggregation often turns out to be a major

bottleneck for the development of antibody-based biotherapeutics, and effective strategies are needed to improve aggregation resistance [12].

3.2.3.2 Fab and Fv

Fab comprises one complete light chain (V_L and C_{L1}) and a fragment of the heavy chain with its variable and first constant domains (V_H and C_{H1}, respectively), which are connected through an interchain disulfide bond. Figure 3.4a shows an overall Fab structure depicting the arrangement of the four domains and location of CDRs from light and heavy chains. In the Fab, the V_H domain closely associates with the $V_{L,}$ and the C_{L1} domain with C_{H1}, and these strong domain interactions create compact globular modules Fv and Fb, respectively. The two globular modules are connected by a pair of short segments of polypeptide chains, namely the switch residues, between variable and constant domains of the both light and heavy chains. The relative orientation of the V_H-V_L and C_L-C_{H1} modules is described by the elbow angle between those V_H-V_L and C_L-C_{H1} pseudo-dyad axes [13]. The elbow angle of Fab is one of the structural parameters routinely calculated to assess the relative disposition of Fv and Fb. A survey of the calculated elbow angles for 365 different Fab fragments revealed a wide range of values with very large elbow angles over 195° for Fabs with λ light chains as compared to their κ light chain counterparts [14]. This suggested that the elbow angles could be influenced by their light chain classes. The changes in elbow angles between liganded and unliganded Fabs might indicate the response to antigen binding or simply the flexibility of the switch region. In the case of Fab structures in isolation

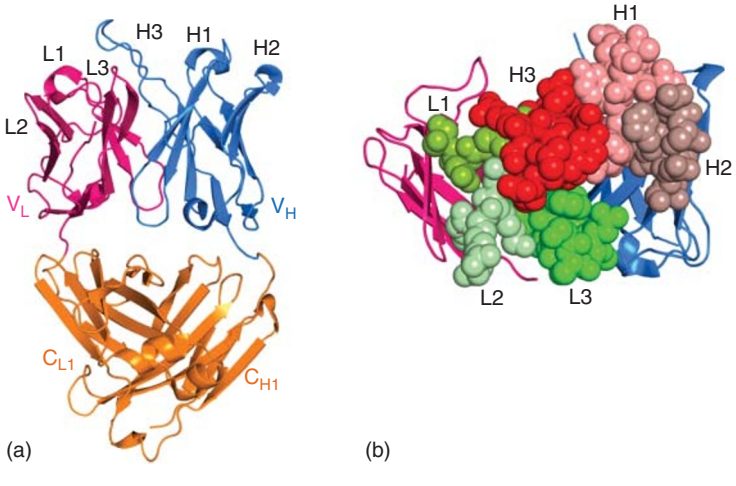

Figure 3.4 (a) Crystal structure of a Fab depicted in ribbons. The variable domains of heavy (V_H) and light (V_L) chains at the N-terminal are shown blue and purple, respectively, while the constant domains are in orange. (b) Close-up view showing the antigen-binding site as formed by the six CDR loops. The CDR H3 is located centrally and forms the primary antigen-binding loop of all CDRs. Note that CDR H3 makes significant interactions with other CDRs.

and in a complex with SARS-CoV glycoprotein receptor binding domain (RBD), a significant change in elbow angle was observed; the ligand-free Fab had almost a straight elbow angle (173.5°), whereas the antigen-bound Fab was markedly bent and in the lowest range for a human antibody (125.1°) [15].

The six CDRs extending from both of the variable domains of heavy and light chains at the edges of β sheets provide an ideal location on the top of Fab to reach antigens (Figure 3.4b). Note that the heavy chain CDR, H3, is located centrally and supported by other CDRs. This facilitates the CDR-H3, which is generally the longest CDR loop, to make significant contact with epitopes. Also, this type of arrangement of CDRs with different lengths and sequences contributes to the structural diversity of paratopes or antigen binding sites [16]. Fvs, most of the time, have the same three-dimensional structures and binding characteristics as Fabs as long as those V_H and V_L chains of the Fv fragments are held together by appropriate peptide linkers. Crystal structures of numerous Fab and Fv molecules that are specific to different antigens have been determined, both alone and in complexes with antigens [17]. Detailed analyses of those structures of Fabs and Fvs are useful in understanding the structure–function relationships and develop methods for engineering of functional antibody fragments.

3.2.3.3 Fc Structure and Its Fragments

Fc fragments are generated entirely from the heavy chain constant domains of an antibody, except C_{H1} that resides in the Fab. Fc contains two or three C domains depending on the isotype (Figure 3.2). The Fc region of an antibody mediates its serum half-life and effector functions, such as complement-dependent cytotoxicity (CDC), antibody-dependent cellular cytotoxicity (ADCC), and antibody-dependent cell phagocytosis (ADCP). The first X-ray crystal structure of the human IgG1 Fc region was determined about 40 years ago [18]. Currently, more than 100 crystal structures of Fc and its fragments, as well as in various glycosylated forms and Fc complexes, are known [4]. Various Fc-based fragments have been generated using structural knowledge available from the crystal structures (Table 3.3). Figure 3.5 shows the crystal structures of intact IgG Fc and its various fragments. In the IgG Fc structure, the two constant domains, C_{H2} and C_{H3}, of each of the heavy chains are packed together, and interactions between C_{H2} and C_{H2} and C_{H3} and C_{H3} domains occur. The $C_{H3}-C_{H3}$ interaction resembles that of the constant domains of the Fab, $C_{H1}-C_{L1}$, in exhibiting tight association via noncovalent hydrophobic interactions but in different extents. A structure-guided design with the phage display library approach has been recently used to enhance heterodimerization between the C_{H1} and C_{L1} domains of a novel CD4–antibody fusion protein against HIV-1 [19]. The stabilized $C_{H1}-C_{L1}$ domains could be useful as a new heterodimerization scaffold with favorable pharmacokinetics for the generation of bispecific and multispecific antibodies for therapeutic applications.

Previously, a biochemical and mutational study of single-chain C_{H3} dimer had revealed interface residues between the C_{H3} domains contributing to the stability of C_{H3} domain homodimers [20]. In contrast, there are no protein–protein

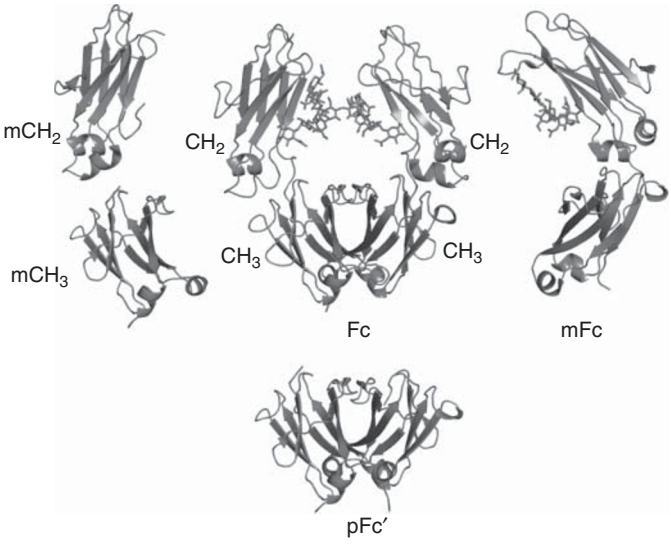

Figure 3.5 Ribbon diagrams of different IgG Fc fragments. X-ray crystal structures of human IgG Fc fragment (PDB 1H3X), monomeric Fc (PDB 4J12), monomeric and unglycosylated CH2 domain (PDB 3DJ9), monomeric CH3 (PDB 4B53), and CH3 dimer (PDB 5HSF).

interactions between C_{H2} domains, as carbohydrates are attached at the interface connecting the C_{H2} domains together. The absence of protein–protein interactions between the two C_{H2} domains enabled the crystal structure of unglycosylated monomeric C_{H2} domain without any mutation at the interface [21], which has encouraged C_{H2} scaffold development as the smallest Ig-based independently folded unit [22]. Other functional Fc-based fragments, monomeric fragment crystallizable (mFc) [23] and monomeric C_{H3} (mC_{H3}) [24], using structure-based rational approach and phage display, as well as glycan-modified mFc (glycan engineering at the $C_{H3}-C_{H3}$ interface of IgG Fc) [25], were successfully generated as potential scaffolds. Currently, the use of antibody Fc and its domains has reached a new dimension in the development of scaffolds for antigen binding as well as bispecific binding of both antigen and cellular receptors [26]. Also, a simple structure-based physical concept of electrostatic steering effect was cleverly engineered through the antibody Fc region with selected mutations in the C_{H3} domain so that the engineered Fc proteins preferentially form heterodimers for applications in bispecific molecules and monovalent IgG [27].

3.3 Antibody Diversity

Antibody diversity is primarily generated through the recombination of multiple gene segments from different locations of chromosomes encoding the

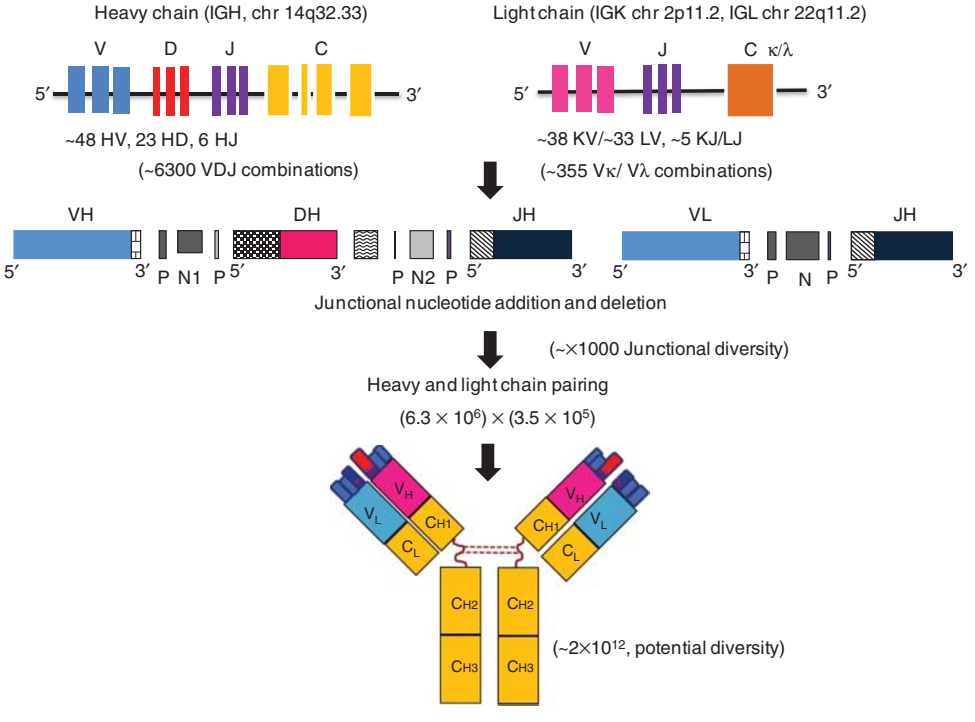

Figure 3.6 Antibody diversity generated by genetic recombination, junctional mechanisms, and heavy/light chain pairing. Theoretical diversity could be estimated as of up to 10^{12} different antibodies based on the number of germline gene segments contributing to functional antibodies and junctional diversity.

variable heavy and light regions and those of constant regions followed by somatic mutation [28]. The genetic assortment of a large number of these gene segments is also enhanced by random addition and or deletion of nucleotides at their junctions [29]. As shown in Figure 3.6, antibody diversity is potentially generated by 6300 VDJ and 355 V_L (V_κ and V_λ) combinations with a limited junctional diversity of 10^3, which can generate about 2 billion antibodies upon all possible $V_H - V_L$ pairing of antibodies. However, the real number of antibody diversity could be still many orders of magnitude higher than this estimate because of the somatic hypermutation in mature B cells and other secondary mechanism of diversification such as V(DD)J recombination or D–D fusion, SHM-associated insertions and deletions, affinity maturation, antigen contacts to FR regions of the antibodies [30], and receptor editing [31]. Further diversity can arise from post-translational modifications, such as glycosylation and tyrosine sulfation [32].

Here we describe how antibody diversity is generated by the rearrangement of germline segments of heavy and light chains, which is augmented with junctional diversity and $V_H - V_L$ pairing.

3.3.1 VDJ/VJ Recombination

Antibody gene rearrangements occur in the early stages of B-cell development prior to antigen exposure. The heavy chain gene segments are located in chromosome 14. Gene segments encoding antibody light chain κ are found on chromosome 2, while those encoding light chain λ are found on chromosome 22. According to the IMGT database, in the heavy chain locus 14q32.33, there are up to 129 IGHV gene segments, of which 43–48 are able to produce functional arrangements; out of 27 IGHD gene segments, 23 are functional; and out of 9 IGHJ genes, 5 are functional. To generate a productive heavy variable chain (V_H), one of the 48 functional IGHV genes is combined with one of several IGHD and IGJH genes. Therefore, there are about 6300 VDJ combinations that could form productive heavy chains (Figure 3.6). For light chain rearrangements, IGKV locus 2p.11.2 encodes 76 IGKV genes (38 functional) and 5 IGKJ genes (5 functional); IGLV locus 22q11.2 encodes 74 IGLV genes (33 functional) and up to 11 IGLJ (5 functional). These give rise to about 355 unique V_κ/V_λ combinations. During these processes, recombinase enzymes remove introns and some exons from the DNA and splice segments into functional antibody V_H, V_κ, and V_λ genes.

3.3.2 Junctional Diversity

Junctional diversity results from the imprecise joining of gene segments and from the addition of nucleotides randomly to the DNA sequence by TdT enzyme at splice sites during antibody gene recombination process. The V–D–J junction diversity results from the adding palindromic "P" nucleotides (P region), exonuclease trimming, and addition of "N" nucleotides (N region) at the V → D (N1) and D → J (N2) junctions (Figure 3.6). Next-generation sequencing (NGS) of antibodies from naïve cord blood libraries has shown that at the V–D–J junction, P addition was observed at the 3′V, 5′D, and 5′J ends but no P region was found at the 3′D, whereas exonuclease trimming was observed at the 3′V, 5′D, 3′D, and 5′J ends [33]. In the case of antibody light chains, N segment insertion as well as the addition and deletion of nucleotides at the V–J junction were also observed [34]. Thus, the VDJ/VJ junctions are imprecise and able to generate much additional variability in the V-region sequences. It should be noted that, as the junctional diversity affects predominantly CDR3, it is more significant. However, the junctional changes are often difficult to quantify since they can also result in nonproductive rearrangements [33].

3.3.3 CDR H3 Diversity

The rearranged CDR3 sequences, defined by positions 105–117 according to the IMGT unique numbering system, result from the V–D–J/V–J rearrangements

and largely determine the diversity of functional V_H and V_L repertoires. For heavy chains, CDR3 diversity mostly comes from the IGHD genes, as they constitute the major part of the CDR3 sequences. The reading frame usage of IGDH genes is also an important factor in determining the repertoire diversity [35]. Of the six reading frames of IGHD genes, certain reading frames are restricted or preferred, based on the amino acid contents [36]. In humans, D–D fusion is found in approximately 5% of antibodies and can contribute more reading frame choices [37]. The major mechanism accounting for the unusually long CDR3 loops found in some heavy chains could be the usage of D–D fusion. By creating extra-long CDR3s and unusual amino acid combinations, these D–D fusions add further to the diversity of the antibody repertoire. Moreover, addition and/or deletion of nucleotides at the junctions increase the diversity in lengths and amino acid compositions of CDR3. The length of the heavy chain CDR3 ranges from 4 to 34 amino acids as seen in the 454 sequencing analysis of IgM repertoire of normal individuals [38]. Previously, statistical analysis of Ig V-REGION amino acid properties in the context of structural data including the heavy chain CDR3 had been described, showing conserved positions to have specific amino acids and/or shared properties, and differences [39].

3.3.4 Germline V_H–V_L Pairing

Pairing of V_H and V_L provides a significant amount of diversity following the recombination of VDJ and VJ gene segments, and leads to formation of germline antibodies. In a previous study [40], by selecting 545 human antibodies of known paired sequences from the KabatMan database and mapping them onto their corresponding germline sequences, the authors found that V_H–V_L pairing preferences exist for a small proportion of germline gene segments but more germline segments had no paring preferences. In Table 3.4, we analyze the V_H–V_L paring occurrences from the known crystal structures of 766 human antibodies, which shows a significant V_H–V_L paring preference for many of the antibodies. Out of 766 antibodies, 399 had the same germline segment pairing that occurred at least 5 times or more. Notably, the HV1-69 germline preferably paired with light chain germline KV3-20 and also frequently paired with other light chain germlines. The heavy chain HV3-66 germline had the highest frequency of pairing with light chain KV1-39 germline.

Recent advances in NGS technologies, particularly high-throughput, single-cell barcoding for heavy and light chains, have enabled us to characterize V_H–V_L paired antibody repertoires [41]. The information of cognate heavy and light chain pairs of expressed antibodies found in human has enormous applications – from developing antibody-based scaffolds to vaccine applications. For example, an interesting approach identified specific amino acid positions that clustered in the antigen-binding site of both heavy and light chains. When those positions

Table 3.4 Germline V_H-V_L pairing occurrences as observed in 766 human antibodies as derived from the Protein Data Bank.

HV	LV/KV	Occurrence[a)]	HV	LV/KV	Occurrence[a)]
HV3-66	KV1-39	62	**HV1-69**	**LV2-14**	**7**
HV2-5	KV1-13	41	**HV1-69**	**KV1-39**	**6**
HV1-69	**KV3-20**	**29**	HV3-66	KV1-5	6
HV1-69	**KV3-D-15**	**23**	HV3-66	KV1-NL1	6
HV3-21	KV1-5	15	HV3-73	KV2-28	6
HV3-30	KV3-11	13	HV4-b	KV3-D-15	6
HV1-18	KV3-20	13	HV1-2	KV3-NL5	6
HV3-23	KV1-39	11	HV4-b	LV2-8	6
HV1-69	**LV1-44**	**11**	HV4-59	LV3-1	6
HV3-30	LV2-14	10	HV5-51	LV3-1	6
HV1-69	**KV1-5**	**9**	HV4-59	LV3-21	6
HV1-3	KV3-20	9	HV3-23	KV1-13	5
HV3-74	KV1-39	8	HV3-66	KV1-13	5
HV1-2	LV3-21	8	**HV1-69**	**KV1-33**	**5**
HV1-46	KV1-33	7	HV3-23	KV1-33	5
HV3-30-3	KV1-33	7	HV5-a	KV1-39	5
HV1-2	KV3-20	7	HV1-f	KV1-D-12	5
HV1-69	**LV1-51**	**7**	HV3-23	LV2-14	5
HV3-30	LV1-51	7			

a) Only V_H-V_L pairs are that have occurrences ≥5, in which heavy chain germline HV1-69 appeared a total of 97 times and paired with different light chain counterparts, as shown in bold.

were mutated to aspartate or glutamate, they endowed superior biophysical properties onto domains derived from antibody comprising the human germline families V_{H3} and $V_{\kappa 1}$ [42], which is one of the most commonly occurring V_H-V_L pairs among known antibodies in the structural database (Table 3.4). Using an in-depth analysis of the human antibody repertoire, one could devise an effective strategy to discover antibody variable domains with favorable biophysical properties and high-affinity antigen binding and neutralizing ability [43]. It is reasonable to expect that high-frequency V_H-V_L pairs in individuals or those that are frequently shared between individuals, as they occur naturally, might be non-immunogenic and very stable with aggregation resistance and developability, rendering scaffolds suitable for antibody therapeutics. Also, antibody landscape dynamics with the V_H-V_L paired data at different time points and from different vaccinees can be used to measure vaccine immunogenicity and identify antibody clonal families with shared rearrangements as correlates of a vaccine response [44]. As antibodies from clonal populations are enriched for virus binding and neutralization [44a], V_H-V_L paired information at the repertoire level provides potential insights for therapeutic antibody discovery.

3.4 Canonical Structures of CDR Loops

In their earlier study, Chothia and Lesk found that most of the CDR loops have one of a small discrete set of main-chain conformations, which they called "canonical structures" [45]. Generally, similar CDR amino acid lengths and conserved residues at specific sites lead to recurring main-chain conformations for five of the CDR loops (H1, H2, of V_H and L1, L2, L3 of V_L). In further studies, more canonical structures for the five CDR loops were found [46], and antigen contacts made by different canonical loops were analyzed [47]. Recently, a clustering method was developed using a dataset of more than 300 nonredundant antibody structures to classify CDR loop conformations, which resulted in 28 CDR-length combinations for L1, L2, L3, H1, and H2 and 72 clusters representing unique canonical conformations [48]. Table 3.5 shows these different cluster types along with sequence logo for the most common CDR cluster. This method is implemented in a database of antibody CDR structural classifications called PyIgClassify [49].

The CDR loops have only a limited range of conformations except for the heavy chain CDR3. The CDR H3 loops are exceptionally long and diverse because of several genetic mechanisms including V/D/J gene recombination, P, N-nucleotide addition and deletion, and different D gene reading frame usages that contribute to much more variability in loop lengths and sequences. Despite the diversity of CDR H3 loops, attempts have been made to classify them to some extent with general conformational rules [50]. The canonical structures of five CDR loops showing the most common conformation for each loop and the structure of CDR H3 loop with general conformational features are depicted in Figure 3.7. The loop lengths of CDR canonical structures are specified according to the recent clustering method [48].

3.4.1 CDR Loop Conformations

The clustering method [48] classifies canonical CDR loops into three different categories, Types I, II, and III, based on the potential for predicting the CDR conformation from its sequence. For Type I, a particular CDR length combination has one common conformation for the majority of structures, whereas Type II, although predictable, has multiple canonical conformations for any particular CDR length. For Type III, the structure prediction of CDR loops is difficult mainly due to statistical limit in the clustering itself, as those CDR structures occur in low abundance and because of the variety in certain structural contexts such as interactions between CDRs, frameworks, or antigens.

3.4.1.1 CDR L1

The CDR L1 loop bridges the two β sheets and is found across the top of the V_L domain. The CDR L1 length ranges from 10 to 17 residues, while the majority

Table 3.5 Canonical structures for CDRs H1, H2, L1, L2, and L3 are classified into different CDR clusters as implemented in PyIgClassify.

CDRs	Number of clusters	CDR cluster types with number of unique sequences	Sequence logo for the most common CDR cluster
L1	16	L1-10-1 (36), L1-10-2 (4), L1-11-1 (179), L1-11-2 (88), L1-11-3 (24), L1-12-1 (29), L1-12-2 (12), L1-12-3 (3), L1-13-1 (47), L1-13-2 (8), L1-14-1 (15), L1-14-2 (19), L1-15-1 (52), L1-15-2 (5), L1-16-1 (98), L1-17-1 (57)	
L2	7	L2-8-1 (441), L2-8-2 (58), L2-8-3 (5), L2-8-4 (29), L2-8-5 (15), L2-12-1 (3), L2-12-2 (2)	
L3	17	L3-7-1 (2), L3-8-1 (26), L3-8-2 (6), L3-8-cis6-1 (2), L3-9-1 (25), L3-9-2 (50), L3-9-cis6-1 (1), L3-9-cis7-1 (442), L3-9-cis7-2 (16), L3-9-cis7-3 (9), L3-10-1 (17), L3-10-cis7,8-1 (8), L3-10-cis8-1 (1), L3-11-1 (55), L3-11-cis7-1 (2), L3-12-1 (3), L3-13-1 (1)	
H1	17	H1-10-1 (4), H1-12-1 (2), H1-13-1 (659), H1-13-2 (15), H1-13-3 (23), H1-13-4 (27), H1-13-5 (14), H1-13-6 (9), H1-13-7 (7), H1-13-8 (2), H1-13-9 (3), H1-13-10 (4), H1-13-11 (2), H1-13-cis9-1 (1), H1-14-1 (26), H1-15-1 (31), H1-16-1 (1)	
H2	15	H2-8-1 (1), H2-9-1 (189), H2-9-2 (2), H2-9-3 (6), H2-10-1 (417), H2-10-2 (180), H2-10-3 (38), H2-10-4 (11), H2-10-5 (6), H2-10-6 (39), H2-10-7 (4), H2-10-8 (4), H2-10-9 (4), H2-12-1 (43), H2-15-1 (1)	

of loops are 11 or 16 residues long. The length variation involves the addition or deletion of residues at the site of residue 27 or after residue 30 in the Kabat numbering scheme [46]. Many of the CDR L1 loops are of Type I, suggesting that a single standard conformation of L1 is predominantly found [48]. Several of the CDR L1 loops also belong to Type II, having alternate conformations predictable by their CDR sequences. For example, L1-11 belongs to Type II category with three alternate conformations, and L1-11-1, L1-11-2, and L1-11-3, forming three distinct clusters and the sequence logo indicate amino acid distribution for the most common cluster L1-11-1 (Table 3.5). The L1-11-1 and L1-11-2 clusters share very similar amino acid distributions originating from V_κ chains, while the L1-11-3 sequences all come from V_λ chains. The structural difference between L1-11-1 and L1-11-2 clusters is due to a single amino acid difference in the framework at position 71, which packs against the loop. The position 71 is predominantly a Phe residue for L1-11-1 and Tyr residue for L1-11-2. The structural conformation of L1-11-1 is shown in Figure 3.7a. Although other

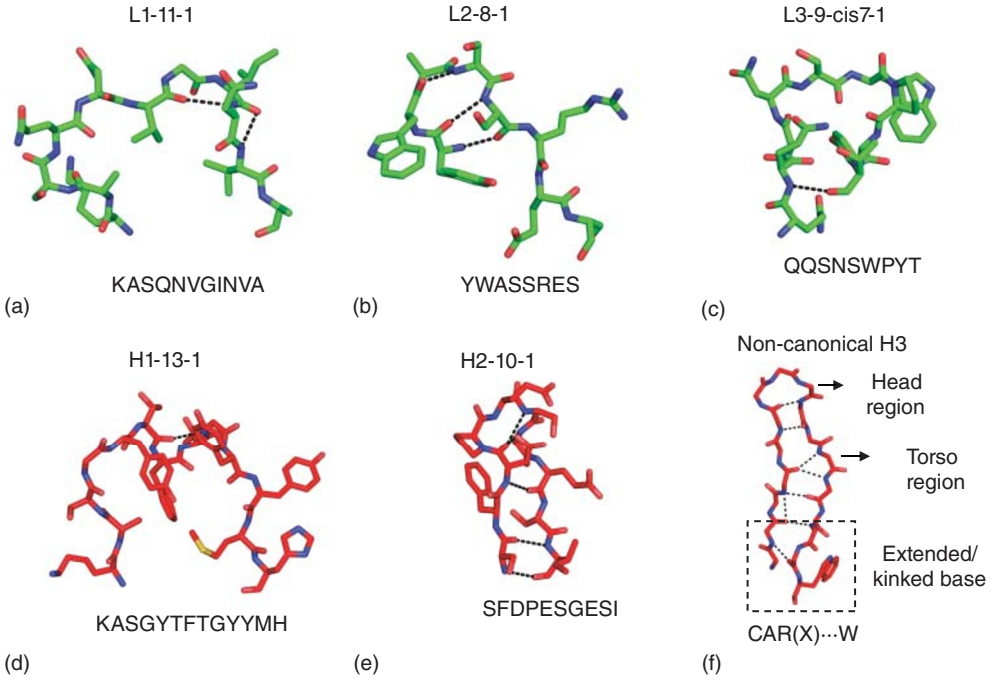

Figure 3.7 Five of the six CDR loops (L1, L2, L3, H1, and H2) with standard conformations, called canonical structures. The CDR H3 is highly variable in length, sequence, and structure, and therefore does not have any canonical structures. (a–e) The most abundant canonical structures for the five CDRs are shown. (f) For CDR H3, it is generally divided into three conformations – base, torso, and head (tip or crown region). The base region has always a kinked conformation or sometimes an extended conformation, and the torso region is either budged or non-bulged. The head region generally forms a classical β-turn motif or different conformations based on the torso region as well as its interactions with antigen and/or other CDRs.

L1 lengths with different canonical structures occur in low abundance, many of them have specific residues at certain positions or sequence motifs that are characteristic to pertinent clusters to which they belong [48].

3.4.1.2 CDR L2

CDR L2 is found in the hairpin loop that connects the C′ and C′′ strands. Almost all CDR L2 canonical structures comprise 8 amino acid residue length with a few exception having the length of 12 residues. Therefore, CDR L2-8 belongs to Type I, as it has mostly one conformation in the cluster (Table 3.5). The three residues at the tip of the L2 loop form a classic γ trun (Figure 3.7b).

3.4.1.3 CDR L3

The CDR L3 loop is at the hairpin loop connecting the F and G strands. The CDR L3 loop length ranges from 7 to 13 amino acids. The CDR L3 canonical structure

with the length 9 is the most commonly observed. The largest cluster of L3–9 among the CDR loops with the length 9 has a cis-proline at position 7 and designated as L3-9-cis7-1, whose structure is shown in Figure 3.7c. There are other L3 clusters with the length of 9 that contain cis7 as well as all-trans. So, L3-9 is Type II and has generally predictable structures; for example, L3-9 loops with Pro7 is most likely to be in the cluster L3-9-cis7-1. Other CDR L3 loops with different lengths such as 7, 12, and 13 rarely occur and they belong to Type III.

3.4.1.4 **CDR H1**

The CDR H1 loop appears across the top of V_H domain and connects the two β sheets. The CDR H1 loop lengths range from 12 to 16, and also include the length 10. The H1-13-1 cluster is the largest category and belongs to Type I as a single canonical conformation, as shown in Figure 3.7d, shared by 442 structures. The other CDR H1 loops have different amino acid lengths; most of them have fewer residues, and therefore those CDR loops are of Type III (Table 3.5).

3.4.1.5 **CDR H2**

The CDR H2 loop forms between the C′ and C″ strands. The CDR H2 loops come in different lengths including 8, 9, 10, 12, and 15 amino acid residues. However, H2-9 and H2-10 are commonly observed and have multiple clusters with different canonical conformations. The Type H2-10-1 is the most abundant, and its structure is shown in Figure 3.7e. There are many hydrogen bonds that cross-link the residues in the CDR H2 loops. The H2-10 loops are grouped into two larger clusters and seven smaller clusters (Table 3.5). For H2-10-1, there is a sequence pattern involving the Gly and Pro residues in the middle of the loop, and also there is a framework residue at position 71 which affects the conformation of CDR H2 loops [51]. The nature of the residue in that position determines the H2-10 cluster types [48]. The other H2-9 and H2-12 types have larger clusters with 189 and 43 structures, respectively, and belong to Type I (Table 3.5). H2-8 and H2-15 types have only one structure and are therefore of Type III.

3.4.1.6 **CDR-H3**

The CDR H3 region in the hairpin loop linking the F and G strands is far more variable in length and sequence than all other CDRs, and therefore there are no canonical conformations for the CDR H3 loops. Several studies have been carried out to help improve our understanding of the relationship between CDR H3 sequences and structures that could be helpfulin classifying and predicting their conformations [47, 50, 52]. The CDR H3 lengths in normal humans can be up to 30 residues or more, with the average length being about 14 [33, 38]. The CDR H3 loop regions are found in between Cys92 and Trp103, which are generally divided into three structural parts – the kinked or sometime extended base, the torso region, and the head, which is also referred as the tip or crown region (Figure 3.7f). The H3 rules were defined to identify the structure of CDR H3 from its sequence; this would enable the prediction of the base either as a kinked or an extended form and also the β-hairpin features of torso regions with and without

bulges [50a]. In another study, the conformations of head regions with respect to the corresponding torso regions were analyzed in detail [52b], which found many interactions including H3 and other framework residues of the heavy chain and interchain interactions with the light chain and or antigens that could determine the conformation of head regions. The H3 rules and other methods for the prediction of structural conformations for longer CDR H3 loops are not effective. However, systematic analysis based on large databases of antibody sequences and structures, including antigen-free and antigen-bound forms, and developing novel algorithms to detect sequence and structural motifs and antigen interaction sites of CDR H3 loops will help find new methods for defining canonical CDR H3 conformations. Such database searches and bioinformatics analysis methods for the CDR H3 sequences combined with structural modeling efforts will continue to be refined and will improve the predictability of CDR H3 conformations [53].

3.5 Crystal Structures of Antibody–Antigen Interactions

The crystal structures of several antibody–antigen complexes have now been solved at high resolution [17]. In earlier reviews, the structures of antibody–antigen complexes were analyzed and compared, which enabled us to gain insights into the structural basis of immune recognition [54]. Particularly, those studies explained how antigen-binding sites could be varied considerably in size, shape, and charge distribution in spite of the invariant antibody framework structures, and helped in the understanding antibody–antigen interactions, epitope mapping, and different structural mechanisms involving the CDR loop usages and conformational changes such as induced fit and elbow angle variations. Here, we discuss some recent examples of crystal structures of human antibodies in complexation with viral proteins, highlighting structural insights as relevant to the antibody-based protein therapeutics development.

3.5.1 Human Antibody Complexes with Viral Envelop Glycoproteins

Recent technological advances have led to the discovery of fully human antibodies, which resulted in many exciting new crystal structures of antibodies in complex with their antigens such as HIV-1, influenza, and other infectious diseases [55]. Figure 3.8 shows the complex structures of human antibodies in complex with different viral envelop glycoproteins from HIV-1 [56], SARS CoV [15], Hendra virus (HeV) [57], and MERS CoV [43a]. All these fully human antibodies were identified from nonimmune antibody phage display libraries and co-crystallized as Fabs with their cognate antigens. In the structure of Fab X5 in complex with gp120 and CD4 (Figure 3.8a), a large structural difference was observed for the CDR H3, with the maximum displacement of up to 17 Å, when the structures of free X5 [58] and bound X5 [56] were compared. The 22 amino acid residue long

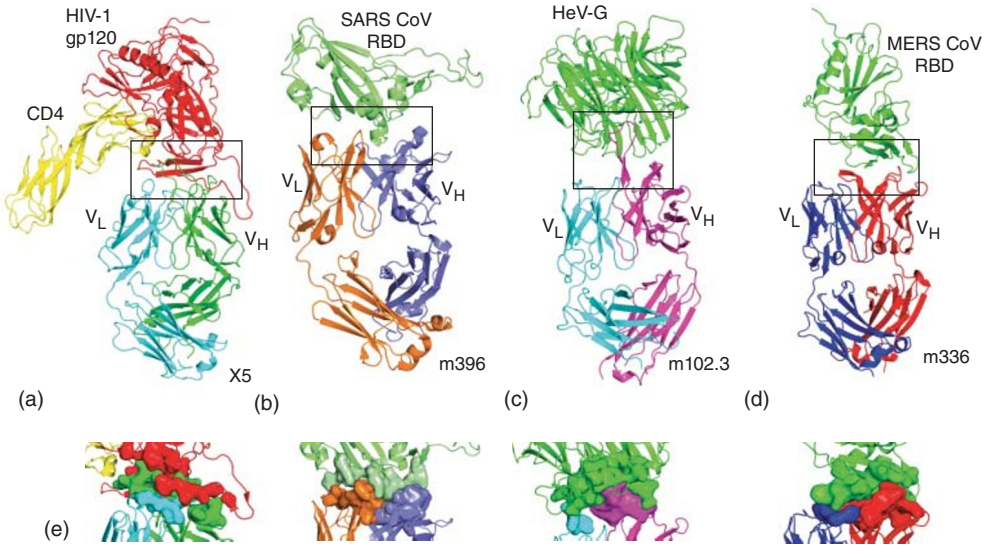

Figure 3.8 Co-crystal structures of fully human antibody fragments (Fabs) with viral envelope glycoproteins: (a) HIV-1 gp120-Fab X5 (PDB 2B4C), (b) SARS CoV RBD-Fab 396 (PDB 2DD8), (c) HeVG-Fab m102.3 (PDB XXX), and (d) MERS CoV RBD-Fab m336 (PDB 4XAK). The antibodies against different infectious diseases were generated by the selection of human antibody fragments from *in vitro* libraries. Structural analysis revealed neutralizing epitopes on the viral envelope glycoproteins or its receptor binding domains (RBDs) recognized by CDRs of those antibodies (paratopes) as indicated by rectangular boxes. (e) Close-up views of amino acid residues that make up the interacting paratope and epitope surfaces.

CDR H3 protruding from the antigen-combining site was found critical for the broadly cross-reactive HIV-1-neutralizing activity. The crystal structure of Fab m396 in complex with the SARS CoV RBD (Figure 3.8b) was the first human antibody structure solved [15] to provide a structural rationale for the function of a major determinant of SARS CoV immunogenicity, mechanisms of neutralization, and the development of therapeutics based on the m396 paratope. It was found that the ligand-free m396 Fab had almost a straight elbow angle (173.5°), whereas the RBD-bound Fab was highly bent (125.1°). The elbow angle for the RBD-bound Fab m396 is the lowest for a human antibody [14]. This significant conformational change supports an early notion that elbow bending in antibodies may occur upon antigen binding and that this could play a role as a signal transfer mechanism [59].

The crystal structure of HeV attachment G glycoprotein in complex with a potent cross-reactive neutralizing human monoclonal antibody, m102.3 [57], as shown in Figure 3.8c, provides structural insight into the mechanism of HeV neutralization; it is similar to that of its variant m102.4, which is used as a therapeutic agent [60]. The antibody–antigen interface involves Fab residues mostly located on CDR H3, and only three CDR H2 residues, one CDR H1 residue, and one CDR L1 residue. The 23-residue, protruding CDR H3 adopted a β-hairpin

conformation, which reached the hydrophobic HeV-G receptor-binding cavity. The CDR H3 of m102.3 binds to HeV-G utilizing a very high affinity lock-and-key mode without inducing conformational changes in HeV-G. Interestingly, the m102.3 antibody also has potent neutralization capacity against NiphaV-G with much higher affinity than for HeV-G. The success of m102.4 *in vivo* as an effective post-exposure treatment against henipavirus disease in two different well-characterized animal models [60b] has been published. These animal data, along with biochemical and structural findings explaining its superior cross-reactive neutralizing activity, have recently led to the approval for human use on compassionate basis for treating accidental exposure to HeV or NiV infections in Australia.

In the complex structure of a human anti-MERS-CoV antibody, m336, with MERS-CoV RBD (Figure 3.8d), the antibody heavy chain dominated in RBD binding and provided >85% binding surface. The CDR H3 has a noncanonical disulfide bond that forms between Cys98 and Cys100c (the Kabat numbering definition), which stabilizes the HCDR3 into a "twisted" loop conformation. The m336 antibody is almost germline with only one somatic mutation in the heavy chain. The complex crystal structure clearly demonstrates the structural role of the V(D)J recombination-generated junctional and HV1-69 allele-specific residues for achieving high affinity of binding at such low levels of somatic hypermutation. It is interesting to note that all the four antibodies described here and most of the other antiviral antibodies reported consistently utilize the human HV1-69 germline [38].

The antibody–antigen interaction sites comprising antibody paratopes and viral antigen epitopes are compared in Figure 3.8e. Generally, the antigen-binding region of antibodies forms a claw-shaped surface, except for m102.3, which grips the RBS, while certain residues from different CDR loops penetrate into the antigen surfaces. For m102.3, the CDR H3 protrudes well out of the antigen-combing site, penetrating into the antigen surface in a lock-and-key fashion. Structural studies of these antibody complexes are useful in mapping the epitopes and elucidating mechanisms of viral neutralization, particularly as compared to the corresponding receptor complexes, and have the potential for the development of antibody-based therapeutics.

3.6 Glycosylation

Glycosylation is an important component of the antibody molecule for its structural and functional requirements [61], and the type and extent of glycosylation varies for different Ig subtypes (Table 3.2). N-linked glycosylation occurs when carbohydrates are attached to asparagine residues, particularly at the conserved Asn297 of all mammalian IgGs and the homologous sites in other Ig subtypes including IgM, IgD, and IgE. O-linked glycans are most commonly attached to serine or threonine residues through the *N*-acetylgalactosamine

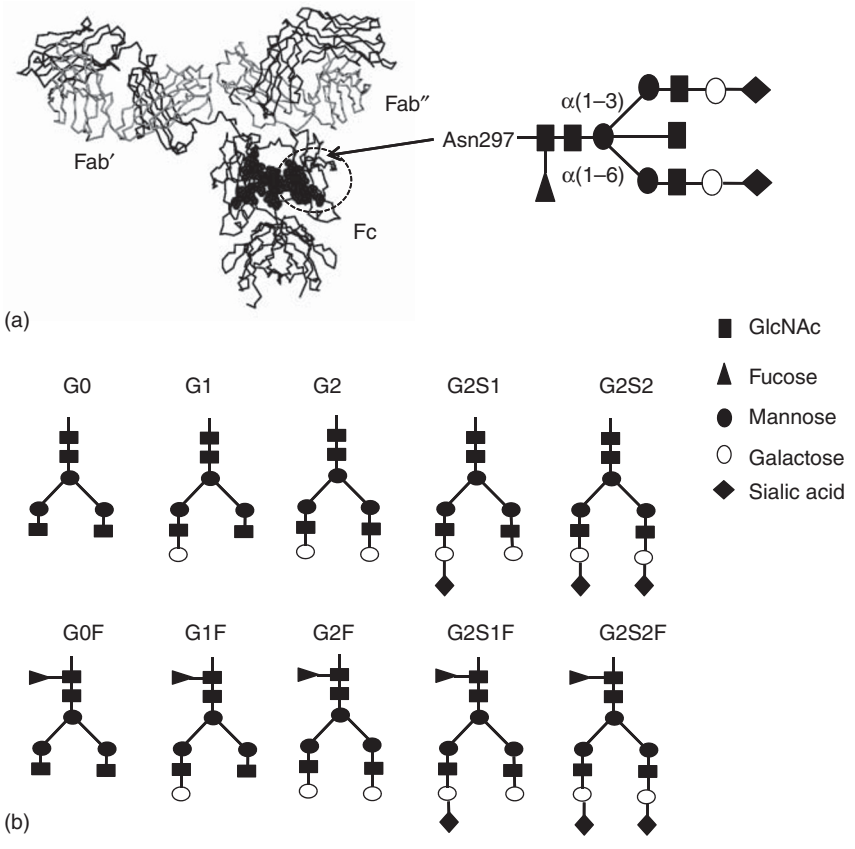

Figure 3.9 (a) View of human IgG with a representative oligosaccharide structure attached to the N-glycosylation site at Asn297. (b) Commonly observed oligosaccharides on recombinant IgGs produced by standard cell lines.

residue and appear only in two Ig subtypes, IgD and IgA1. Apart from the Fc region, there are N-glycosylation sites in the V regions of both heavy and light chains as reported in the germline sequences of the IMGT database [62]. The potential N-glycosylation sites of the germline sequences are found in the heavy chain V genes of IGHV1-8*01, IGHV1-38-4*01, IGHV3-16-*01, IGHV3-19*01, IGHV3-35-1*01, and IGHV5-10-1*01 and the light chain V genes of IGKV5-2*01, IGKV7-3*01, IGLV3-12*01, IGLV3-13*01, IGLV3-22*01, and IGLV3-32*01. These N-glycosylation sites predominantly appear in the CDR2 region and framework region 3 (FR3). Additional N-glycosylation sites in the V regions can be created during somatic hypermutation [63]. The effects of N-glycosylation at different sites of the V regions on antibody expression and affinity for antigen have been reported [64].

Previous studies on IgG Fc deglycosylation and truncated glycoforms have demonstrated the role of N-glycosylation in binding to Fcγ receptors.

N-deglycosylation of IgG1-Fc results in a loss of its capacity to bind FcγRs and failure to initiate effector functions [65]. *N*-Glycans of the Fc region are involved in important effector functions by interacting with Fcγ receptors. The oligosaccharide of polyclonal IgG, as attached through the ASN297 residue of the CH2 domain in Fc region, has a core heptasaccharide composed of N-acetylglucosamine (GlcNAc) and mannose, which could be extended by adding fucose to the primary N-GlcNAc residue, galactose to the outer arms of GlcNAc residues, and a bisecting GlcNAc. The galactosylated oligosaccharides may be further extended by the addition of sialic acid [66]. Figure 3.9a shows a typical core structure observed for human IgG, an octasaccharide Fuc-GlcNAc2-Man3-GlcNAc2 that is extended by the addition of galactose and sialic acid residues. It has been shown that different host cell lines such as Chinese hamster ovary (CHO) and murine myelomas could contribute to different glycoforms in recombinant antibodies. In Figure 3.9b, different IgG Fc glycans of fucosylated and unfucosylated core complexes of biantennary types are shown. While the fucosylated glycoforms G0F, G1F, and G2F are predominantly observed along with sialylated glycoforms, a small percentage of fucosylated glycans with or without a bisecting GlcNAc residue also exists [67]. The biologic significance of Ig glycosylation can be seen from studies demonstrating that IgG from patients with defective galactosylation is associated with rheumatoid arthritis, systemic lupus erythematosus, and Crohn's disease as well as a variety of chronic inflammatory diseases [68]. Antibody glycosylation, which impacts biological functions as well as the pharmacokinetic and pharmacodynamic properties of antibodies and Fc-fusion proteins, can be engineered and optimized using modern technologies for the development of therapeutics [67b].

3.7
Role of the Fc and Fc Receptors

The three-dimensional structure of human antibodies elegantly supports dual functionality, in which the two Fab units are involved in antigen binding while the Fc region interacts with effector molecules such as FcRs and component C1q. Much of the biological activity of the antibody is mediated through interactions between Fc and specific FcRs found on immune cells important for host defense. Previously, human antibody–FcR interactions were reviewed in the light of crystal structures of antibody–FcR complexes available at that time [69].

Different antibody isotypes exert different effector mechanisms through interaction between Fc regions and specific FcRs (Table 3.2). There are four FcRs that bind to the Fc regions of different antibody classes – FcγR to IgG, FcεR to IgE, FcαR to IgA, and FcRα/μ to both IgA and IgM. For the IgG class, ADCC and ADCP are governed by binding of the Fc region with a family of receptors called FcγRs [70]. In humans, the FcγR family includes FcγRI (CD64); FcγRII (CD32) and its isoforms FcγRIIa, FcγRIIb, and FcγRIIc; and FcγRIII (CD16) with its isoforms FcγRIIIa and FcγRIIIb [71]. These FcγRs differ from one another in their cellular

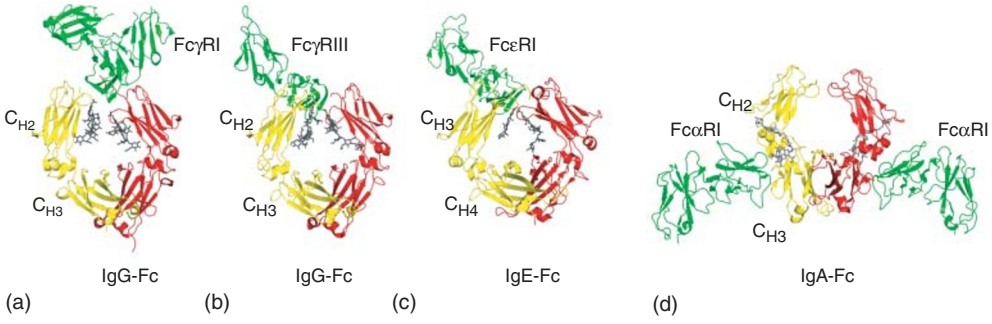

Figure 3.10 Comparison of the complex crystal structures of (a) IgG Fc–FcγRI (PDB 4ZNE), (b) IgG Fc–FcγRIII (PDB 1T83), (c) IgE Fc–FcεRI (PDB 1F6A), and (d) IgG Fc–FcαR1 (PDB 1OW0). The extracellular domains of the Fc receptors are shown in green, whereas one heavy chain of each Fc region is shown in yellow and the other in red. Carbohydrates are shown as gray sticks.

distribution, function, and binding to IgG Fc [72]. Also, there are other FcRs, two known forms of FcεR, as well as FcRα and FcRα/μ with one expressed form [72]. In the FcγRII subfamily, the phenotypically different receptors, namely FcγRIIa, FcγRIIIa, and FcγRIIIb, bind to different IgG subclasses [73]. Among different IgG subtypes, IgG1 and IgG3 bind to FcγRs with higher affinity than IgG2 and IgG4 [74]. Despite the similar profile of IgG1 and IgG3 binding to FcγRs, IgG3 antibodies do not mediate phagocytosis of antibody-coated red blood cells as much as IgG1 antibodies do. However, the ADCC activity of IgG3 is better than that of IgG1 [75], and Fc glycosylation could impact on the binding of FcRs resulting in different IgG-dependent effector functions, which determine therapeutic effectiveness [76].

Here, we highlight the molecular mechanisms of the Fc–FcR interactions using complex structures of two most prevalent antibody isotypes, IgG and IgA, and allergen-specific IgE. Figure 3.10 shows cartoon ribbon diagrams of known antibody Fc–FcR complexes, with glycans depicted as sticks.

3.7.1 IgG–FcγR Interaction

The crystal structure of a human IgG1 Fc fragment bound to wild-type human FcγRI was recently reported [77] and is shown in Figure 3.10a. The structural analysis with biochemical data showed that FcγRI D2 is the most important domain to confer high-affinity binding to Fc. The carbohydrates of IgG Fc are not involved in the FcγRI interactions. However, it is known that aglycosylated IgG molecules exhibit very weak to no binding to FcγRI. Therefore, Fc glycans could play an important indirect role in the Fc–FcγRI interaction by maintaining a favorable Fc conformation for engaging FcγRI. The structure of IgG Fc in complex with FcγRIIIB [78] is shown in Figure 3.10b. The interface of the complex consists of

the hinge loop between the D1 and D2 domain as well as the BC, C′E, and FG loops, and the C′ β strand of the receptor. On the Fc part of the complex, interactions with the receptor FcγRIIIB are dominated by residues Leu234–Pro238 of the lower hinge. Although FcγRIIIB does not make any direct contacts with carbohydrates attached to the conserved glycosylation residue Asn297 on the Fc, it probably stabilizes the conformation of the receptor-binding epitope on Fc. Structural comparison between Fc and FcγR complexes could provide insight into subtle differences at a molecular level accountable for differential recognition by high- and low-affinity receptors. For example, previous structural-based calculations using the Fc–FcγRIII complex have been informative for the successful design of Fc variants with optimized binding affinity [79].

3.7.2 IgE–FcεRI Interaction

The initiation of IgE-mediated allergic responses requires the binding of IgE antibody to its high-affinity receptor FcεRI. Figure 3.10c shows the complex crystal structure of FcεRI with the $C_{H3}-C_{H4}$ fragment of IgE3 [80]. The C_{H3} domains of the heavy chain dimer of IgE bind along one side of the D2 domain and at the top of the D1–D2 interface of FcεRI, defining two distinct binding sites. This includes the interactions of one of the C_{H3} domains with the C–C′ region of the receptor D2 domain centering around the receptor residue Y131 and second C_{H3} domain with the D1–D2 interface, and involves the cluster of four surface-exposed tryptophan residues W87, W110, W113, and W156 [80]. It was noted that IgG Fc–Fcγ receptor complexes are structurally similar to the IgE Fc–FcεRI complex, and they could potentially form analogous interactions. Compared with the IgE, binding of IgG to its FcRs is more sensitive in the presence of the N-linked carbohydrate of Fc [65a]. The IgE Fc–FcεRI complex provides a model for understanding the function of other IgE-mediated FcRs and could suggest new ways for intervention of diseases mediated by IgE.

3.7.3 IgA1–FcαRI Interaction

IgA antibodies are important components of the first line of human defense against pathogens, and the targets that are opsonized by them are removed by FcαR-mediated phagocytosis. FcαR shares a high structural similarity with FcγRII and FcεRI receptors in spite of low sequence similarities. Earlier studies had identified that FcαR bound at the interface of the C_{H2} and C_{H3} domains of IgA along one loop in the C_{H2} and in another loop in the C_{H3} [81]. These showed the binding mode of IgA with its receptor FcαR, which would not follow the pattern in either FcγRII and FcεRI receptors. Later, the crystal structure of IgA Fc in complex with FcαRI confirmed the binding sites and revealed molecular details of the FcαRI–IgA1 Fc interface [82]. As illustrated in Figure 3.10d, FcαRI has two Ig-like domains that are oriented at approximately right angles to each other. IgA

Fc generally resembles those of IgG and IgE, but has differently located interchain disulfide bonds and external rather than interdomain N-linked carbohydrates. The N-linked carbohydrates do not play any role in the IgA – FcαRI interaction, as opposed to the IgG – FcγR interactions, which can be modulated by carbohydrate composition of the Fc region. The detailed structural comparison of antibody and FcR interactions as well as their binding modes relevant for physiological mechanisms will be helpful to design effective antibody therapeutics. Recently, a tandem IgG1/IgA2 antibody format in the context of a trastuzumab variable domain has been designed, which showed enhanced ADCC and ADCP capabilities [83]. The IgG1/IgA2 tandem Fc format retained IgG1 FcγR binding and FcRn-mediated serum persistence which was augmented with myeloid-cell-mediated effector functions through FcαRI – IgA Fc interactions.

3.8 Conclusions and Outlook

Antibodies are nature's paradigm for protecting our bodies against invasion by pathogens and other foreign antigens. Their elegant structural features are capable of binding to virtually any target of different shapes, sizes, and properties. Harnessing this structural variety provides huge potential for the development of effective protein therapeutics. In this chapter, we have discussed the fundamentals of antibodies as related to their sequences, immunogenetics, structures, and functions based on currently available data and literature information. We have exemplified the current state of knowledge of complex immunogenetics including recombination of germline genes, junctional mechanisms, and $V_H - V_L$ pairings. The modern clustering of CDR loops with canonical conformations, CDR H3 features, and structural insights from antibody – antigen interactions were elaborated. The patterns of antibody glycosylation and structural details of Fc – FcR interactions were also highlighted with implications for antibody structure and function. Overall, this chapter provided the framework necessary for understanding human antibody structure and function, and provided the background in aiding the discovery and development of antibody-based therapeutics, fragments, and scaffold design for protein therapeutics.

The prospect for human antibody-fragment-based scaffold development has been already realized [84]. Novel antibody scaffolds based on variable (V_H, V_L) and constant domains (C_{H2} and C_{H3}) have been successfully engineered, which have raised the scope for therapeutic applications [26a, 85]. Recent technological advancements have brought us a plethora of human antibody sequences and structures, which have been publically stored and annotated with immunogenetic properties and CDR canonical structure classifications (Table 3.6). These resources can be exploited along with antibody structure modeling to perform computer-aided antibody design [53a] for scaffold engineering applications. To manage enormous amounts of antibody repertoire sequences and structural data and support therapeutic developments, relational database system and

Table 3.6 Databases and tools for analyzing antibody sequence and structural data.

Resources	Website	Description
Protein Data Bank	http://www.rcsb.org	Structural data repository for biological macromolecules including antibodies and complexes from X-ray, NMR, and EM
IMGT	http://www.imgt.org/IMGTrepertoire/http://www.imgt.org/HighV-QUESThttp://www.imgt.org/3Dstructure-DB/	IMGTrepertoire for Ig germline database; IMGT/HighV QUEST for Ig repertoire immunoprofiling; and 3Dstructure-DB for Ig structural data and immunogenetics annotation
NCBI SRA	http://www.ncbi.nlm.nih.gov/sra	Sequence read archive (SRA) that includes raw sequencing data and alignment information from next-generation sequencing (NGS) of antibodies
PyIgClassify	http://dunbrack2.fccc.edu/pyigclassify/	Database and web server for classification based on a clustering method and identification of CDRs of antibodies structures
SAbDab	http://opig.stats.ox.ac.uk/webapps/sabdab-sabpred/Welcome.php	The structural antibody database, curated from the PDB, V_H-V_L orientation analysis, CDR clustering methods
KabatMan	http://www.bioinf.org.uk/abs/simkab.html	GUI to the Kabat Sequence Database
abYsis	http://www.bioinf.org.uk/abysis/	An integrated database system for antibody sequence and structure analysis
IgBLAST	http://www.ncbi.nlm.nih.gov/igblast/	IgBLAST analysis of immunoglobulin variable domain sequences
PIGS	http://circe.med.uniroma1.it/pigs/	Antibody Fv modeling based on canonical structure method
RosettaAntibody	http://antibody.graylab.jhu.edu/	Antibody Fv homology modeling with *de novo* loop modeling for CDR H3 loops

novel bioinformatics algorithms should be specifically developed. Such database system applications will enable big data analysis for finding patterns in antibody sequences and structures that relate to properties and function, for example, predominant structural configurations involving selected CDRs combinations [86], redundant mutations in CDRs as motifs [43b], convergent or public clones with shared gene usages, specific CDR sequence features, and certain V_H-V_L clonotypes among individuals [87]. These types of advanced, novel bioinformatics analyses at the molecular and immunogenetic level will be the key to future successful developments toward biotherapeutics and beyond [44b].

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4
Antibodies from Other Species

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4.1
Introduction

Immunoglobulins are the molecular basis of humoral immunity. Across different species, these macromolecules maintain a common quaternary structure, which is typically comprised of two identical heavy chains with covalently attached oligosaccharide groups and two identical non-glycosylated, light chains. These glycoprotein molecules recognize and bind a particular antigen in a highly complex and exceedingly specific immune response. Antibodies are the primary protective molecules elicited by most vaccines, and recombinant antibodies are now a major class of therapeutics for multiple diseases. The earliest antibody therapeutics were derived from serum of nonhuman species. In particular, horse serum served as anti-venom yet had substantial toxicity (serum sickness) due to the immune response against the nonhuman antibody protein [1, 2]. Other antibody preparations such as anti-thymocyte globulin produced in rabbit had therapeutic benefit but also had significant toxicity. The use of alternative species for these therapeutic preparations was largely due to ease of production, as they were developed prior to the advent of modern molecular biology techniques, which have enabled rapid discovery and engineering of recombinant antibodies. Thus, most current approaches for producing recombinant antibodies rely on humanizing antibodies derived from other species, usually mice, or beginning with human scaffolds engineered into libraries or transgenic "humanized" mice.

Recently, however, novel features of antibodies derived from other species have sparked interest in developing antibodies that may have particular unique features in binding certain antigens or epitopes [3–7]. For example, several heavy chain only antibodies (HCAbs) originally derived from camelids are now in

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clinical trials [8]. Other unique features of antibody paratopes have been found in cows, chickens, and shark. These paratopes may be a reflection of the fact that diseases, and their susceptibility, vary greatly among the various species. This reflects the staunch differences in genetic background, physiology, phylogeny, lifestyle, immune systems, and environment that exist in nature. These differences have clearly driven the evolution of the adaptive immune system. In this chapter we review the genetic and structural features of the antibody system of several diverse species, with an emphasis on those with social or economic importance to humans, but also including unique examples of novel antibody genetics or structure that have been identified in evolutionarily important organisms (Figure 4.1).

Although antibodies maintain a common structure, they exist in various isotypes, which differ in their biological function, structure, and tissue distribution. There are five major immunoglobulin isotypes in mammals: IgM, IgD, IgG, IgA, and IgE. IgM is widely conserved throughout vertebrates, with the potential unique exception of the coelacanth [9]. Additionally, IgM, IgD, and IgA, or its analog, IgX, have been described in nonmammalian tetrapods. Birds, reptiles, and amphibians express IgY, a likely evolutionary precursor to IgG and IgE. More extreme differences are present in some species. An example is the development of unique immunoglobulin isotypes, such as IgX in amphibians or IgW in sharks. Variability also exists in the type and usage frequencies of light chains. In certain species, the κ and λ light chains are utilized equally, while in others one or the other is preferred. The diversity of the immunoglobulin repertoire depends on several factors, and different species have evolved distinct mechanisms to generate antibody diversity. In particular, novel structures have evolved that may have unique functions in binding antigen in different species, such as antibodies devoid of light chains in camelids and sharks, ultralong complementarity-determining region 3 of the heavy chain (CDR H3) antibodies in cows, and unusual isotypes for both heavy and light chains in various organisms (Figures 4.1 and 4.2; Tables 4.1 and 4.2).

4.2 Mammals

4.2.1 Rat and Mouse

4.2.1.1 *Passive Transfer*

A unique feature of mammals is the passive transfer of immunoglobulins from mother to offspring. IgG is transferred from maternal serum into the offspring by the neonatal Fc receptor (FcRn) expressed in placenta and/or the infant intestine. In rats, this occurs both prenatally through the placenta as well as postnatally via the colostrum through the intestines. However, the majority of antibody transport occurs through the consumption of colostrum and milk after birth [10].

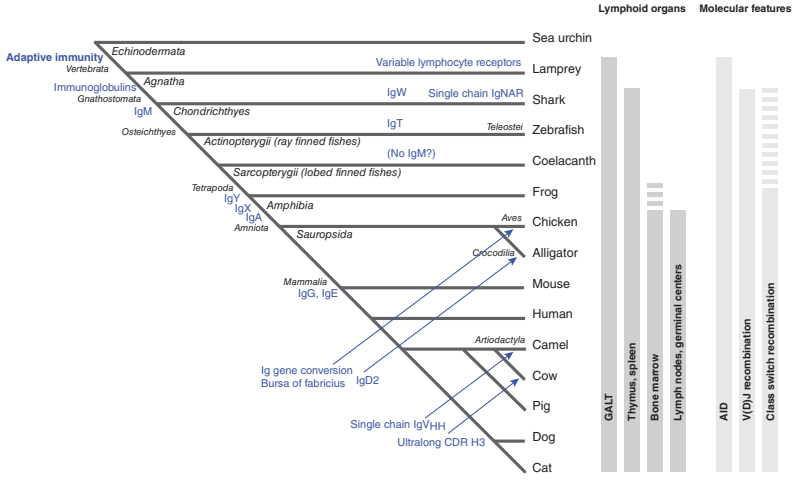

Figure 4.1 Phylogenetic tree of species of immunological interest. Appearance of unique features, isotypes, and binding structures are indicated in blue. Appearance of lymphoid organs and molecular mechanisms involved in antibody diversity are indicated in the bars on the right. AID stands for the activation-induced cytidine deaminase and GALT for gut-associated lymphoid tissue.

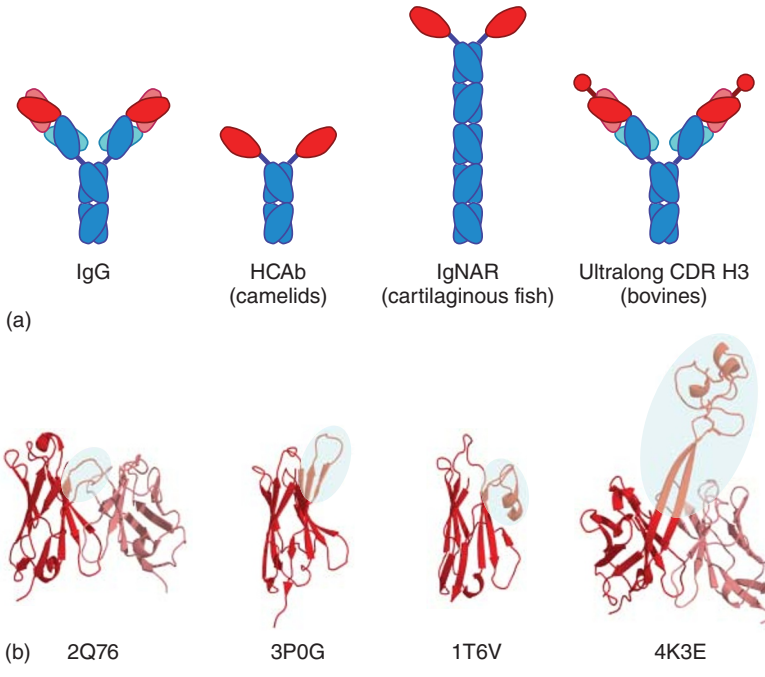

Figure 4.2 Comparison of different species' antibody structures. Common IgG antibody structure found in most species. The heavy chain only antibodies, or HCAbs, are found in camels, which only possess C_H2 and C_H3. The heavy chain only antibodies, IgNAR, are found in sharks, have 3–5 C_H (C_H1–5) regions. The stalk and knob structure found in ultralong cow antibodies. Cartoon (a) of the antibody and ribbon (b) diagrams of the variable region of each antibody type are shown. The CDR H3 regions are highlighted in each ribbon diagram. The PDB codes of the structures are indicated below each graphic.

Table 4.1 Different immunoglobulin isotypes found in each species.

	IgM	IgD	IgG	IgA	IgE	IgD2	IgNAR	IgT	IgW	IgX	IgY	HCAbs
Rat/mouse	X	X	X	X	X							
Cat/dog	X	X	X	X	X							
Pig	X	X	X	X	X							
Cow	X	X	X	X	X							
Camel	X	X	X	X	X							X
Chicken	X			X							X	
Sauropsida	X	X		X		X					X	
Xenopus	X									X	X	
Teleost	X	X						X				
Shark	X						X					

Table 4.2 Presence and use of light chains in various species.

	λ	κ	σ	σ-cart
Rat/mouse	X	X*		
Cat/dog	X*	X		
Pig	X	X		
Cow	X*	X		
Camel	X	X		
Chicken	X*			
Sauropsida	X	X		
Xenopus	X	X		X
Teleost	X	X		
Shark	X	X	X	X

Asterisks indicate known preferential use.

4.2.1.2 Lymphoid System

The immune system of rodents has been very well characterized, as these animals have been major model systems in immunology. As with most mammals, the lymphoid system can be organized into two regions: primary and secondary. The primary lymphoid organs, where lymphocytes are formed and mature, comprise the bone marrow and thymus. The secondary lymphoid organs are the spleen, lymph nodes, Peyer's patches, and mucosa-associated lymphoid tissue. Similar to other mammals, in rodents, the majority of B-cell development occurs in the bone marrow.

4.2.1.3 Antibody Organization

Like in other mammals, antibody diversity in the rat and mouse is created by the combination of heavy and light chain gene segments. The heavy chain locus is comprised of a variable (V_H), diversity (D_H), joining (J_H), and constant region genes. The light chain locus only contains a variable (V_L) and joining (J_L) region plus a constant region. Diversity is generated by the combination of different VDJ and VJ regions in the variable domain genes of the antibody. Addition of non-templated (N) or palindromic (P) nucleotides at the V–D, D–J, or V–J joints and exonuclease-mediated deletions greatly increase the diversity within CDR 3 regions. The constant region encodes the immunoglobulin isotype. The variable regions are encoded by a series of duplicated exons. In rodents, approximately 100 V_H, 4–30 D_H, and 4–6 J_H exons for the heavy chain region and 50 V_L and 4–5 J_L segments for light chains are present [11].

4.2.1.4 Antibody Isotypes

The rodents contain five immunoglobulin isotypes: IgM, IgD, IgG, IgA, and IgE, which are equivalent to their human homologs (Table 4.1) [12]. In the rat, the

IgG isotype contains four subisotypes: IgG1, IgG2a, IgG2b, and IgG2c, while IgG1, IgG2a, IgG2b, and IgG3 are present in the mouse [13]. There is significant homology between these subisotypes in the rat and mouse, but they are not direct homologs to their human counterparts IgG1, IgG2, IgG3, and IgG4. For example, human IgG1 appears to be a functional homolog of mouse IgG2a rather than mouse IgG1. Thus, it is important to remember that rodent IgG1 and human IgG1 are not interchangeable and therefore do not harbor the exact same functionality. Similar to other mammals, only one IgA subtype exists in mice and rats; however, two IgA subtypes, IgA1 and IgA2, exist in humans. In rodents, as well as rabbits, the light chain that is highly preferred is κ (Table 4.2) [14].

4.2.2 Cat/Dog

4.2.2.1 Antibody Organization

Because of the popularity of dogs and cats as companion animals and their excellent ability to serve as models for human diseases, some research on the immune systems of the dog and cat has been performed, yet detailed examination of the genetics and structural biology of their antibodies has not been published. Recent work has provided some information on the immunologenetics of the dog. Eighty variable (V_H) genes, six diversity (D_H) genes, and three joining (J_H) genes have been mapped to chromosome 8 in the dog [15]. The heavy chain locus in the dog appears to be made up of three variable gene families. However, only approximately half of the variable region genes are potentially functional. Additionally, the complementarity-determining region 3 of the heavy chain (CDR H3) in the dog is longer than in the mouse, which averages around 10 amino acids in length, but still shorter than their human counterpart, which averages 15 amino acids in length [16].

4.2.2.2 Antibody Isotypes

The five major isotypes (IgA, IgG, IgM, IgD, IgE) and two forms of light chain (κ and λ) are present in dogs and cats (Table 4.1). In both species, the λ light chain is more commonly utilized (Table 4.2) [11]. In the dog, four subisotypes for IgG have been identified, IgG1–4; however, only three have been recognized in the cat to this point (IgG1–3) [17]. IgA is typically found as a monomer in serum and in a dimeric form called secretory IgA in mucous secretions. Unlike in other species, in the cat, IgA is present as a dimer in serum and mucosa, while in most species IgA is only present as a dimer in its secreted form [18]. In both species, there is little transfer of immunoglobulins across the placenta. The majority of passive immunity occurs via the postnatal consumption of colostrum and milk [19]. Given that both species serve as important model systems in toxicology and infectious diseases, in addition to their role as companion animals, more research is warranted in the basic biology of their antibody systems.

4.2.3 Pig

4.2.3.1 Antibody Organization

Porcine antibody genes are arranged in the same way as in other mammals. Current characterizations of the porcine immunoglobulin heavy chain locus include 15 variable (V_H) genes, 2 functional diversity (D_H) genes, 1 functional joining (J_H) gene, and the constant genes [20–22]. Complementary DNA evidence indicates that additional variable genes may exist upstream from the 15 that are characterized [20]. Additionally, swine appear to only utilize a small number of V_H genes to form the majority of their antibody repertoire [22]. Thus with only 15–17 V_H genes, 2 D_H genes, and 5 J_H genes, the combinatorial potential of the heavy chain is relatively small compared to that of other mammals. A robust antibody repertoire is a result of somatic hypermutation within a small number of V_H genes [22].

Pigs use the κ and λ light chains in equal proportions (Table 4.2) [23, 24]. The porcine κ and λ light chain loci are located on chromosomes 3 and 14, respectively [23, 24]. The kappa locus contains at least 14 variable (V_L) genes, 5 joining (J_L) genes, and a single constant gene. Of the 14 κ V_L genes, 9 are proposed to be functional and can be divided into five families. The lambda locus contains 23 annotated V_L genes arranged in two clusters, while the constant and J_L genes are arranged in four sets of tandem cassettes. The 23 lambda V_L genes can be separated into seven families; IGLVl, IGLV2, IGLV3, IGLV5, IGVL7, IGLV8, and a poorly defined Group III [24, 25]. Ten of these genes appear functional and belong to either the IGLV8 or IGLV3 families. Deep sequencing indicates that there is considerable variation from one individual pig to another, including the absence of the λ V_L genes 3–6 in some animals and truncated versions in others [25]. Interestingly, it has been suggested that antibody gene variation at the population level may compensate for reduced diversity within an individual with regard to pathogen protection [26].

4.2.3.2 Antibody Isotypes

All five mammalian immunoglobulin isotypes are present in the pig (IgA, IgG, IgM, IgD, IgE) (Table 4.1). And, similar to many higher mammals, with the exception of humans and rodents, there appears to be no trans-placental transfer of maternal antibodies [27]. In humans and rodents, IgD cannot be expressed by class-switch recombination. Instead, alternative splicing occurs to produce either the IgD or IgM isotype. However, at the DNA level, class switching to IgD is possible in cattle and perhaps in porcine due to their unique IgD switch region [28].

4.2.4 Cow

An unusual paradigm for creating both genetic and structural diversity in antibodies is present in the cow. Antibodies in the cow can have an unusually

long complementarity-determining region 3 of the heavy chain (CDR H3), which can reach lengths of nearly 70 amino acids long [29–32]. Approximately 10–15% of the repertoire is comprised of these ultralong CDR H3s. On a structural level, these ultralong CDR H3 regions form β-ribbon "stalk" and disulfide-bonded "knob" mini domains (Figure 4.2). In addition to the ultralong antibodies, cows generate a shorter repertoire that is still significantly long compared to other species. The average cow CDR H3 sequence is between 20 and 40 amino acids in length, which, in comparison to the average length in rodents and humans (10–15 amino acids), are still considered unusually long.

4.2.4.1 Antibody Organization

As seen in several ungulate species, cows have a limited number of heavy chain variable (V_H) genes. Twelve V_H regions are thought to comprise one highly homologous heavy chain family [33–35]. There are nine diversity genes and six joining genes [36]. Cows perform V(D)J recombination similar to other species; however, the ultralong subset of cow antibodies appears to preferentially use a single V_H (termed V_HBUL) gene and an ultralong D_H2 gene [37]. Cows preferentially use the λ light chain (Table 4.2) [38]. The genomes of many species (human, mouse, rat, pig, dog, cat, etc.) including the cow have been published [39]. However, the difficulty in assembly and annotation of the heavy and light chain loci leaves open the possibility that additional highly homologous regions can still be discovered.

Unlike in rodents and humans, cows activate somatic hypermutation during development of the primary repertoire, compensating for the limited V(D)J combinatorial diversity [34, 40]. An unusual feature of cow germline D_H regions is that they encode multiple cysteines. In the D_H2 region, used in the formation of the ultralong CDR H3s, amino acids in repeats of Gly-Tyr-Gly or Gly-Tyr-Ser are encoded by the uncommon codons GGT (for Gly) and AGT (for Ser) and TAT (for Tyr). Each of these can be mutated to cysteine with one nucleotide change [37]. Frequent RGYW somatic hypermutation hot spots are present throughout the D_H, which may drive a high frequency of mutation. Thus this D_H region may be easily mutated to cysteine through somatic hypermutation. This was confirmed with deep sequencing, which identified mutations both to and from cysteine in antibody sequences undergoing somatic hypermutation [37]. These results indicate that cysteine mutations can alter the disulfide patterns in the ultralong CDR H3s of the cow, resulting in wholesale changes in loop structures and compositions, suggesting a novel mechanism for structural diversity generation unique to the cow [37].

Wang *et al.* solved the structures of two cow antibody Fab fragments, which contained ultralong CDR H3s [37]. Although the sequences of these two ultralong CDR H3s were highly dissimilar, their structures shared common features. Both structures were composed of a β-ribbon "stalk" and a distal disulfide bonded "knob" that rested upon the stalk. These structural features have never been seen in antibodies derived from other species. Each CDR H3 had six cysteines that were not conserved between their linear amino acid sequences. The structures clearly revealed different disulfide bonding patterns in the two knobs. Despite

the unique "stalk" and "knob" features of the two antibodies, all other attributes were very different. The sequences, disulfide bonding pattern, surface shape and charge, and loop lengths within the knob were dissimilar between the two antibody fragments. The remaining five CDRs and variable region framework regions were almost identical in structure and sequence, indicating that the diversity of these cow antibodies appears to reside solely within the ultralong CDR H3 region. Deep sequencing also revealed that most sequences contained an even number of cysteines at different positions, further strengthening the hypothesis that disulfide bonds, and their associated loops, are an important component of the structural repertoire. Significantly, all of the antigen binding was found to reside within the ultralong CDR H3 "knob" domain in a model antibody [37].

Three more ultralong CDR H3 antibody crystal structures were solved by Stanfield *et al.* [41]. Each contained the characteristic "stalk" and "knob" architecture; however, additional conserved features were identified. Despite having very little sequence conservation in the knob regions, each knob contained a very short three-stranded β sheet. This conserved β sheet is structurally similar to other small disulfide-bonded domains. The closest match was Kalata B1, which plays a role in pathogen protection in plants. A conserved disulfide in the antibody knobs was identified that utilized a germline-encoded cysteine, but the bonding partner cysteine was not conserved. Furthermore, the loops between the strands were different in length and amino acid content, suggesting that the short loops within CDR H3 may be functionally analogous to the CDRs of a traditional antibody.

4.2.4.2 Antibody Isotypes

The biological function of ultralong CDR H3 antibodies remains unknown. The heavy chain locus of the cow is unusual compared to those of other mammals, with a duplication that results in two IgM genes [42]. Interestingly, ultralong antibodies seem to be completely associated with rearrangement to the IgM2. The biological roles, if any, of the two IgM genes remain to be determined.

4.2.4.3 Therapeutic Applications

Serum-derived bovine immunoglobulin/protein isolate (SBI) is a protein powder composed of immunoglobulin and other serum proteins, similar to those found in colostrum and milk but do not contain any milk products such as lactose, casein, or whey. The use of SBI has been shown to be effective for the prevention and nutritional treatment of childhood malnutrition and gastrointestinal disease, including acute diarrhea and necrotizing enterocolitis. Well-established applications for the use of SBIs include HIV-associated enteropathy and diarrhea-predominant irritable bowel syndrome. The use of SBI could become important components of the treatment regimen for inflammatory bowel disease, conditions associated with the depletion of circulating and luminal immunoglobulins, and in critical care nutrition [43]. Currently, there are several ongoing clinical trials examining the effects of SBI on these diseases. In a similar vein, Avaxia Biologics is investigating polyclonal anti-TNF antisera as an oral formulation for inflammatory bowel disease [44]. Sevion Therapeutics (formerly Fabrus, Inc.)

is now developing recombinant monoclonal humanized cow antibodies with ultralong CDR H3s, with a lead molecule targeting the ion channel Kv1.3, which is a very challenging target for traditional antibodies.

4.2.5 Camel

4.2.5.1 Antibody Organization

Camels possess two populations of circulating antibodies. The first comprises 25% of total circulating antibodies and is composed of the conventional heterotetrameric antibodies with identical heavy chains paired with identical light chains (Table 4.2) [45]. In the second population, termed HCAbs, antibodies are similar to the conventional IgG molecule but have identical heavy chains that lack the C_H1 domain and do not pair with light chains (Figure 4.2). A simple point mutation from G to A that disrupts a consensus splicing sequence may be the cause for HCAbs lacking a C_H1 domain [46]. These HCAbs represent a significant fraction of the Igs in the serum, constituting up to 75%, and are significantly smaller than a conventional IgG molecule. The HCAbs have a molecular weight of about 90 kDa compared to the typical IgG molecular weight of about 150 kDa [47]. Different isoforms of these HCAbs have been identified and are classified by the length of the hinge region sequence between the variable domain and the C_H2 domain. Shorter hinge length isoforms are referred to as IgG3 and the longer hinge regions as IgG2 [47]. Alpacas and llamas also have HCAbs that are very similar to those found in camels [48].

HCAbs have a dedicated variable domain referred to as the V_{HH} domain, which is structurally and functionally similar to a typical IgG F_v fragment. The V_{HH} domains only have three CDR variable loops, which define the antigen binding surface. The CDR H3 of the V_{HH} contains long loops (Figure 4.2b), which may enable V_{HH} regions to interact with and inhibit unusual targets or epitopes not available to the flat binding surface of conventional antibodies, such as enzyme active sites or other recessed crevices [49].

Camel HCAbs share the same gene locus as their conventional IgG tetrameric counterpart [50]. In addition, both HCAbs and IgGs have dedicated variable region genes encoded in germline sequences and undergo classical V(D)J recombination [51]. HCAbs can be encoded by over 30 unique variable region (V_{HH}) sequences, possible unique splicing events of the mRNA, and promiscuous V genes that can produce either V_H (which will also pair with V_L molecules) or V_{HH} domains, each of which can undergo somatic hypermutation to produce further diversity [51].

4.2.5.2 Therapeutic Applications

The HCAbs found in camels are being researched for multiple pharmaceutical applications and have the potential for use in the treatment of acute coronary syndrome, cancer therapies, and Alzheimer's disease. A Belgium biopharmaceutical company, Ablynx, has spearheaded many of these technologies coining the term

"nanobody" when referring to the HCAbs found in camels, llamas, and alpacas. Because of their small size and conformational stability, these antibodies are able to access difficult epitopes, making them excellent targets for diagnostic and therapeutic applications. As an example of their diagnostic capabilities, HCAbs were immobilized on sensor surfaces sensing human prostate-specific antigen (hPSA) and tested. They outperformed the classical antibodies in detecting clinically significant concentrations of hPSA [52]. Therapeutically, HCAbs have been shown to suppress the replication of influenza A virus subtype H5N1 *in vivo* and neutralize cytopathic effects of toxin A and toxin B of *Clostridium difficile* in fibroblasts *in vitro* [53, 54]. Furthermore, several clinical trials have been completed using HCAbs as therapeutics for psoriasis (anti-IL-17A/F), acquired thrombotic thrombocytopenia purpura (anti-von Willebrand Factor), rheumatoid arthritis (anti-IL6R), and rotavirus diarrhea in infants. Additionally, the biopharmaceutical company arGEN-X uses llamas for human therapeutic antibody discovery. arGEN-X is developing a human anti-CD70 antibody and a human antibody against c-Met proto-oncogene, which have entered into clinical trials [55].

4.3 Reptiles

4.3.1 Chicken

4.3.1.1 Lymphoid System

Birds are often classified as a subgroup of reptiles [56]. The chicken is the most primitive vertebrate species with lymph nodes, true IgA, and pronounced affinity maturation. B lymphocytes mature in the bursa of Fabricius, and then migrate to other body tissues. The bursa is a blind sac that extends from the dorsal side of the cloaca, the common portal of the reproductive, urinary, and digestive systems, and then atrophies around the time of sexual maturation. The key role of the bursa of Fabricius in B-cell development was revealed when bursectomized chicks failed to produce antibodies [57, 58].

4.3.1.2 Antibody Organization

Chickens produce a diverse repertoire of antibodies that is unique compared to development of antibodies in humans or mice. Chicken antigen receptor genes undergo a single VDJ recombination event followed by gene conversion utilizing multiple upstream V-region pseudogenes [59]. Rearranged variants of the pseudogenes can further diversify the complementarity-determining region of the heavy chain 3 (CDR H3) by inserting sequence into the D_H region. Interestingly, the gene conversion process is dependent on the activation-induced cytidine deaminase (AID) enzyme [60], the same factor that is required for performing somatic hypermutation and class-switch recombination in several

other species [61]. Framework diversity is limited in chicken antibodies. This is most likely a result of the need for DNA homology, which allows for efficient gene conversion. The potential diversity of creating different CDR combinations and content through multiple theoretical gene conversion recombination events is enormous [62].

As is the case with many species, few detailed structural studies on chicken antibodies have been performed. In repertoire analysis by gene sequencing, Wu *et al.* analyzed the amino acid content of chicken heavy chains [62]. Interestingly, the cysteine content was substantially higher in chicken CDRs (9.4%) than in mice (0.25%) or humans (1.6%). Six families of putatively different disulfide patterns were identified, which may include disulfide bonds within CDR H3, or between CDR H3 and CDR H1 or CDR H2. Tyrosine is an important and abundant amino acid in antibody CDRs [63–65]. This amino acid is found less frequently in chicken CDR H3 (9.2%) compared to humans (16.8%). While, on average, chicken CDR H3s are not longer than most mammals, certain CDR H3s may form longer and unique disulfide-stabilized structures [62]. In structural studies of two chicken scFvs, Conroy *et al.* identified unique canonical classes of CDR L1 and a disulfide-bonded CDR H3 [66]. The data strongly suggests that chickens may have a novel repertoire of paratopes.

4.3.1.3 Antibody Isotypes

In chickens, B lymphocytes produce three classes of antibodies. Chickens have serum IgM, IgA, and IgY, the first two being homologs of their mammalian counterparts; however, they do not have IgE or IgD (Table 4.1) [67]. IgY appears to be related to both mammalian IgG and IgE [59] and may be an evolutionary common ancestor to both [68]. Similar to amphibians, immunoglobulins switch from IgM to IgY. Similar to mammals, IgA is found primarily in the mucus secretions of the eyes, gut, and respiratory tract. In chickens, the light chain repertoire is derived solely from one isotype, which is similar to the λ isotype in other vertebrates (Table 4.2) [69, 70].

4.3.1.4 Therapeutic Applications

Monoclonal antibodies are an essential tool in the treatment of many diseases. However, limitations exist with antigen recognition when mammalian hosts are used because of the evolutionary relationship to humans. The company Crystal Bioscience is working to develop transgenic chickens expressing human antibody genes. This could allow the access of human epitopes that have been difficult in mammalian hosts due to the tolerance to conserved proteins. By the expression of human immunoglobulin variable regions in a chicken DT40 B cell line and further diversification of these genes by gene conversion, Crystal Bioscience has demonstrated that a diverse pool of human antibody sequences in chicken B cells is produced, which suggests that a functional repertoire of chimeric antibodies will be expressed in transgenic chickens [71]. Additionally, others have performed clinical trials examining the effects of IgY on diseases of the alimentary track. Oral administration of IgY has been successfully used to prevent or treat specific diseases including dental caries (*Streptococcus mutans*), infant rotavirus diarrhea, gastritis (*Helicobacter pylori*), and periodontitis (*Porphyromonas gingivalis*) [72–75].

4.3.2 Sauropsida

4.3.2.1 Antibody Isotypes

Reptiles, including avian and non-avian species, evolved in parallel to mammals [76]. The Testudine species is one of the oldest reptile groups in existence, older then the crocodilians. This group of animals provides important insight into evolutionary immunology. A complex immunoglobulin heavy chain locus has been identified in two Testudine species [77]. The heavy chain locus of these species encodes an IgM gene similar to that found in all vertebrates, a gene coding for an IgD immunoglobulin with 11 exons, genes for 7 IgY isotypes, as well as 5 additional immunoglobulin D2 (IgD2) genes [78]. Similarities exist between the IgD2 heavy chain genes and avian IgA, but it does not appear that IgD2 is a true ortholog to IgA; rather, IgD2 and IgA may share a common ancestor similar to the amphibian IgX, further indicating the complexity in the evolution of the immunoglobulins [79].

By studying the full genome sequences, the structure and content of immunoglobulin heavy chain loci has been analyzed from two crocodilian species: *Alligator mississippiensis* and *Crocodylus porosus,* which originated from the evolutionary lineage that led to birds [80]. In the examined loci, IgD2 and IgA encoding genes were found in addition to IgM, IgD, and IgY genes (Table 4.1) [81]. Thus, an ancestral immunoglobulin may have given rise to IgX in amphibians, IgA in reptiles and mammals, and IgD2 in reptiles by recombination, leading to a chimeric IgD/IgA chain. Similar to most mammals, both κ and λ light chains are present in non-avian reptiles (Table 4.2).

Some of what is known about the immunology of crocodilians is that they do possess class switching, somatic hypermutation, and affinity maturation. Yet, their evolutionary history and unusual isotype repertoire begs for further investigation. Merchant *et al.* have presented some enticing data suggesting that there is a wide spectrum of antibacterial, antiparasitic, and antiviral properties of alligator serum [82–85]. Their data suggest that alligators have evolved a high-,affinity humoral immune response.

4.4 Amphibians

4.4.1 Xenopus

4.4.1.1 Lymphoid System, Antibody Organization, and Antibody Isotypes

The major lymphoid tissues in amphibians are the thymus and spleen [86]. As in mammals, the antibody diversity in this species is generated via somatic rearrangement and combinatorial joining of multiple V, D, and J elements within immunoglobulin heavy and light chain loci [87]. There are three isotypes of immunoglobulin heavy chain constant region genes: (i) IgM [88], (ii) IgY, a homolog of mammalian IgG [89], and (iii) IgX, which is preferentially expressed

in the gut and is orthologous and analogous to mammalian IgA (Table 4.1) [90]. Three light chains are present in Xenopus: κ [91], λ [92], and σ (Table 4.2) [93]. The σ light chain is quite different from the λ and κ light chains. The latter two have longer CDR L1s and shorter CDR L2s; whereas, the σ light chains have longer CDR L2s and shorter CDR L1s [94]. Somatic hypermutation and affinity maturation are present in Xenopus. Interestingly, compared to mammals, the antibody serum response is slower in Xenopus than in mammals, being weeks compared to days, which is consistent with other poikilotherms [95].

Because amphibians undergo a unique metamorphosis from the water-bound tadpole to a terrestrial adult, their immune systems undergo a distinctive maturation unlike other vertebrates. Major changes during metamorphosis occur, yet there is a lack of autoimmunity. All three immunoglobulin isotypes, IgM, IgY, and IgX, are present in both the tadpole and the adult. However, different antibody repertoires are present in pre- and post-metamorphosed Xenopus [11]. Sequential rearrangement of the heavy chain variable genes occurs in the developing tadpoles, and by day 13 all heavy chain variable families can be used [96]. In the early stages of tadpole development, all heavy chain diversity and joining genes are randomly expressed. However, by about day 40, certain genes are overexpressed. At approximately day 10, the Xenopus tadpoles are capable of producing specific antibodies. Antibody memory is transferred between the tadpole and adult [97]. Most of the larval and young post-metamorphic immunoglobulin gene samples have a complementarity-determining region of the heavy chain 3 (CDR H3), which is between 3 and 10 acids in length. This is 2 amino acids shorter than the adult CDR H3s, which are between 5 and 12 amino acids long [98]. Amphibians are the most primitive vertebrate with an immunoglobulin isotype switch [99]. There is a switch from IgM to IgY, which can be prevented by thymectomy of Xenopus tadpoles. The switch from IgM to IgX is not hindered in thymectomized tadpoles [100]. This process of class switching in Xenopus is also highly dependent on temperature, and appears to be less efficient at the larval stage. Class switching from IgM to IgY is more pronounced after metamorphosis is complete [101].

4.5 Fish

4.5.1 Teleost

4.5.1.1 Lymphoid System

Bony fish comprise ~96% of the world's fish population with over 20 000 species, while cartilaginous fish, such as the shark, only comprise 3.7%. Jawless fish make up the remaining percentage of fish species in the world [102]. The immunology of the bony fish, teleosts, is similar to that of higher vertebrates with some differences. The major lymphoid organs in this group are thymus, kidney, spleen, and gut-associated lymphoid tissues. The four main mucosal immune compartments

found in bony fish are (i) the gut-associated lymphoid tissue with the lamina propria and intraepithelial compartments, (ii) the skin-associated lymphoid tissue, (iii) the gill-associated lymphoid tissue, which includes the gills and the interbranchial immune tissue, and (iv) the nasopharynx-associated lymphoid tissue [103], which is composed of a diffuse network of immune cells. Similar to cartilaginous fish, they lack lymph nodes and germinal centers.

4.5.1.2 Antibody Isotypes

Immunoglobulins have been identified in several teleost species through the discovery of heavy and light chain genes [104–107]. Similar to cartilaginous fish, four light chains are present in bony fish: κ, λ, σ, and σ-cart (Table 4.2) [94]. The latter two are closely related and are early light chains that differ significantly from the λ and κ light chains, specifically in the variable region [94]. The immunoglobulin heavy chain isotypes in the teleost are IgM, IgD, and IgT (Table 4.1) [108–110]. IgT has been reported in gut mucosal immunity; however, IgT and IgA are phylogenetically distant, suggesting that their similar functions are a result of convergent evolution [111]. IgT (also referred to as IgZ) is found in some, but not all, teleosts. Unlike in mammals, where IgM forms a pentamer, in teleost IgM forms a tetramer by varying the degree of disulfide polymerization of monomer subunits [112]. The prevalent serum immunoglobulin in most teleosts is a high molecular weight (600–850 kDa) antibody corresponding to tetrameric IgM [112]. A low molecular weight immunoglobulin was identified over 40 years ago [113]; however, the molecular nature of this protein remains a mystery. Teleost antibodies are found in the skin [114], intestine [115], gill mucus [116], bile [117], and systemically in the plasma. No class switching at the DNA level occurs in this species, and they lack a secondary response. However, AID is present in both cartilaginous and bony fish, and they are able to undergo somatic hypermutation [118]. In fish, the catalytic domain and carboxy-terminal region in AID differ from those seen in the AID in other species [118]. Recombinational class switching is dependent on switch regions and multiple constant regions, which are lacking in fish. In cartilaginous and teleost fish, different isotype production is driven by the constant regions being dedicated to certain V, D, or J segments, which exist in various arrangements upstream of the constant region [119]. In some cases, the V–D, or D–J rearrangement event can be instructive of isotype lineage commitment [120]. Given the enormous diversity of teleost species, with relatively few studied immunologically at significant depth, it would not be surprising if further unusual characteristics are uncovered.

4.5.2 Shark

4.5.2.1 Lymphoid System

Nearly half a billion years ago, the adaptive immune system evolved in cartilaginous fish (reviewed in Ref. [121]). The major lymphoid tissues are the well-developed thymus and spleen, which in the shark are their earliest

phylogenetic appearance. Because sharks are cartilaginous, they lack bone marrow and lymph nodes; however, they possess unique lymphomyeloid tissues with similar immune functions. These include the epigonal organ [122] that surrounds the gonads, and the Leydig organ [123] that surrounds the esophagus. These organs participate in red blood cell production and immune function. Gut-associated lymphoid tissues are also present in the shark [124].

4.5.2.2 Antibody Organization

Antibody diversity in shark is created through V(D)J recombination of immunoglobulin genes like other jawed vertebrates, and it is one of the earliest vertebrates with this ability (reviewed in Ref. [125]). Their immunoglobulin loci exist in a multiple cluster organizations throughout the genome [126]; yet sharks are still able to utilize class switching as well as haplotype exclusion [127]. A unique feature of cartilaginous fish is that some of the immunoglobulin loci are inherited from their parents in a partially (VD–J) or completely (VDJ) joined state [128]. The fusion of V, D, and J elements in the germline, as opposed to a somatically developing lymphocyte, may be a product of gonadal RAG expression in sharks [129]. RAG expression has been demonstrated in shark gonad, explaining how some of the many shark immunoglobulin loci can become V(D)J rearranged in the germline. One specific germline-joined IgM locus is preferentially used in young sharks [130]. Although shark IgH arise from simpler loci with fewer elements, sequence differences between the multiple genomic loci in the shark "multi-cluster" (as opposed to translocon of tetrapods) organization and junctional diversification by non-template and palindromic additions produce a repertoire thought to be as diverse as other vertebrates [131, 132].

In cartilaginous fish, like shark, the immunoglobulin new antigen receptor (IgNAR) is a unique heavy chain-only antibody. In young sharks, low serum levels of IgNAR are present. These serum levels slowly rise during the shark's first year of life. The affinity maturation of the molecule has suggested that this isotype serves a similar role in the shark's immune system as the mammalian IgG does in mammals and that it may be T-dependent [133, 134]. A memory response by the IgNAR isotype is characterized by specific antigens and is clearly present in the nurse shark [135]. Additionally, upon antigen exposure, IgNAR is a target of significant somatic hypermutation, leading to affinity maturation [136].

Surface IgNAR has one amino terminal variable domain. This single variable domain antibody's general quaternary structure has independently evolved at least twice in the vertebrate natural history: once in cartilaginous fish, and again in camelids (Figure 4.2) [137]. IgNAR also contains three or five constant domains determined by alternate splicing. IgNAR commonly exists as a monomer, but it has been found to multimerize in some species such as the spiny dogfish [138]. Similar to the structure found in C1-type immunoglobulin superfamily domains, the complementarity-determining region 2 of the heavy chain (CDR H2) in IgNAR can form a belt around the side of the domain instead of projecting away from the constant domain with the other CDR [139]. Additionally, IgNAR CDR H2 can have selected hypermutations and can be important for antigen binding [140].

The IgNAR variable region is 12 kDa, making it the smallest antigen-binding variable region known in the animal kingdom [141].

4.5.2.3 Antibody Isotypes

Significant differences exist between the shark and mammalian immunoglobulin isotypes (Table 4.1). IgM is found in sharks and nearly all jawed vertebrates, with the coelacanth being the only known exception [9]. IgW is present in sharks and is orthologous to IgD of other vertebrates. Good evidence exists for an unusual form of class-switch recombination between IgM and IgW [142]. Lastly, IgNAR is a lineage-specific isotype of cartilaginous fish that does not associate with light chains (Figure 4.2) [143]. However, in cartilaginous fish, IgM and IgW have four light chains to pair with: κ, λ, σ, and σ-cart (Table 4.2) [94]. Currently it is unclear whether the additional light chains serve to enhance diversity of the immune repertoire or whether they have evolved for other specific functions.

4.5.2.4 Therapeutic Application

Because of its small structure and binding ability, the single variable domain from the IgNAR represents an opportunity to bind different epitopes than traditional antibodies. Ossianix Inc. is targeting several different targets with their VNAR platform. Utilizing the transferrin receptor, Ossianix Inc. has also demonstrated that they can transfer their VNAR antibodies across the blood–brain barrier [144].

4.6 Conclusions

Significant differences in antibody genetics, structure, and function exist across species. These distinctions may provide a clue to evolutionary relationships as well as to the pathogens that may have provided selection pressure on the immune system. Evolution has led to incredible diversity among the immune systems of modern species, yet comparatively little research has been done to provide an understanding of the unique differences. Interestingly, IgM is widely conserved among vertebrates, with the interesting exception of the coelacanth. The majority of mammals possess the five major immunoglobulin isotypes IgM, IgD, IgG, IgA, IgE; however, unique immunoglobulin isotypes and structures are also present in other species (Table 4.1). Although the cow contains all five major immunoglobulin isotypes, within these isotypes is a subset of ultralong antibodies that comprise a “stalk” and “knob” domain structure not found in other species [37]. Camels and sharks provide insight into convergent evolution. In cartilaginous fish and camels, the single variable domain antibody structure appears to have independently evolved in each species [137]. The immunoglobulin IgNAR in sharks is encoded at dedicated loci, and camels use an IgG variant that has evolved to encode certain structural modifications; yet, both antibody types lack an associated light chain [47]. An additional example of convergent evolution is

present in teleost. Bony fish lack IgA yet possess the mucosal antibody IgT [111]. In avian species, which lack IgG, IgD, and IgE, the IgY isotype appears to be evolutionarily related to both mammalian IgG and IgE [59]. In ancient reptiles, similarities exist between IgD2 and avian IgA; however, it does not appear that IgD2 is a true ortholog to IgA, but rather IgD2 and IgA may share a common ancestor similar to the amphibian IgX [79]. Additionally, Xenopus contain three light chains (κ, λ, σ) as opposed to κ and λ found in most species (Table 4.2) [91–93]. Yet, in teleosts and sharks, a fourth immunoglobulin light chain has been described, σ-cart, that is an ortholog of the amphibian σ light chain [94]. While the function of some of these unusual constant regions, like IgY and IgT, can be surmised, others like IgD2, IgW, and so on, have not been studied well. The function of these unusual constant regions perhaps could shed light on new mechanisms of disease resistance or pathogen removal in these species.

In addition to the differences in isotype evolution, some species like camel, shark, cows, and perhaps chickens have evolved antigen-binding structures that are atypical compared to traditional antibodies derived from mice or humans. Camelid antibodies have been produced with novel paratopes targeting G-protein-coupled receptors (GPCRs). Given the small size and protruding nature of the antibodies devoid of light chains (camels and sharks), these antibodies may have evolved the ability to bind concave epitopes. Similarly, the ultralong CDR H3s of cows provide a remarkably protruding paratope, suggesting that these may also "reach" into cavities or crevices within an antigen surface. Further detailed study on the antigens and epitopes bound by these novel structures could shed further insight into the evolutionary drivers behind their selection. Given the importance of antibodies as research reagents, diagnostics, vaccines, and recombinant therapeutics, unusual antibodies derived from more exotic species might provide an important niche for targeting antigens that are challenging for traditional antibodies derived from other species.

The complexity of the evolution of the immune system is remarkably vast throughout nature. Significant diversity exists in isotype evolution as well as in the genetic and structural properties of antibody binding regions. Remarkably, relatively few species have been studied in detail in this regard, yet many unusual and interesting antibody properties have already been uncovered. The differences and biologically unique approaches to immune defense may provide answers to some of today's most challenging diseases.

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Part III
Discovery and Engineering of Protein Therapeutics

5
Human Antibody Discovery Platforms

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5.1
Introduction to Therapeutic Human Antibody Platforms

Therapeutic monoclonal antibodies (mAbs) are generated by isolating variable heavy and light chain sequences (V_H and V_L, respectively) that together bind selectively and tightly to a known antigen and, upon doing so, confer a biological activity that is desired therapeutically, for example, blocking the activity of the antigen or of its interaction with other proteins [1, 2]. Over the past 30 years, four primary sources of variable sequences have been used to construct therapeutic mAbs: (i) antibodies isolated from hybridomas of immunized rodents, usually Balb/c mice, producing mouse antibodies that can be manipulated by chimerization [3] or humanization [4]; (ii) transgenic mice producing human antibodies [5–8]; (iii) phage display libraries of human antibodies [9–11]; and (iv) antibody genes cloned from human B cells [12–14].

Whatever the source of the V_H and V_L sequences, they must meet some basic requirements. They must be compatible with human constant light (C_L) and (C_H) heavy regions, and they must confer upon the resultant IgG reasonable "developability" characteristics, such as proper folding, good solubility, absence of aggregation, biochemical stability, and lack of post-translational liabilities such as variable region glycosylation, deamidation, oxidation, and so forth [1]. While V_H/V_L sequences from a variety of animal sources, for example, mice, rats, rabbits, camels, and nonhuman primates, can and have been used extensively as the primary source of variable chains for therapeutic antibody candidates [2], the best source is the human antibody genes themselves. Human V_H/V_L sequences do not need to be humanized to reduce their potential for immunogenicity, and they are most likely to be compatible with human constant regions. Nevertheless, even fully human antibodies are subject to some level of immunogenicity, mostly due to the rearrangements made in CDRs (complementarity-determining

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regions), especially CDR H3, which can lead to the presence of novel human T-cell epitopes [15]. Many of the human antibodies on the market today (e.g., Stelara®, Benlysta®, and Yervoy®) exhibit very low rates of immunogenicity in patient populations (typically <5%) as described in their prescribing information packages.

Human therapeutic mAbs, as defined here, are antibodies whose variable chains are either derived from the human antibody genetic repertoire or made synthetically to simulate the human immune repertoire. There are three general approaches to obtain fully human V_H/V_L genes to produce therapeutic antibodies: (i) directly isolating the genes from human B cells [12–14]; (ii) generation of transgenic animals in which the human antibody V_H/V_L gene repertoire has been cloned and is expressed in place of the endogenous antibody genes [5–8]; and (iii) generation of human antibody libraries that can be sampled and candidates isolated via one of several display and panning technologies [9–11]. One important nuance is that even those antibodies that have been derived from human V_H/V_L sequences but then affinity-optimized *in vitro* using random or potentially nonhuman mutations are still considered as fully human antibodies [1, 2]. Interestingly, XBiotech, a company that sources antibodies directly from human B cells, claims on their website (www.XBiotech.com) that only antibodies directly sourced from human B cells and made into therapeutics without change are truly human antibodies.

The first fully human mAb to be approved for marketing is Humira® (adalimumab), which was derived from the scFv phage displayed human antibody library built by Cambridge Antibody Technology (C*a*T) [16]. Humira® was approved for marketing by the FDA in the United States in 2002 [1]. This particular antibody, however, was not isolated *de novo* from the C*a*T human antibody library, but rather was generated through a process known as "guided selection," whereby the human antibody was derived by chain switching, first the light chain, and then the heavy chain, from the original murine antibody binder [17].

Now, 14 years after the approval of Humira® by the FDA, 17 additional fully human antibodies have been approved for marketing in at least one of the major markets (US, EU, Japan; Table 5.1). Based on the 14 (of 17 total) fully human antibodies for which sales data were available, the market worth of fully human antibodies at end of 2015 was $25 820 MM, led by Humira® at $14 375 MM [18] (Table 5.1). This number should increase dramatically over the next few years, however, as three of the antibodies in Table 5.1 and another 28 fully human antibodies currently in Phase III clinical trials (Table 5.2) were not included in the dataset. Based on the number of fully human antibodies already approved as well as those in Phase III clinical trials at this time, the number of approved fully human antibodies should more than double to >40 total by 2020. As can be seen in Figure 5.1, most of the approvals of fully human mAbs have occurred since 2009. These human mAbs make up 30% of the marketed mAbs and 39% of those mAbs currently in Phase III clinical trials (Table 5.2). Of the fully human mAbs that are approved for marketing, 12/17 (~71%) are derived from transgenic

Table 5.1 Human antibodies approved for marketing in major markets as of January 2016.[a)]

US trade name (generic name)	Approval year (US)	Target	Primary indication	Source of variable domains	Antibody format	Company	2015 Total sales ($MM USD)[b)]
Humira® (adalimumab)	2002	TNF-α	RA, Crohn's disease	Human phage display library	Human IgG1κ	Abbott/C*a*T	14 375
Vectibix® (panitumumab)	2006	EGFR	Colorectal cancer	Transgenic mouse producing human antibodies	Human IgG2κ	Amgen	712
Simponi® (golimumab)	2009	TNF-α	RA	Transgenic mouse producing human antibodies	Human IgG1	J&J (Janssen R&D)	2135
Stelara® (ustekinumab)	2009	p40 (IL-12 & IL-23)	Psoriasis	Transgenic mouse producing human antibodies	Human IgG1	J&J (Janssen R&D)	2474
Ilaris® (canakinumab)	2009	IL-1β	CAPS	Transgenic mouse producing human antibodies	Human IgG1k	Novartis	236
Arzerra® (ofatumumab)	2009	CD20	CLL	Transgenic mouse producing human antibodies	Human IgG1k	Novartis/ GenMab	80 (2014 data)
Prolia® and Xgeva® (denosumab)	2010	RANK-L	Osteoporosis; bone cancer	Transgenic mouse producing human antibodies	Human IgG2	Amgen/GSK	2717
Benlysta® (belimumab)	2011	BLyS	Lupus (SLE)	Human phage display library	Human IgG1λ	GSK/HGS	329
Yervoy® (ipilimumab)	2011	CTLA4	Malignant melanoma	Transgenic mouse producing human antibodies	Human IgG1k	BMS (Medarex)	1126
Abthrax® (raxibacumab)	2012	*Bacillus anthracis* PA toxin	Anthrax biodefense	Human phage display library	Human IgG1	GSK/HGS	NA

(*continued overleaf*)

Table 5.1 (*continued*)

US trade name (generic name)	Approval year (US)	Target	Primary indication	Source of variable domains	Antibody format	Company	2015 Total sales ($MM USD)[b]
Cyramza® (ramucirumab)	2014	VEGF-R2	Gastric cancer	Human phage display library	Human IgG1	Eli Lilly	384
Opdivo® (nivolumab)	2014	PD-1	Advanced melanoma	Transgenic mouse producing human antibodies	Human IgG	BMS	942
Cosentyx® (secukinumab)	2015	IL-17a	Plaque psoriasis	Transgenic mouse producing human antibodies	Human IgG1k	Novartis	261
Praluent® (alirocumab)	2015	PCSK9	Treatment of high cholesterol	Transgenic mouse producing human antibodies	Human IgG	Sanofi	11
Repatha® (evolocumab)	2015	PCSK9	Treatment of high cholesterol	Transgenic mouse producing human antibodies	Human IgG	Amgen (Astellas in Japan)	38
Darzalex® (daratumumab)	2015	CD38	Multiple myeloma	Transgenic mouse producing human antibodies	Human IgG1k	J&J (Janssen R&D)/GenMab	NA
Portrazza® (necitumumab)	2015	EGFR	NSCLC	Human phage display library	Fully human IgG1	Eli Lilly/Dyax	NA

Abbreviations: BLyS, B-lymphocyte stimulator; BMS, Bristol-Myers Squibb; CAPS, cropyrin-associated periodic syndrome; C*a*T, Cambridge Antibody Technology (now part of AstraZeneca); CD, cluster of differentiation; CLL, chronic lymphocytic leukemia; CTLA4, cytotoxic T-lymphocyte associated protein-4; GSK, GlaxoSmithKline; EGFR, epidermal growth factor receptor; HGS, Human Genome Sciences; IgG, immunoglobulin G; IL, interleukin; J&J, Johnson & Johnson; NSCLC, non-small-cell lung cancer; PCSK9, proprotein convertase subtilisin/kexin type 9; PD-1, programmed cell death protein-1; RA, rheumatoid arthritis; RANK-L, receptor activator of nuclear factor κ-B ligand; RSV, respiratory syncytial virus; SLE, systemic lupus erythematosus; TNF-α, tumor necrosis factor alpha; VEGF-R2, vascular endothelial growth factor receptor-2.

a) Data obtained from prescribing information released by the manufacturers and company websites.

b) Rounded off to nearest million (MM); NA, no data available. Data for Cosentyx®, Praluent®, and Repatha® are based on partial year sales. Data for Arzerra® are from 2014 full year sales. Data obtained from La Merie [18] or from company reports.

Table 5.2 Source of variable sequences for approved and Phase III clinical candidate mAbs.[a)]

Source of variable sequences	Antibodies		
	Marketed mAbs	Phase III mAbs	Totals
Total fully human mAbs	17	28	45
From *in vitro* libraries[b]	−5	−8	−13
From transgenic animals expressing human antibodies[b]	−12	−16	−28
From human B cells[b]	0	−4	−4
Humanized	25	34	59
Chimeric	9	4	13
Murine or other	5	5	10
Totals	56	71	127

a) Data from WR Strohl database as of March 2016.
b) Separated subsets of fully human antibodies based on source.

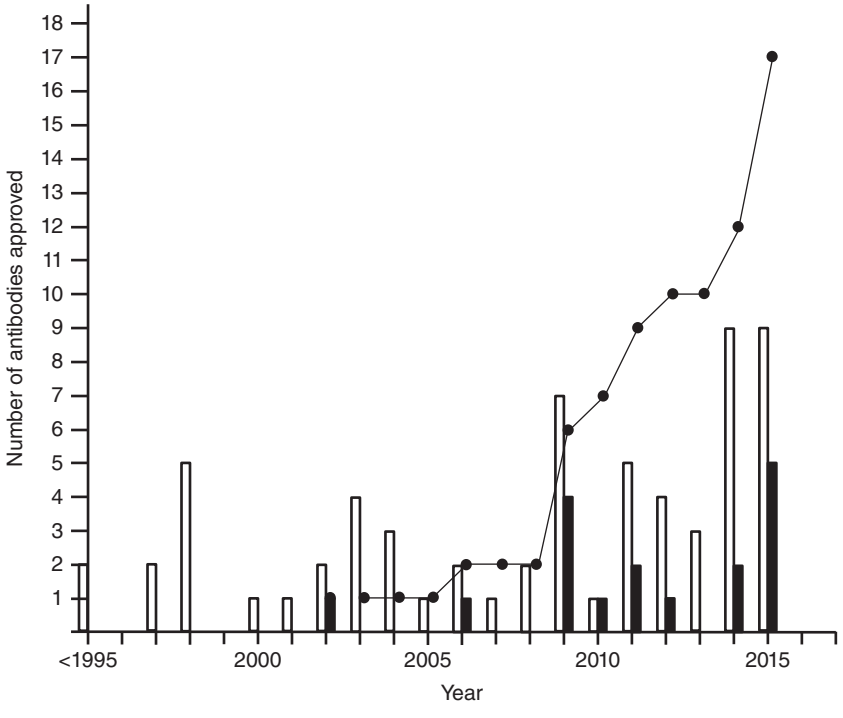

Figure 5.1 Total and human monoclonal antibodies approved by the United States Food and Drug Administration (US FDA) by year. Clear bar: total number of antibodies approved in a given year; Solid bar: number of human antibodies approved in a given year; Line with solid circles: accumulated total of human antibodies approved over time.

animals producing human mAbs and 5/17 (~29%) are derived from human antibody libraries (Table 5.2). None of the currently approved human antibodies was derived directly from human B cells, but four of the therapeutic antibody candidates currently in Phase III clinical trials were originally derived directly from human B cells. Moreover, in the earlier phases of clinical development, at least 23 fully human antibodies have been derived directly from human B cells, indicating an increase in use of that approach.

The percentage of combined marketed and Phase III fully human mAbs has remained relatively constant from 33% (18/55) in 2010 to 35% (45/127) in 2016 (data not shown). This is quite a change from data spanning 1990–1999, a period in which very few fully human mAbs entered the clinic, versus 2000–2008, during which 45% of the antibodies entering the clinic were human [19].

Today, as detailed throughout this chapter, there are numerous sources of fully human V_H/V_L sourcing options that are widely available to companies or labs trying to build and develop fully human therapeutic mAbs. Virtually any organization, large and small, can access a fully human mAb platform at a relatively reasonable cost. Thus, in many ways the generation of fully human mAb leads has become commoditized, leading to a significantly higher level of competition on each target and in each field of study. Hopefully, this level of competition also will lead to better therapeutic antibodies as well. An entire book was published recently covering many aspects of fully human monoclonal antibodies [20], so additional information specifically on human antibodies may be found there.

The generic names of fully human mAbs end in the suffix "-umab" (e.g., adalimumab, panitumumab, ustekinumab, golimumab), although there is a move to change the definitions from "human-derived" to more of a "percent human amino acid"-based system [21].

5.2 Properties of Human Antibody Genes

5.2.1 Human V_H and V_L Genes

Antibody variable heavy (V_H) and light chain (V_κ or V_λ) sequences define the paratope, specificity, and affinity of antibodies. Thus, the organization, rearrangements, and expression the variable chains are critical features for understanding how to manipulate them optimally. The structure and assembly of human variable genes has been described multiple times [1, 20], so this will not be covered here in detail.

In brief, humans generate a diverse pre-immune (or non-antigen driven) repertoire of functional heavy immunoglobulin (Ig) chains from a combination of

IGHV, IGHD, and IGHJ genes [22, 23]. The light Ig chains are derived from either κ variable (IGKV) genes or λ variable (IGLV) genes. These V genes are grouped on the chromosome into families [24, 25].

The human IGH (Ig heavy) gene locus is comprised of 170–176 genes, approximately 76–84 of which are functional (IMGT/GENE-DB; www.imgt.org; [26, 27]). Of these functional genes, 5–9 are functional Ig heavy constant (IGHC) genes [28–30], 38–46 (perhaps as high as 51) are functional Ig heavy variable (IGHV) genes, 23 are functional Ig heavy diversity (IGHD) genes, and 6 are functional Ig heavy joining (IGHJ) genes (Table 5.3). Polymorphisms and presence of pseudogenes dictate that the actual numbers of functional V, J, and D genes are likely to differ among individuals [30].

Humans express two different types of light chains, κ and λ, encoded by genes within the IGK and IGL loci, respectively (www.imgt.org; [1, 24, 25]). The IGK locus consists of 82 Ig κ genes. There are 76 Ig κ variable (IGKV) genes, and about 31–36 of the IGKV genes are considered to be functional (www.imgt.org; [1, 24]). Additionally, the locus includes five Ig κ joining (IGKJ) segments, and one unique Ig κ constant (IGKC) gene (24). The IGL locus has 87–96 genes, 73–74 of which are Ig λ variable (IGLV) genes, 7–11 are Ig λ joining (IGLJ) genes, and 7–11are Ig λ constant (IGLC) genes. Not all of these IGL genes are functional: only 29–33 of the IGLV genes, 4–5 of the IGLJ genes, and 4–5 of the IGLC genes are thought to be functional (www.imgt.org; [1, 25]).

Table 5.3 Use of genes in formation of serum Igs.

Gene family	Characteristic	Properties of human genes
IGHC	Number of functional HC genes	5–9
IGHV	Number of functional V_H genes	~38–46
	Number of functional D_H genes	23
	Number of functional J_H genes	6
	Mostly highly expressed V_H genes	V_H3, ~36%; V_H1, ~26%; V_H4, ~15%; V_H5, ~12%; ~V_H2, 4%; ~V_H6, 3%
IGKV	Number of functional C_κ genes	1
	Number of functional V_κ genes	31–36
	Number of V_κ families	6
	Number of J_κ genes	5
	Human usage of V_κ genes	~60%
IGLV	Number of functional C_λ genes	4–5
	Number of functional V_λ genes	~29–33
	Number of functional V_λ families	11
	Number of functional J_λ genes[a)]	4–5
	Human usage of V_λ genes	~40%

a) Each associated with a separate C_λ locus; Refs [1, 24, 25, 30–32].

5.2.2 Human V_H, V_κ, and V_λ Gene Expression

When making human libraries, generating transgenic animals to produce human antibodies, or accessing human B cells directly for antibodies, it is important to understand which germlines are most highly expressed and used in antibodies that are matured in response to antigen stimulation. Human antibody variable genes are not expressed equally, which results in a bias of variable sequences that are incorporated into circulating IgMs and IgGs. Based on reverse transcriptase-polymerase chain reaction (RT-PCR) amplification and sequencing of Ig genes from B cells of multiple human donors, the V_H families V_H1, V_H3, V_H4, and V_H5 comprise most of the functional V_H segments [33–35]. The most widely utilized V_H genes were shown to be IGVH3 at ~36%, IGVH1 at ~26%, IGVH4 at ~15%, IGVH5 at ~12%, IGVH2 at ~4%, and IGVH6 at ~3% [33]. In the construction of a phage display library of human antibody genes recovered by PCR from 654 healthy human donors, Glanville *et al.* [36] found very similar frequencies, with IGVH3, IGVH1, IGVH4, and IGVH5 (in descending order of usage) as the most predominant IGVH genes recovered. In a study on human IGV genes expressed in baby cord blood, IGVH families 1, 3, and 4 combined to make up nearly 92% of the expressed V_H population observed, and IGHV families 2, 6, and 7 were underrepresented, indicating a slight shift in the repertoires of babies versus adults in those studies [37].

Approximately 60% of light chains incorporated into human serum Igs are κ, with the other 40% being λ [35, 38, 39]. In cord blood samples, the ratio was slightly higher, with ~77% of human serum Igs sequences recovered containing κ, with the other ~23% being λ [37].

For both κ and λ V regions, families 1, 2, and 3 tend to dominate those expressed [31, 32]. In one study of IGKV gene usage, IGKV families 1, 3, 2, and 4 were expressed 41%, 38%, 10%, and 9%, respectively [31]. Of the IGK genes recovered from cord blood, IGKV families 1, 2, 3, and 4 accounted for 76% of the sequences, with IGKV3 alone accounting for >48% [37]. For IGLV gene usage, IGLV families 2, 1, and 3 were expressed 36–49%, 28–30%, and 15–20%, respectively [32]. Similarly, from cord blood, V_L1, V_L2, and V_L3 were the most highly expressed VL families [37].

V_H–V_L pairing appears to be stochastic and strictly dependent on the expression levels of the V_H and V_L genes in both natural B cells [35] and in recombinant libraries [36]. For example, in one study of B cells, the most predominant V_H–V_L pair recovered was VH3-23/Vk3-A27, both being the most highly expressed gene segments [35]. In a highly detailed study of V_H–V_L pairing from isolated B cells, DeKosky *et al.* [40] showed recently that the highest percentage of pairings were donor-specific, but that the highest number of pairings occurred with those sequences most recovered, supporting the contention that the pairing process *in vivo* is largely stochastic. In these cases, IGKV1-39 and IGKV3-20 were the highest V_L sequences recovered, and IGH3-23 was, on average, among the highest V_H sequences recovered [40].

5.3
New Technologies Driving Changes and Improvements in Human Antibody Discovery

5.3.1
Introduction

There are several newer technologies that have been around for the past 5–10 years, but are now hitting their stride as common-use technologies to discover new antibodies. A few of the key technologies being used cross-platform, especially next-generation sequencing (NGS) and single-cell technologies, are transformational and in some cases potentially disruptive. In many cases, these technologies are combined, such as for the isolation of antigen-specific single antibody-producing B cells or hybridomas using flow [41], microfluidic [42, 43], or microarray [44, 45] technologies, followed by single-cell analysis [46–50], single-cell sequencing [50–52], and recovery of the antibody genes from single cells or micro-clones [53, 54]. Combined, these technologies can be incredibly powerful to isolate, analyze, and characterize antigen-specific B-cell-produced antibodies, regardless of the source.

5.3.2
Next-Generation Sequencing Approaches to Antibody Discovery

Next generation sequencing (NGS; also called massively parallel sequencing and deep sequencing), which allows the generation of huge gene datasets that can be compared with advanced gene mining tools, has been hugely disruptive in genomics, translational research, and related areas [55, 56]. It was noted recently that it took about four decades to sequence ~30 000 antibody genes prior to the use of NGS, but now, with NGS approaches, that number of sequences or more can be obtained in a single run [57]. With this power of detailed sequence information, NGS has become transformational for determining antibody repertoires [36, 56–58] and, more recently, for the isolation and characterization of therapeutic mAbs [59]. There are several limitations for this approach, including short read lengths, high error rates, unknown biases from using multiplex PCR methods, and reading errors, but many approaches are being used to correct those issues [55, 56, 60, 61].

There are multiple sequencing systems that are potentially useful for sequencing antibody repertoires or single-cell antibody sequences. Two detailed comparisons for most widely used NGS platforms, namely the Illumina MiSeq, IonTorrent, Roche 454, and PacBio RSII sequencing systems, have recently been reviewed in detail [59, 60]. At a high level, the PacBio system can give long reads up to 8500 bp, but with a very high error rates. With its high error rate, the PacBio system does not work for reading antibody genes unless shorter reads of ~850 bp are done multiple times to generate a consensus sequence [60]. The Roche 454 and Illumina MiSeq paired-end sequencing approaches are low throughput, and are capable of reading either a V_H or V_L gene but not both at the same time. The IonTorrent

system is high throughput but typically yields shorter reads of less than 300 bp, which gives information only on HCDR3 and some upstream sequence [59, 60]. A key to making NGS work is the ability to analyze large datasets in an automated manner. There are several bioinformatics programs that can analyze NGS data, including IMGT's HighV-QUEST, which can analyze up to 5 000 000 NGS IGV domain sequences with full annotation [62–64]. Glanville *et al.* [60] reviewed several additional software packages for analyzing NGS data, including the LANL Antibody Mining toolbox, iHMMunalign, ImmunediveRsity, and MiXCR. Additionally, D'Angelo *et al.* [65] recently described the "AbMining ToolBox," an open source software for detecting and aligning antibody variable sequences for analysis.

NGS can be used in two fundamentally different ways to assess the antibody repertoire in antibody discovery. In the first approach, NGS can either enhance or supplant screening of hybridoma supernatants or phage display outputs. Sequencing and analysis of first-round binders from a given display-based panning approach [59, 66] or of pooled bulk B cells after immunization with a specific antigen [67] can be used to identify bins of particular sequences and their relatives (i.e., a few amino acids different) that are in higher relative abundance than others, indicating those sequences that should be interest for further analysis. For example, from a hypothetical analysis of 100 000 antibody genes, a bin containing a particular sequence and its relatives observed several hundred times would be of more interest than a sequence found only once or a few times. Relying more on the antigen-specific sequence binning as opposed to additional rounds of panning or binding analyses can result in a broader and more inclusive panel of potential hits for analysis [59]. At Janssen R&D, we have found that incorporation of NGS approaches with fewer rounds of phage panning has resulted in different and higher quality leads than relying on multiple rounds of phage panning alone.

Similarly, NGS coupled with repertoire mining of B cells from immunized animals has been used to improve the antigen-specific output after immunization. Antigen-specific antibodies produced by immunized animals have been traditionally recovered by the fusion of immortal myeloma cell lines with B cells to form hybridomas, followed by the analysis of the hybridoma-produced antibodies for antigen binding. Hybridoma technology has been known for decades as highly inefficient, capturing only a small fraction of the total antigen-specific B-cell repertoire [68]. One method widely used in recent years to improve the recovery of antigen-specific B cells has been to generate combinatorial libraries of V_H and V_L sequences from pooled B cells, followed by phage or other *in vitro* panning on the desired antigen [69]. This latter approach helps in recovering additional antigen-specific sequences, but may suffer from the fact that not all antibodies may express well in phage display libraries [69, 70]. Recently, Saggy *et al.* [70] compared the use of a combinatorial phage library approach to a 454 NGS approach to analyze the antibodies produced by B cells from immunized mice. They found that, while both strategies resulted in large antigen-specific binder pools, they were, in fact, complementary. Phage library hits often were represented as low-abundance

sequences in NGS, while high-abundance sequences often did not express well in the phage libraries and therefore were not recovered by those methods [70]. In another example, Reddy *et al.* [67] sequenced the antibody-producing bone marrow plasma cells (BMPCs) recovered from immunized mice, then reconstructed the most abundant V_H and V_L gene pairs for expression, and found that 78% of the resulting clones were antigen-specific. Thus, in this case, NGS replaced the screening approach and allowed access to a B-cell population (BMPCs) that could not be immortalized [67].

The biggest weakness of the sequence binning approach is moving from bins of similar sequences, especially if the available sequences include only CDR H3 or V_H sequences, as is typical due to the short length of sequences typically covered by available NGS sequencing methods [59, 60, 65], to the rescue of specific antibody clones that can be screened for biological activity. D'Angelo *et al.* [65] recently described a rapid inverse PCR method for recovering full scFv clones for screening after binning of the HCDR3 sequences within scFvs using Ion Torrent sequencing. Similarly, Murugan *et al.* [52] developed a barcoded set of matrixed primers for separately rescuing V_H and V_L genes from B cells after single B-cell sequencing. These and other methods recently described in the literature are making it easier to recover antibody genes even after obtaining only partial sequences using an NGS binning strategy.

The second approach for using NGS in antibody discovery is in the analysis of antibody genes from isolated B cells after immunization or exposure to a novel antigen. In this case, antibody-producing B cells are isolated, the V_H and V_L genes are sequenced, the antibodies they produce are analyzed for binding to a specific antigen, and, in some cases, neutralization or activity assays can be run to determine antibody functionality [49, 55]. The advances in single-cell technologies include high-throughput sorting, single-cell sequencing, and single-cell assays, which allow binding or even functional data to be obtained on an antibody produced out of a primary B cell or recombinant cell clone. For example, McDaniel *et al.* [71] recently described a method to sequence antibody and T-cell receptor (TCR) repertoire in a high-throughput manner. Their technology consists in encapsulating single B or T cells by a flow focusing device, followed by use of magnetic beads for RNA capture, reverse transcriptase synthesis of DNA with overlap PCR to adjoin the heavy and light chain genes, and sequencing using paired-end Illumina sequencing. Their process, which can be carried out by a single individual in 12 h, results in sequences for millions of paired IGHV and IGLV antibody genes with >97% precision [71].

In another example, Tsioris *et al.* [49] recently utilized single B-cell analysis coupled with NGS sequencing to isolate West Nile virus-specific neutralizing antibodies from B cells of infected subjects. Methods used for single-cell V_H-V_L sequencing included cell barcoding, overlapping RT-PCR to physically link the genes encoding V_H and V_L, and droplet RT-PCR [55]. Single-cell sequencing is a powerful new adaptation of NGS, which maintains V_H and V_L pairings [72] and, when combined with high throughput single-cell functional assays, can form the basis of a single B-cell-based antibody discovery paradigm [50, 55].

Imkeller *et al.* [72] recently developed the sciReptor software analysis package to assist in the analysis of immunoglobulin sequences obtained from single B-cell sequencing. Some potential limitations in sequencing single B cells or clonal B cells is that ~50% of them may have multiple rearranged Ig gene loci, PCR and/or sequencing bias may create sequence errors, and many B cells produce multiple light chains [55]. Indeed, only a small subset of the sequences of the anti-West Nile virus antibodies described previously contained good reads of both heavy and light variable regions [49].

5.3.3
Single-Cell Cloning and Manipulation

One of the most significant advances in recent years has been the ability to obtain binding and genetic data with single antibody-producing cells [46–50]. The field has progressed by a marriage of engineering technologies, such as microfluidics, nanowell technologies, and high-resolution optical devices [42, 43], with biological techniques such as microscale enzyme-linked immunosorbent assay (ELISA)-like analyses, RT-PCR, and NGS [45, 48, 50]. While advanced single-cell analysis is still in its infancy [42], techniques have evolved to the point where single B cells can be captured efficiently [41–45, 50], the antibodies analyzed for antigen binding and/or isotyping [45–49], and single-cell sequencing carried out of the antibody genes from those cells of interest [50–52]. In one example, a microengraving technique has been developed to capture single hybridoma cells for high-throughput analyses [44]. This technology was then adapted to capture single B cells, followed by isotyping the antibodies produced, testing for antigen binding, and, ultimately, RT-PCR and sequencing of antibody encoding genes [45, 48]. Finally, antibody genes from B cells expressing antibodies of interest can be recovered by RT-PCR methods and expressed for further analyses [48, 51].

5.4
Antigen-Specific Human mAbs from Human B Cells

5.4.1
Introduction to Recovery and Isolation of Human V_H/V_L Sequences from Humans

In theory, the best source of a human antibody is from a human B cell directly [2]. One would expect these antibodies to have low immunogenicity, good developability characteristics, and reasonable expression in mammalian cell culture. There are, however, several reasons for why only a limited number of antibodies have been sourced from human B cells directly. The first is that most of the human antibodies on the market or in the clinic today are against human antigens, to which humans would naturally have tolerance [1]. B cells that recognize self-antigens are selected in the B-cell maturation process and are programmed

for apoptosis [1]. For the most part, this limits the antigens to those not typically found in circulation or on cell surfaces.

Second, historically, the generation of stable and useful human hybridomas has been technically difficult, making that approach less favorable than it is with rodents [73]. Third, for safety and regulatory reasons, humans cannot be immunized with just any protein. Thus, the pool of antigens from which to isolate an antigen-specific human antibody from human B cells is limited to those generated by vaccines that have been approved by regulatory authorities, or to those from convalescing individuals who have been infected or exposed to foreign antigens naturally. Examples of cases in which gene encoding mAbs have been isolated from the B cells of individuals who have been vaccinated include anti-anthrax PA antibodies [74, 75], influenza [46, 76, 77], and the Lewis tumor antigen [78].

5.4.2
Selection of B-Cell Types for Antibody Discovery

The first issue to consider when cloning antibody genes directly from B cells is the population of B cells from which to retrieve antibody genes. Based on a very recent analysis, there are three types of circulating B cells that produce antibodies: $CD20^{+}CD27^{+}CD38^{lo/int}CD43^{+}$ B1 cells, $CD19^{+}CD20^{-}CD27^{hi}CD38^{hi}$ plasmablasts (PBs), and a newly described population of $CD20^{+}CD27^{hi}CD38^{hi}$ B cells that has properties of a pre-PB cell population [79]. PBs, which can be formed either from newly generated naive B cells or from memory B cells, are often the B cells of choice for isolating antibody genes associated with an immune response [55]. As pointed out earlier, Reddy *et al.* [67] analyzed the antibody gene repertoire from mouse antibody-producing $CD45^{-}CD138^{+}$ BMPCs, a significant population of B cells producing a large fraction of circulating antibody that cannot be sampled through standard hybridoma technologies [67].

The concept of determining the antibody repertoire of tissue-specific B-cell populations to determine the targets of their locally produced antibodies has been intriguing for many years. The presence of intratumoral B cells has been associated with improved prognosis [80–82], suggesting that they may be providing antitumor effects such as creating an inflammatory environment [82] and producing antitumor antibodies [81, 82]. Antibody activity against tumor antigens has been detected in the presence of intratumoral B-lymphocytes [81]. To that effect, intratumoral B cells have been isolated and tested using NGS for genes encoding antibodies directed against tumor antigens [82]. In another approach, tumor infiltrating B cells were captured by laser-assisted microdissection, and the genes encoding the antibodies they produced were recovered by RT-PCR and expressed for analysis [83]. While these approaches have not yet yielded novel antitumor antibodies or revealed novel targets, intratumoral B cells may become a more widely used source of antibody genes in the future as the understanding of the role of B cells in the tumor microenvironment increases [82].

5.4.3 Strategies for Retrieving Antigen-Specific Antibody Genes from B Cells

After immunization with an approved vaccine, or during convalescence from an infection or other disease state, the desired antibody-producing B cells can be obtained and the antibodies can be isolated and analyzed for their utility using a variety of different approaches, such as generation of human hybridomas or heterohybridomas [73, 84], B-cell immortalization using Epstein–Barr virus (EBV) transformation [53, 85], generation of small immune phage-displayed libraries of pooled B cells [69, 74, 86], recovery and cloning of expressed antibody genes using RT-PCR methods [46, 51, 53, 54, 76], using NGS to find the most highly expressed antibody sequences [67], retroviral transduction of human B cells with BCL6 and Bcl-xL [87, 88], or isolation and analysis of single B cells as highlighted in Section 5.3.3. Many of these technologies have been described in greater detail elsewhere [12–14, 89].

5.4.4 Antigen-Specific mAbs in Clinical Trials from Human B Cells

Table 5.4 shows examples of antibodies currently in clinical trials that were obtained from human B cells. In all, at least 27 antibodies currently in clinical trials (i.e., ~4% of the total mAbs in clinical development) have been sourced directly from human B cells. These include the four Phase III candidates: the anti-IL-1α mAb Xilonix™ (MABp1, 18C3) from XBiotech, Inc.; roledumab (LFB-R593), an anti-RhD factor from the French Pharmaceutical Group LFB [90]; aducanumab, an anti-amyloid-β mAb isolated at Neurimmune and currently in development by Biogen; and sevirumab (MSL-109), which recognizes glycoprotein H of the human cytomegalovirus (CMV) (Table 5.4). Of the 27 known antibodies in clinical development derived directly from human B cells, 12 are associated with infectious diseases, 7 are cancer antigens, 4 are for neurological disorders, and 4 are non-cancer, non-neurological human antigens. A set of examples from the various disease areas is shown in Table 5.4.

There are several companies that specialize in retrieving human antibodies from human B cells to discover therapeutic mAb candidates. For example, Neurimmune, which utilizes the Reverse Translational Medicine™ (RTM™) platform in which antibody genes are recovered from memory B cells with IgG-specific primers, discovered the anti-amyloid-β antibody aducanumab as well as the Phase I anti-α-synuclein candidate, BIIB054, both of which are in clinical development by Biogen (Table 5.4). XBiotech, Inc. used their "High Stringency Antibody Mining" (SHSAM™) technology and proprietary direct cloning approaches to isolate the "True Human Antibody™," Xilonix™. AIMM Therapeutics developed a sophisticated molecular approach to the immortalization of B cells by virally transducing them with BCL6/Bclxl [87, 88] and used this approach for the discovery of the anti-RSV mAb MEDI-8897 (Table 5.4) and the anti-influenza antibodies CR8020 [77] and CR8043 [94]. Scientists at

Table 5.4 Examples of antibodies in preclinical and clinical trials that have been sourced from human B cells.

Antibody	Phase of clinical development	Target	Indications	Technology and technology company	Development company or institute	Comments and/or references
Aducanumab (BIIB037)	Phase III	Amyloid-β	Alzheimer's disease	Neurimmune RTM™ platform	Biogen	NCT02484547[a)]
Xilonix™ (18C3, MABp1)	Phase III	IL-1α	CRC and many others	XBiotech "True Human™" Antibody platform	XBiotech, Inc.	NCT01767857
Sevirumab (MSL-109)	Phase III	Cytomegalo-virus (CMV)	CMV infection in transplant patients	NAIAD	Johns Hopkins	NCT00000135
Roledumab (LFB-R593)	Phase III	RhD factor	Rhesus incompatibility factor between a mother and her fetus	French Biopharma-ceutical Group LFB	French Biopharma-ceutical Group LFB	NCT02287896, [90]
MEDI-8897	Phase II	RSV F-protein	RSV-caused respiratory disease	AIMM Therapeutics	MedImmune	NCT02290340
Pritumumab	Phase II trials in Japan	Vimentin	Cancer indications	Nascent Biotech, Inc.	Nascent Biotech, Inc.	NA, [91]
514G3	Phase I/II	*Staphylococcus aureus* surface protein	Staphylococcal infection	XBiotech "True Human™" Antibody platform	XBiotech, Inc.	NCT02357966
CSJ-148	Phase I/II	CMV	CMV infection in transplant patients	Humabs Biomed Cellclone platform	Novartis	NCT02268526
MEDI-8852	Phase I/IIa	HA stem region shared by 18 Flu A subtypes	Influenza	Humabs Biomed Cellclone platform	MedImmune (AstraZeneca)	NCT02350751
HuMab 5B1	Phase I	Sialylated Lewis (a) antigen	Pancreatic cancer; other solid tumors	MabVax	MabVax	NCT02672917, [78]
MorAb-022	Phase I	GM-CSF (aka CSF2)	RA	Morphotek	Morphotek/Esai	NCT01357759, [92]
VRC01	Phase I	CD4 binding site on gp120	AIDS	NIAID (RT-PCR)	NIAID	NCT02599896, [93]
BIIB054	Phase I	α-Synuclein	Parkinson's disease	Neurimmune RTM™ platform	Biogen	NCT02459886

Abbreviations: AIDS, acquired immune deficiency syndrome; Ang, angiopoietin; CD, cluster of differentiation; CMV, cytomegalovirus; CRC, colorectal cancer; GM-CSF, granulocyte macrophage-colony stimulating factor; HA, hemagglutinin; IL, interleukin; NA, not applicable; NIAID, National Institutes of Allergy and Infectious Diseases; NIH, National Institutes of Health; RA, rheumatoid arthritis; RhD, Rh-factor D antigen; RSV, respiratory syncytial virus; RTM, Reverse Translational Medicine™; RT-PCR, reverse transcriptase-polymerase chain reaction.

a) NCT numbers refer to clinical trial numbers registered at www.Clinicaltrials.gov.

MabVax fused human B cells from vaccinated cancer patients with the murine myeloma cell line P3X63Ag8.653 to immortalize them, followed by RT-PCR of antigen-specific antibody genes to retrieve the anti-sialyl Lewis A antigen-specific antibody, 5B1 [78], which is now in Phase I/II clinical trials (Table 5.4). Morphotek has utilized their "Morphogenics™" platform in conjunction with the myeloid cell line K6H6/B5 to immortalize human B cells to discover the anti-GM-CSF antibody, MorAb022 [92], which is currently in Phase I clinical trials (Table 5.4). Additionally, several other companies, such as Aridis, with their MabIgX® platform (obtained from Kenta Therapeutics), Theraclone and their I-STAR™ platform, and HuMabs with their Cellclone™ platform, also have generated clinical candidates.

5.5 Human Antibody Libraries

5.5.1 Introduction

The concept of a display system is to couple the gene encoding an antibody or other binding protein to the cell, nucleic acid, or other entity that displays that protein in a form capable of binding a cognate antigen. The power of display systems is having the gene in hand when an attractive binding or functional result is observed [9–11, 95, 96]. Human antibody libraries can be made using virtually any display system available, including phage, nucleic acid-based, yeast, bacterial, and mammalian. The most widely use display system historically has been the phage display system based on the filamentous bacteriophage (f, fm, M13), although in recent years various yeast and mammalian display systems have become increasingly popular [12–14, 97–101].

5.5.2 Display Formats

Human antibody display strategies should encompass certain properties such as (i) linkage of the gene sequence encoding the antibody with the displayed antibody [9–11, 95, 96]; (ii) the ability to construct a large library of antibodies [1, 9–11, 16, 102–105]; (iii) the ability to select antibodies that bind to the target of choice while excluding those that do not bind [11, 105]; (iv) the ability to amplify target-specific antibody genes, a process that may include the generation of additional diversity; and (v) the ability to produce a soluble form of the antibody for functional characterization [11, 105]. There are many display systems available that have been used to build human antibody libraries, including phage display [9–11], *in vitro* display [106], bacterial display [107], yeast display [98], and mammalian display [99–101].

5.5.2.1 Phage Display

Phage display has been the most widely used display technology to date, largely due to its relative simplicity, robustness, and convenience for construction of large human antibody libraries that may be suitable both for *de novo* discovery of antigen-specific binders and for the optimization of desired characteristics [9, 11, 105]. Three different proteins on the filamentous phage, namely PIII (the most widely used), PVIII, and PIX [108], have been used for fusion of the antibody proteins for display purposes. One practical consideration of phage display libraries is that, above 10^{11}, total diversity is limited by bacterial culture volumes required to create larger libraries, the efficiency of bacterial transformation, and the practical limit of phage particle density ($\sim 10^{13}$/ml). Several recent reviews have detailed both phage display technologies and construction of human antibody libraries in phage-based systems [9–11, 96]. At least 75 antibodies currently in clinical trials, and likely many more that are not publicized, are derived from phage display human antibody libraries.

One of the biggest advances in recent years for phage display is the incorporation of NGS with one or a few rounds of panning to increase the diversity in the early lead pool [59, 66, 109–111]. There are multiple advantages to this new approach, including a vastly wider array of potential sequences to improve the breadth of epitopes covered, the generation of phylogenetic trees of the hits as well as structure–function comparisons of similar sequence hits, and, finally, the ability to detect binders to difficult antigens such as cell surface antigens [59] or antigens and epitopes found in serum [112].

Other new technologies associated with phage display that are improving the processes include methods for rapid conversion from scFvs or other fragments into IgGs or similar constructs (e.g., scFv-Fc fusion) for functional screening [110, 113, 114] and the use of phage–antibody constructs to bind to specific cells and tissues on slides directly out of biopsies [115–117]. This latter exciting new development allows closer linkage of the discovery process with clinically relevant targets and epitopes [115–117].

5.5.2.2 In Vitro Display

In vitro display is a second type of display system, which actually comprises several different technologies. Kawasaki [118] and Mattheakis *et al.* [119] first demonstrated the power of ribosome display of DNA-encoded peptide libraries to isolate peptides that are bound to a target of choice. For ribosome display, the transcribed mRNA product of a gene lacking its stop codon remains tethered to the ribosome, resulting in a complex of the antibody and the mRNA encoding it [106, 118]. Other *in vitro* display systems include the mRNA display in which the *in vitro* translated peptide or protein is covalently bound to the mRNA through a puromycin linker [106], polysome display [119], cis display [120], and DNA display [121]. A major advantage to these display systems is that display libraries with diversity reaching 10^{12}–10^{13} can be generated [106]. Additionally, mutational approaches such as error-prone PCR [122, 123] or DNA shuffling [124] can be used with *in vitro* display technologies to increase the diversity for optimization.

Recently, Groves *et al.* [125] described an integrated phage and ribosome display paradigm to improve the affinity of an anti-IL-1RI antibody by 3700-fold. New *in vitro* display libraries have recently been described, including a monovalent mRNA display system [126] and a novel FAb-based *in vitro* display platform that links only one of the chains to maintain the genotype–phenotype linkage [127].

Ribosome display has been used for the maturation of at least one late-stage clinical candidate, Tralokinumab (CAT-354, BAK1.1; [128]), which is currently in Phase III clinical trials for the treatment of asthma. Two potential disadvantages of *in vitro* display systems are that the sequences must typically be in a single open reading frame (ORF), single chain (scFv), or single domain (VHH) type of format [106], and that it is difficult to select these types of libraries on cells or complex systems because the RNA molecule is highly labile. Another potential issue with the randomized (e.g., typically error-prone PCR) mutagenesis used in *in vitro* display [122] is the greater potential for introducing immunogenic sequences or mutations that could potentially decrease the developability of the antibody candidate.

5.5.2.3 Bacterial Display

Microorganisms also have been used for display purposes. Bacterial display, using both Gram-positive [129] and Gram-negative [107, 130–134] bacteria, has been used to generate and select from human antibody libraries, although it has not yet caught on as a widely used technology in the field. Advantages to bacterial display are short replication times, high transformation frequency, and ability to secrete proteins [130, 134]. On the negative side, the bacterial cell wall adds an extra layer of complexity when dealing with surface-displayed proteins. A few bacterial display systems have recently emerged that may help solidify this approach. In the *Escherichia coli* display system, "anchored periplasmic expression" (APEx), scFvs can be displayed on the cytoplasmic membrane of spheroplasts via fusion to a lipoprotein A fragment or bacteriophage gene 3 coat protein (pIII) [130, 132]. APEx also can be used to display full-length, aglycosylated IgG ("E-clonal" antibodies [131, 133]), followed by selection of those antibodies binding fluorescently labeled antigens using fluorescence-activated cell sorting (FACS) analysis [133]. Recently, Lombana *et al.* [134] described a bacterial antibody display (BAD) system in which an lpp mutant of *E. coli* was used to display proteins in the periplasm. Treatment of this mutant with ethylenediaminetetraacetic acid (EDTA) results in the ability of fluorescently labeled antigen to enter through the outer membrane, allowing antibody–antigen interaction in the periplasm. Importantly, after sorting, the cells could be recovered and expanded, allowing multiple rounds of panning in a short time. The BAD system can be used with antibody fragments, whole IgGs, and even bispecific antibodies, and was shown to distinguish between antibodies with subtle expression or binding differences [134].

5.5.2.4 Yeast Display

Yeast display has become one of the most powerful tools for the generation and panning of human antibody libraries and/or for affinity maturation of antibodies

[97, 98, 135]. There are several advantages to yeast display over phage and *in vitro* display, including the use of multicolor FACS to quantify both antibody expression on the yeast surface and binding of fluorescently labeled antigen to it [97, 98, 135], and the expression and secretion of antibodies in cells, which may help to select for both higher expressing, better folding, and properly secreted clones [98]. Two advantages over bacterial display are the ability to display on the surface without having to manipulate the cell wall, and the yeast secretion system which is much more similar to the mammalian secretion system [97, 98]. Yeast display can accommodate all forms of antibodies and fragments, including domain antibodies (dAbs), scFvs, FAbs, and even IgGs, which are secreted and captured [98].

The most widely used system for yeast display is the *Saccharomyces cerevisiae* Aga1p/2p α-agglutinin system, which relies on disulfide bonds to link the GPI-anchored Aga1p protein with the displayed antibody [98, 135, 136]. Boder *et al.* [98] have tabulated the affinities of several antibodies isolated and/or affinity-matured using FACS-based yeast display. Very high affinity antibodies, including some affinity-matured to the sub-picomolar range, have been obtained using a combination of yeast display and FACS [98, 135, 136]. A significant advantage of using a FACS-based system for selections is that the criteria for selections can be manipulated by changing the flow parameters and boundaries, which allows the optimization of binding parameters including, for example, focusing on off-rates [97, 98]. FACS also allows sorting of individual clones and isolation of the single yeast cells, providing the best signals. This display system has been optimized in multiple ways, including the incorporation, in some cases, of magnetic-activated sorting (MACS) to enrich libraries prior to FACS sorting [98, 137]. One down side to yeast display coupled with FACS sorting is dominance by a few FACS-positive clones [138]. Bidlingmaier and Liu [139] have incorporated a high-throughput exon-sequencing step with the FACS-sorted yeast to sample a wider array of clones. Other yeast display systems that have been developed but, at least to this point, not yet widely adapted include an adapter-directed system [140] and an Fc-Sed1p system for *Pichia,* which allows both the display and secretion of the antibody simultaneously [141].

Additionally, human antibody library sizes displayed on yeasts have historically been somewhat limited ($\sim 10^7 - 10^9$ total diversity) due to transformation efficiencies and FACS analysis, which has a maximum processing rate of $\sim 2 \times 10^8$ cells/h [97, 98]. Recently, however, improved technology for electroporation-based transformation of yeasts has increased efficiencies up to 1.5×10^8 transformants/μg of DNA, which is large enough to build libraries with up to 10^{10} diversity [142]. The yeast haploid/diploid life cycle offers another way to get past this limitation, whereby two different yeast strains, one carrying a heavy chain library and the other carrying a light chain library, can be mated to generate combinatorial diversity [143, 144]. Several reviews [97, 98, 135] and a book [145] have been written recently describing details about yeast display, so additional information can be obtained from them.

As mentioned previously, yeast display has been used both to generate antibody leads from libraries and for affinity maturation. Adimab LLC has combined yeast display technology with a well-designed human antibody library to become one of the premier antibody discovery companies. The diversity of their libraries is focused on CDR H3 (example of their H3 diversity, 2.11×10^8) and CDR L3 (example or their L3 diversity, 1.2×10^5) (Table 5.5) and they have strived to make libraries that mimic the pre-immune antibody repertoire [153]. Their library is expressed and displayed as full IgGs, and outputs are FACS-selected followed by sequencing of individual clones [155]. They have developed methods to generate data on developability of their leads [156].

5.5.2.5 Mammalian-Cell-Based Display

Mammalian display would seem to be the most relevant type of display to use for human antibodies, especially because this system would be the most natural system for the expression and secretion of human antibodies, favoring natural folding, stability, lack of aggregation, and similar developability-related features [100, 101]. Additionally, similar to the advantage with yeasts noted previously, FACS analysis and sorting is typically used both to analyze the antibodies on the surface of mammalian cells and to sort and isolate those cells displaying the antibodies providing the best signals [100]. Historically, however, mammalian display has been hampered by the issues of transfection frequency, library sizes, and difficulty in generating clonal sequences in single cells [99–101, 154]. With these limitations, the original primary use of mammalian display was to optimize antibodies using relatively small library sizes [99–101, 154]. In the past few years, however, mammalian-based libraries substantial enough to provide *de novo* hits and leads have been built and used as discovery engines [99–101, 154]. A variety of mammalian display systems now have the capability to generate human antibody leads for discovery programs, a few of which will be described further in the following.

AnaptysBio AnaptysBio has built a mammalian display and antibody optimization system built around the capabilities of activation-induced cytidine deaminase (AID), the enzyme responsible for carrying out somatic hypermutation (SHM) (SHM-XEL™ system) [99]. Initially, the platform was perhaps best used for optimizing and humanizing antibodies that already existed as leads [99, 101]. More recently, however, they have expanded their platform to include the AnaptysBio evolving library of mAbs (ABELmAb Library; Table 5.5) by generating a library of variable gene segments joined with rearranged D regions from naive human donors. Their heavy (6×10^5 diversity) and light (5×10^5 diversity) chain libraries are in episomal vectors that are transfected separately, resulting in a functional library capable of yielding moderate binders that are matured using their SHM platform [101]. Additionally, they designed in the ability of the cells to have displayed IgGs as well as secreted IgGs for assays. Their positive hits typically resulted in cells carrying only a single heavy chain (HC) but two or more light chains (LCs) [101].

Table 5.5 Examples of human antibody libraries and associated display technologies.

Company	Library(ies)	Display and antibody format	CDR and framework source	Library size	mAbs currently in clinical trials	Comments and/or references
Morphosys	HuCAL GOLD®, HuCAL PLATINUM®, Ylanthia®	Phage display; FAb (P3)	Synthetic; multiple frameworks	1.6×10^{10} (GOLD); 4.5×10^{10} (PLATINUM); 1.0×10^{11} (Ylanthia)	At least 23	[146, 147]
Dyax (acquired in January 2016 by Shire)	Dyax library	Phage display; FAb (P3)	Human gene-derived CDR H3 and V_L; synthetic CDR H1 and H2	1.0×10^{10}	At least 11	[148]
Cambridge Antibody Technology (now MedImmune Cambridge)	CAT1.0 and CAT 2.0	Phage display; scFv (P3)	Naive donors	1.4×10^{10} (CAT1.0); 1.3×10^{11} (CAT2.0)	At least 18	[16, 149]
BioInvent	n-CoDeR®-scFv, n-CoDeR®-Fab-Lambda, and n-CoDeR®-Fab-Kappa	Phage display; scFv and FAb (P3)	Human gene-derived CDRs; V_H and V_L each single frameworks	$10^9 - 10^{10}$	At least 5	[150, 151]
Janssen library	pIX Library	Phage display; FAb (P9)	Synthetic, based on structures of several antibodies	$\sim10^{11}$	At least 3	[108, 152]
Adimab	Yeast display	Full IgG	Yeast display and FACS sorting	$>2\times10^8$ HC[b)] $>1\times10^5$ LC[b)]	None known	[153]
Anaptys	Mammalian display with SHM-XEL; ABELmAb Library	Full IgG	Mammalian display	6×10^5 HC; 5×10^5 LC	At least 1	[99, 101]
Vaccinex	ActivMAb; library in vaccinia virus	Full IgG fused to surface protein	Mammalian display; Vaccinia display	5×10^6 HC; $\sim10^3$ LC	None known	[154]

Abbreviations: CDR, complementarity-determining region; FAb, fragment antigen binding; HC, heavy chain; IgG, immunoglobulin G; LC, light chain; scFv, single chain fragment variable; V_H, variable heavy; V_L, variable light.

a) With virtually all large pharma and biopharma companies, as well as many academic institutes, possessing their own human antibody libraries, there are far too many examples to list. Thus, the human antibody libraries included here represent some of those that are either available for licensing or have antibodies currently in clinical trials.

b) Diversity focused on CDR H3 and CDR L3.

Vaccinex Using a method called "trimolecular recombination," Vaccinex has developed large cDNA heavy chain and light chain libraries inserted into the thymidine kinase gene in a vaccinia virus vector platform [154]. For mammalian display, they utilized larger heavy chain libraries (5×10^6 diversity) in conjunction with smaller ($\sim 10^3$ diversity) light chain libraries to obtain hits based on the HC. Once hits were obtained, the fixed heavy chains were then paired with larger light chain libraries to generate leads. Another approach, which takes advantage of the fact that they are using vaccinia virus as the vector, is to carry out a very large *in vitro* bead-based panning of the library as a virus, followed then by infection of mammalian cells and mammalian display of those antibodies for maturation [154]. Vaccinex first used these technologies to convert murine IgGs to human IgGs using a process they call "V gene replacement" [154], essentially the same process as "guided selection," the process C*a*T used to isolate the fully human antibody adalimumab (Humira®), using a murine mAb as the original template [17]. An example of this process was the V chain replacement of a murine4 anti-CXCL13 mAb to generate the fully human anti-CXCL13 IgG1 5261 [157]. More recently, they have used their platform, collectively called "ActivMAb," to generate *de novo* fully human hits and lead for antibody discovery [154].

Immunologix (Now Intrexon) Tonsils are known to be an excellent source of B-lymphocytes. Duvall *et al.* [14, 158] used EBV-immortalized tonsil B-lymphocytes, most of which were $CD45^+$ $CD19^+$ $CD20^+$, obtained from tonsil tissue of non-vaccinated subjects, to generate "naive" libraries of human IgG antibodies displayed by the B cells. The natural IgG libraries produced in these B cells was diverse enough to be used to screen against targets for isolation of *de novo* discovery antibodies [14, 158].

Antibody Retroviral B-Lymphocyte Display "Retrocyte Display®" Breous-Nystrom *et al.* [159] have described an IgG-mammalian display system built around Abelson murine leukemia virus (A-MuLV)-transformed $CD79a^+$/$CD79b^+$ pre-B cells. They generated separate libraries of variable heavy chain sequences derived from naive donors and a germline-based κ light chain library, which were sequentially used to infect pre-B cells, resulting in a combined library with diversity in the 10^9 range. They used the library to screen for mAbs targeting DR6 and demonstrated the ability to obtain sub-nanomolar human IgG hits and leads [159].

Transpo-mAb Display Very recently, a new IgG-mammalian display system was described by scientists at NBE Therapeutics AG, which utilizes the large cargo capacity *PiggyBac* transposon [160] to generate libraries in $CD79a^+$/$CD79b^+$ A-MuLV clone L11 transformed pre-B cell clone 63-12 derived from RAG-2 knock-out mice with approximately 10^8 diversity [161]. Besides diversity, another problem they experienced, which is common to mammalian display systems, was having a single antibody clone per cell. By adjusting plasmid concentrations and transformation conditions, they were able to optimize the system, which was used to humanize and murine IgGs against multiple target antigen [161].

Innovative Targeting Solutions (ITS) ITS has built into HEK293 cells the natural antibody rearrangement and maturation system normally expressed in B cells, including V(D)J recombination and SHM [162]. With this platform, called HuTARG™ technology, they have been able to generate both hits and leads against difficult targets such as G-protein coupled receptors (GPCRs) and T-cell receptors, as well as optimize antibodies focusing only on CDRs. This platform, which is still relatively new, has the potential to be a very exciting mammalian displayed human antibody library platform for antibody discovery in the future.

5.5.3 Human Antibody Libraries

In the early 1990s, it was found that combinatorial libraries derived from the B cells of naive human donors with a diversity of 10^7 or more antibody clones yielded antibodies that would bind to human proteins; thus, these were the equivalent of "self-antibodies" [102, 163, 164]. This led to the concept of building libraries so that antibodies could be isolated *in vitro,* thereby "bypassing" the immunization step [102]. Human antibody libraries are generally sourced from one of four types of sources: (i) antibody genes recovered by PCR from human antibody-producing B cells taken from non-immunized subjects [9, 16]; (ii) antibody genes recovered by PCR from human antibody-producing B cells taken from immunized subjects [69, 74, 86]; (iii) synthetic libraries constructed on the sequences, structures, or combinations of both sequence and structure of human antibodies [9, 104]; or (iv) combinations of synthetic and natural gene approaches [9, 103]. Because theoretical antibody diversity could be as great as 10^{20} [95], limits to library size and functional diversity are generally dictated by the limitations and physical size of the display system used.

5.5.3.1 Human Antibody Libraries of Natural Antibodies from B Cells

For the first human antibody libraries, McCafferty *et al.* [165] used PCR technology to recover antibody variable genes from hybridomas and B cells, which were then inserted as single-chain Fv (scFv) fragments into the pIII gene of fd phage for display on the tip of the phage. Marks *et al.* [102] built a combinatorial library of $>10^7$ with heavy and light chain antibody genes, which was used to demonstrate that immunization of animals, and hybridoma technology could be bypassed completely. Since then, multiple human antibody gene-derived libraries have been built using naive donors [16, 163, 166]. Hoogenboom *et al.* [164] also demonstrated that libraries of Fab fragments could be displayed as alternatives to scFvs. The most significant observation in the early 1990s was that combinatorial antibody libraries from naive human donors with at least 10^7 diversity were sufficient to yield specific antibodies that would bind to human proteins ("self-antibodies") [102, 164].

Human antibody libraries generated by RT-PCR of antibody genes from B cells of immunized subjects or convalescent patients, specifically seeking antigen-specific antibodies, also have been used, mostly to generate human

antibodies against pathogens or cancer antigens as described in Section 5.4.3 [74, 86].

5.5.3.2 Synthetic Libraries

Once it was demonstrated that human antibody genes could be used to build *in vitro* antibody libraries for panning, several groups designed synthetic antibody libraries based on antibody genes, rearrangements, and maturation for the same purpose [104, 108, 146, 147, 152, 167–169]. Finally, antibody libraries also have been designed and built using a combination of natural antibody gene segments and synthetic segments [148, 150, 151]. Fellouse *et al.* [169] showed that a synthetic CDR H3 with diversity dictated mostly by just two amino acid residues, serine and tyrosine, was enough to generate reasonably strong binders.

It is generally considered that the probability of finding novel antibodies in a library increases in proportion to its size [170], although arguments have been made that quality, antibody-like sequence diversity [153], and percent of productive clones [147] are critical factors in the library's success. One of the premier libraries today would likely be the Ylanthia library at Morphosys, which has 1×10^{11} diversity [147] (Table 5.5).

At the other end of the size spectrum, one of the most widely accessed libraries today is the relatively modest-sized Adimab yeast display library, which focuses its diversity on CDR H3 ($>2 \times 10^8$ diversity) and CDR L3 ($>1 \times 10^5$ LC diversity; Table 5.5 [153]) and utilizes powerful FACS-based selections to isolate the best antibodies. Thus, size, quality, and the ability to select positive clones are all intermingled when comparing library success.

There are too many new human antibody libraries to mention them all here, but a few employ novel technologies or concepts that are highly worthy of mention. The most interesting of these are the pH-dependent antibodies that have been engineered using synthetic libraries [171–174]. Igawa *et al.* [171] demonstrated the power of their "sweeping antibodies," which were designed to bind targets in circulation at neutral pH and then release those targets for degradation in the reduced pH of the endosome [171, 172]. They found that their sweeping antibodies could remove >50–1000-fold more antigen from circulation than a conventional antibody [171, 172]. Additionally, these antibodies functioned to remove the antigen from circulation even when conventional antibodies could not do so. Critically, this astounding discovery was achievable only using synthetic library approaches. Other investigators also have reproduced these findings [173, 174], and Schröter *et al.* [174] have developed a His-scanning library approach in conjunction with yeast display to isolate new pH-dependent antibodies.

Human antibody libraries have been formatted in several different way, including as scFvs [16], FAbs [146, 147], single-chain FAbs [175], and full IgGs [131, 133, 153, 154]. The biggest issue with the use of scFvs and FAbs is that, for most testing, they need to be converted to full-length IgGs [11]. While there are several newer and improved methods for making these conversions quickly and efficiently [113, 114], they still take time and effort. There are advantages and disadvantages to each format, and arguments have been made for each of the formats, but it is

generally recognized that if an IgG can be displayed with high efficiency, it is likely to be the best format because it saves having to reform the antibody for testing and it provides the paratopes in their most natural format. Additionally, special libraries of human single-dAbs have been built and used for panning [176–178]. Rouet *et al.* [179] recently generated a VHH library that was demonstrated to have properties equal to that of camelid VHH domains, that is, high stability and lack of aggregation. At least four dAb-derived antibodies are now in clinical trials, including GSK 2862277, an anti-TNFRI dAb Phase II candidate for acute lung injury; BMS-986090 and BMS-986004, anti-PD1 and PD-L1 dAbs for immuno-oncology, respectively; and BMS-931699, an anti-CD28 PEGylated dAb for treatment of systemic lupus erythematosus (SLE). Two domain antibody companies, Domantis (now part of GSK) and X-BODY, Inc. (now part of Juno), were leaders in the generation of human dAbs.

5.5.3.3 Advantages of Libraries over Immunization-Based Approaches

The major advantage of *in vitro* human antibody libraries over immunization-based approaches is the ability to manipulate the epitope (antigen), paratope (antibody), and selection conditions to generate antibodies to epitopes, which would be difficult to achieve through immunization [180]. Additionally, *in vitro* selection allows the ability to isolate antibodies against antigens that are not typically immunogenic (e.g., lipids, carbohydrates), to generate antibodies that bind cells and cell-surface targets including GPCRs and ion channels, to modulate panning conditions to recover rare antibodies, to modify libraries and/or selection conditions to favor stable, non-aggregating antibodies [9–11, 181], and to isolate antibodies against antigens or pathogens that might be toxic to animals [9–11].

Perhaps one of the most interesting new technologies developed using human antibody libraries is the "sweeping antibody" technology [171, 172]. This technology has been incorporated into an anti-IL-6-receptor mAB named SA237, and is currently in Phase III clinical trials by Chugai. As noted previously, these types of designer libraries and the technologies associated with them could not be accomplished easily using any type of immunization strategy.

5.5.3.4 Disadvantages of Libraries over Immunization-Based Approaches

There are some disadvantages of human antibody libraries over immunization approaches as well. The first is that initial selection often results in antibodies that are not of high enough affinity, so affinity optimization may be required in many cases [182]. Additionally, as mentioned previously, cycles of conversion of scFv or FAb fragments to full-length IgG take time and effort [11, 113, 114]. Much has been made about the potential for phage-library-derived human antibodies to have a higher aggregation potential [183, 184], but in recent years library quality and the ability to analyze and correct such aggregation tendencies have improved to the point where this no longer an issue. In some cases, now human antibody libraries are being designed specifically with the lack of aggregation tendencies in

mind. Finally, libraries built in phage and other *in vitro* methods of display do not incorporate the natural mechanism of SHM, the process by which B cells affinity-mature antibodies [99–101].

Antibodies derived from cells such as hybridomas, mammalian display, or yeast display have several potential advantages over antibodies from phage and *in vitro* display-based libraries, including the expression, folding, and secretion processes of the cells (e.g., *in vivo* "editing"), which tend to favor those antibodies that fold, express, and secrete well [183, 184]. Cell-based human antibody libraries, such as those made in yeast and mammalian cells, are thought to have the advantage over phage or *in vitro* displayed libraries of generating antibodies that have better solubility characteristics, are less aggregation-prone, and naturally express better [14, 99–101], although, to balance, this selection from *in vitro* systems has evolved to a point where selection and engineering for solubility and expression are becoming routine. Finally, certain cell-based antibody library systems such as those at Anaptys [99, 101] and Innovative Targeted Solutions [162] have been built to take advantage of the natural antibody maturation process of SHM as well as the other features mentioned previously.

5.5.3.5 Clinical and Marketed Antibodies from Human Antibody Libraries

Five antibodies have now been approved that were originally derived from human antibody display libraries: the anti-TNF-α mAb Humira®, the first fully human antibody to be approved, the anti-BLys mAb Benlysta® (belimumab), the anti-anthrax PA antigen Abthrax™ (raxibacumab), the anti-VEGFR2 mAb Cyramza™ (ramucirumab), and Portrazza™ (necitumumab), an anti-EGFR mAb (Table 5.1). Another eight fully human antibodies from phage libraries are currently in Phase III clinical trials (Table 5.2). Thus far, human antibodies derived from transgenic mice harboring human antibody genes have been much more successful than library-derived human antibodies (Tables 5.1 and 5.2). While Humira® was the first fully human antibody to be approved for marketing, a total of 28 human IgGs from transgenic animals are either now approved or in Phase III clinical trial as compared with only 13 approved or Phase III clinical candidates from human antibody libraries (Table 5.2).

In terms of productivity, Morphosys appears to have the most library-derived fully human antibodies in clinical trials, with at least 23 antibodies in the clinic, most of them via partners who have licensed the use of their HuCAL [146, 168] libraries. The Dyax (now a subsidiary of Shire plc) library [148], the C*a*T (now MedImmune Cambridge, a subsidiary of AstraZeneca) libraries [16], and the BioInvent n-CoDeR® libraries [150, 151] also have multiple human phage display-derived antibodies in clinical trials. Other human antibody libraries contributing current clinical candidates include the Swiss Federal Institute of Technology (ETH-2 and ETH-2 GOLD) library [185], the pIX phage human antibody library [108, 152] at Janssen R&D, the Adimab yeast display library [153], the Xoma ADAPT™ platform and library [186], the Micromet library [187], the Domantis (now part of GlaxoSmith Kline [GSK]) domain antibody library [178], and the NovImmune scFv library [188], among others. Including Phase I

through III clinical trials, there are now at least 75 known examples of human phage displayed antibodies currently in clinical trials worldwide. Additional details and information on phage display and strategies for the construction of human antibody libraries can be found in several recent reviews, including Ponsel *et al.* [9], McCafferty and Schofield [10], Zhou *et al.* [11], Nixon *et al.* [96], and Tohidkia *et al.* [105].

5.6
Human Antibodies from Transgenic Animals

5.6.1
Transgenic Rodents Producing Human Antibodies

By far the most widely used and most successful approach to date for obtaining fully human antibodies is through the use of transgenic mice in which human antibody genes have been inserted, replacing the function of the resident rodent antibody genes [5–8, 189]. In those animals, immunization with a foreign antigen results in the generation of antigen-specific human antibodies, derived from the insert human antibody genes [5–8, 189]. The history and genesis of the original "humanized" transgenic mice, the Abgenix "Xenomouse" [190] (now owned by Amgen) and the Medarex "HuMAb mouse" (now owned by Bristol-Myers Squibb; BMS) have been detailed several times [5–8], so will not be covered here. A third line of first-generation transgenic mice producing human antibodies, the "KM mouse" strain [191], also was generated by Kirin and cross-licensed with Medarex (now BMS). The Kirin KM mouse is notable in that all human classes and isotypes, including IgG1-4, IgA1 and 2, IgD, IgM, and IgE, are generated by those mice [191]. Table 5.6 gives an overview of many of the human antibody-producing transgenic animal platforms available today.

A significant limitation of the first generation of transgenic mice producing human antibodies was that the antibodies were entirely human IgGs with human Fc domains [5–8]. It was shown later that the human Fc did not interact optimally with mouse FcγR on the surface of B cells, which resulted in a relatively modest B cell and antibody maturation process [192, 193]. Over the past decade or so, several new second-generation transgenic rodent platforms producing human antibodies have been constructed in which the antibodies possessed the rodent Fc, which leads to greater potential for antibody maturation [192–197]. The leader in this second generation of "humanized" mice was Regeneron, who constructed the VelocImmune mouse [192, 193]. Since then, many other companies have built transgenic rodent platforms producing human antibodies using the same general theme of human variable sequences coupled with native, or homologous, Fc sequences in one format or another (Table 5.6). Several of these companies offer various twists on the concept such as a single light chain to make common light chain bispecific antibodies (e.g., Merus BV's MeMo mouse; OMT's

Table 5.6 Companies with transgenic animal platforms producing human antibodies upon immunization.

Company	Location and date founded	Name/description of technology(ies)	Comments and references
Medarex (now BMS, since 2009)	Princeton, NJ (1987)	HuMAbs, UltiMAbs, TC Mouse, KM Mouse[a)]	Human Fc [6, 7]
Kyowa Hakko Kirin	Tokyo, Japan (1949)	KM Mouse	Produces human antibodies of IgG1,2,3,4, IgA1,2, IgD, IgM, IgE classes; human Fc [191]
Abgenix (now Amgen, since 2006)	Fremont, CA (1996)	XenoMouse	Mice producing specific isotypes, of which the IgG2-producing strains have been most prolific; human Fc [5, 8]
Regeneron	Tarrytown, NY (1988)	VelocImmune Mouse	Mouse Fc; improves Ig maturation in B cells [192, 193]
Open Monoclonal Technology, Inc. (OMT; proposed to be purchased by Ligand Pharmaceuticals in Dec 2015)	Palo Alto, CA (2007)	OmniRat®, OmniMouse®, OmniFlic™ (together, called OmniAb™)	Mouse and rat Fc; improves Ig maturation in B cells [194, 195]
OMTT (now TeneoBio)	Palo Alto, CA (2015)	UniRat, UniAbs, UniDAbs	Spinout from OMT, Inc.
Kymab	Cambridge, UK (2009)	Kymouse HK	Mouse Fc; improves Ig maturation in B cells; common light chains [196]
Merus BV	Utrecht, Netherlands (2003)	MeMo mouse; BicLonics®	Mouse Fc; improves Ig maturation in B cells; common light chains
Trianni, Inc.	Emeryville, CA (2010)	TRIANNI Mouse	Mouse Fc; improves Ig maturation in B cells
Ablexis	San Francisco (2009)	AlivaMab Mouse platform	Mouse Fc; improves Ig maturation in B cells
Crescendo Biologics	Cambridge, UK (2013)	HumaBody™	V_H technology
Crystal Bioscience, Inc.	Emeryville, CA (2008)	HuMab SynV Chicken and GEM Assay	—
Harbour Antibodies BV	Cambridge, MA (started in Rotterdam, Netherlands, 2006)	Human H2L2 mAbs	Normal Human mAbs and V_H-based single chain mAbs (HCAbs) [197]
Creative BioLabs	Shirley, NY	FHAT™, FHAT mice	—
arGEN-X[a)]	Breda, Netherlands	SIMPLE (superior immunodiversity with minimal protein lead engineering)	Camel antibodies that are claimed to have "virtually human sequences and structure" [198]

a) Not human antibodies, *per se,* but close enough in sequence and structure to potentially be claimed as "human-like" antibodies based on sequence identity, which has been proposed to be part of the definition of "human antibodies" in the future [21].

OmniFlic), or heavy chain only (domain) antibodies (e.g., Crescendo Biologics, Harbour) (Table 5.6).

5.6.2 Recovery of Antibodies from Transgenic Animals

For decades, hybridomas have been used to recover antigen-specific antibodies from mice [199] and rats (Y/0 platform [200]) after immunization. The greatest barrier to the generation of human antibodies by species other than mice and rats has been the absence of good hybridoma platforms for those other species. Historically, rabbit hybridomas did not work well until methods to fuse the immortal line 240E-W2 with rabbit B cells were developed [201, 202]. Additionally, the MU-H1 avian myeloma cell line was very recently developed as a hybridoma partner for chicken B cells [203]. Now, however, with the improvement in technologies to obtain antibodies directly from B cells either by RT-PCR cloning or by generation of small immune libraries (see Section 5.4.3), the barrier to generating and retrieving human antibodies from various animals has been lifted. Thus, in theory, one could clone human antibody genes into many different vertebrate species for the generation of antigen-specific human monoclonal antibodies via transgenic animals.

The chicken B cell line DT40 has been modified by Crystal Biosciences to produce human mAbs that are recognized and diversified using the chicken antibody recombination machinery [204]. Leighton *et al.* [204] used deep sequencing to find that the human antibody genes formed in chickens were the result of both SHM and gene conversion, the latter process not occurring in natural human antibody generation. The gene conversion processes were more skewed toward CDR1 and CDR2, whereas SHM was predominant for maturation of CDR3 [204]. Using that information and technology, Crystal Biosciences has now created a first-generation transgenic chicken, called SynV, in which the natural chicken V_H and V_L genes have been replaced with human V_H and V_L genes, respectively (http://www.crystalbioscience.com/). One of the key advantages to this platform is that chicken protein sequences are further away from human protein sequences phylogenetically than are rodent protein sequences. Thus, it would be expected that human proteins should be more immunogenic in chickens than they would be in rodents.

5.6.3 Success with Human Antibodies from Transgenic Rodents

The fully human anti-EGFR mAb, panitumumab (Vectibix®; Amgen), from the Abgenix Xenomouse® platform, was the first transgenic-mouse-derived human antibody to be marketed when the FDA approved it in 2006 (Table 5.1). Currently, there are 12 fully human mAbs derived from transgenic mice that have been approved for marketing by at least one of the major regulatory agencies (Table 5.1).

Of the approved antibodies from transgenic mice, eight are from the Medarex HuMab® platform, three are from the Abgenix Xenomouse platform, and one is from Regeneron's VelocImmune® platform.

Additionally, there are another fully human 16 mAbs in Phase III clinical trials that have been generated in transgenic rodents (Table 5.2); there are also at least 56 more known human mAbs in Phase I or Phase II clinical trials from transgenic animals. Thus, there are at least 84 human mAbs in clinical trials or approved for marketing that have been derived from transgenic animals, and likely many more since many of those in earlier stage of clinical trials often are from unknown sources. Of these, at least 30 of them are derived from Medarex Humab mice, more than 20 are from the Abgenix Xenomouse® platform, more than 15 are from Regeneron's VelocImmune® platform, and at least 5 (and likely, many more) are from the Kirin KM® mouse platform. Of the new second-generation platforms, it is notable that Merus MCLA-128, an anti-Her2 (ErbB2)-anti-Her3 (ErbB3) bispecific antibody with common light chains, is in Phase I clinical trials.

5.6.4 Potential Importance of Transgenic Farm Animals Producing Human Antibodies

The human intravenous immunoglobulin (IVIG) industry, which in 2015 had approximately an $8 billion market size, requires substantial serum from many human donors, followed by purification of the human IgG from the pooled sera. Treatments are very expensive, and the source of serum is sometimes inconsistent [1]. Additionally, when "specific IVIG," that is, IVIG from vaccinated donors or from convalescent patients, is desired, it is generally difficult to source and is limited to those very few individuals who have received the pertinent vaccines or exposure. Moreover, vaccination to generate specific IVIG is currently limited to those vaccines that have been approved for marketing for regulatory and safety reasons. Thus, it has been desired for a very long time to have alternative sources of human IVIG for treatment of individuals for whom other treatments might not be available [205, 206]. This is especially true for the protection of subjects from potential serious pathogens such as Ebola, hantavirus, or similar serious disease threats. To achieve this goal, efforts have been made to generate transgenic rabbits, chickens, pigs, and cattle, in which their normal Ig sequences have been replaced with human Ig sequences [206]. Matsushida *et al.* [207] reported the engineering of cattle with human antibody genes for the production of polyclonal human antibodies. They demonstrated that the cattle could achieve up to 15 g/l of human antibodies in their serum and that antigen-specific antibodies could be recovered after immunization [207]. Even though their effort was for the generation of polyclonal antibodies [207], with modern molecular RT-PCR technologies those antigen-specific genes could be retrieved relatively easily for generation of monoclonal antibodies.

As an example of the use of transgenic cattle to generate "specific Igs," Hooper *et al.* [208] described the generation of transgenic cattle producing human IgGs

that were vaccinated with a hanta virus DNA vaccine. The human antibodies produced by those cows were protective in rodent models. Similarly, Bounds *et al.* [209] described the vaccination of transgenic cattle with a DNA-based Ebola virus vaccine. The antibodies from these cattle also protected rodents in Ebola infection models [209]. These are key advances that should stabilize the supply of both normal IVIG and specific IVIG markets in future years and should help drive down the costs of IVIG treatments. Additionally, specific IVIG to antigens for which humans cannot be vaccinated (e.g., unapproved hanta virus, Ebola virus, Zika virus, etc. DNA vaccines) should help greatly to improve global health.

5.7 Summary and Future Directions

Approximately a third (>200) of the more than 600 mAbs currently either approved for marketing or in clinical development are fully human antibodies, derived either from transgenic rodents expressing human antibody genes or large naive human antibody libraries or directly from human B cells. The number of libraries, transgenic animals, and direct B cell technologies now available has made it relatively straightforward to discover high-quality fully human antibodies for clinical development. There are two significant ramifications of this. (i) First, virtually any company with even modest resources can get into the game and generate fully human clinical candidate mAbs, which will increase the pool of antibodies available and should increase both the quality of those that move forward and the competition in clinical development and marketing. (ii) The commoditization of human antibody discovery will push the field toward a more mature contract-research-like approach similar to what has already happened in the small-molecule space. The emphasis will be even greater to use fully human antibody approaches to access novel biology, to generate more complex platforms (e.g., bispecifics, antibody–drug conjugates, antibody–cytokine fusions, etc.), and to address newly discovered unmet medical needs. Finally, the development of technologies to generate fully human antibody responses in large farm animals may help to stabilize and grow the IVIG market, particularly with specific IVIG derived from vaccinated cattle.

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6
Beyond Antibodies: Engineered Protein Scaffolds for Therapeutic Development

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6.1
Introduction

The rapid rise of protein therapeutics and biologics has been largely due to the development of antibody-based drugs. As described in Chapters 2 and 3, antibodies are robust molecular scaffolds that can be customized to bind a diverse range of extracellular targets with relatively high affinity. Monoclonal antibodies, in addition to their ubiquitous use in flow cytometry, immunohistochemistry, and diagnostics, have become prominent as clinical therapeutics. In particular, antibodies are now part of the primary treatment regimen for many types of cancer, inflammatory bowel disease, rheumatoid arthritis, and cardiovascular disease, among others [1]. As of 2014, there are 47 monoclonal antibody drugs and countless other antibody products available in the United States and Europe, comprising a market of almost US$75 billion [2]. Methods to screen for antibodies and antibody-binding domains, including hybridoma technologies, transgenic animals, and display technologies, have become standard in the pharmaceutical industry, and are described in Chapter 5. However, the limitations and challenges of antibodies have motivated the development of so-called alternative scaffolds. These protein- and peptide-based mimetics can accomplish similar goals as antibodies but possess different molecular sizes and biophysical attributes, conferring alternate pharmacokinetic and pharmacodynamics properties that make them integral to the growing protein therapeutics industry.

More than 20 different alternative scaffolds have been investigated, and this number continues to grow [3]. In this chapter, we will discuss the structural features, advantages, and commercialization path of some of the most prominent non-antibody scaffolds (summarized in Table 6.1). For convenient reference, we have organized the discussed scaffolds by the homogeneity of their secondary structure. We conclude the chapter by presenting considerations for

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Table 6.1 Characteristic qualities of prominent non-antibody scaffolds.

Scaffold	Secondary structure	Example target (binding affinity)	MW (kDa)	Disulfide bonds	Company
Monobodies	β	Abl-SHR (9 nM) [4]	10	No	Adnexus/Bristol-Myers Squibb
		VEGFR2 (11 nM) [5]			
		EGFR (250 pM) [6]			
Fynomers	β	MSA (26 nM) [7]	7	No	Covagen/Cilag GmbH International
		IL-17A (1.8 nM) [8]			
Anticalins	β	Fluorescein (~100 nM) [9]	20	No	Pieris Pharmaceuticals
		PSMA (500 pM) [10]			
		VEGF-A (25 pM) [11]			
Nanobodies	β	Lysozyme (5 nM) [12]	15	Yes	Ablynx
		Carbonic anhydrase (20 nM) [13]			
		IL-6R (0.19 pM) [14]			
DARPins	α	MBP (4.4 nM) [15]	3.5 (ankyrin repeat)	No	Molecular Partners
		BCL-2 (30 pM) [16]			
		HER2 (~100 pM) [17]			
Affibodies	α	Taq polymerase (2 μM) [18]	6	No	Affibody
		HER2 (22 pM) [19]			
		TNF-α (500 pM) [20]			
Avimers	Mixed	IL-6 (25 nM) [21]	4 (A domain)	No	Avidia/Amgen
Kringle domains	Mixed	DR5 (58 nM) [22]	15	Yes	N/A
		TNF-α (3.8 nM) [22]			
Cyclotides/knottins	Mixed	$\alpha_V\beta_3$, $\alpha_V\beta_5$, $\alpha_5\beta_1$ (~1 nM) [23]	< 10	Yes	Various commercial entities
		CTLA-4 (~300 nM) [24]			
		Hdm2/HdmX (~10 nM) [25]			
Kunitz domains	Mixed	***Kallikrein (10 pM)*** [26]	7	Yes	Dyax
GP2	Mixed	Lysozyme (1 nM) [27]	5.2	No	N/A
		EGFR (18 nM) [27]			

Drug targets in bold/italics indicate candidates currently in clinical trials.

the development and application of alternative scaffolds as next-generation engineered protein therapeutics.

6.2 Motivation for Developing Antibody Alternatives

Antibodies are large biomolecules with an average molecular weight of 150 kDa [28]. This considerable size is above the cutoff for glomerular filtration (60 kDa) [29], conferring long blood circulation times which are desirable for therapeutic applications. However, the large size of an antibody can often be a liability for applications such as noninvasive *in vivo* molecular imaging. Effective imaging agents are typically small molecules or proteins that produce a high signal-to-noise ratio by binding to their target with high affinity, with the unbound probe clearing rapidly from the body through liver or kidney filtration and excretion. In addition, a larger molecular size has also been correlated with poor tissue penetration, especially for tumors. The stroma between tumor cells is often filled with dense interstitial matrix molecules that slow down the diffusion of larger particles [30]. In addition to size-related limitations, antibodies can sometimes suffer from production challenges. Correct assembly of antibody chains and overall molecular stability rely on disulfide linkages and post-translational glycosylation [31]. These structurally important disulfide bonds require antibodies to be produced in nonreducing environments. The presence of glycosylation increases batch-to-batch variation and often necessitates expression in mammalian cells that contain the requisite post-translational machinery [32]. In contrast, alternative scaffolds are roughly an order of magnitude smaller than antibodies, their molecular sizes can be tuned by modification, and they can be produced by microbial expression or in some cases chemical synthesis. In addition, they have high thermal and proteolytic stability equal to or surpassing that of antibodies. These properties have garnered great interest in the development of alternative scaffolds as antibody alternatives for diagnostic and therapeutic applications.

Antibodies contain flexible loop structures, termed complementarity-determining regions (CDRs), that are naturally mutated by the immune system to bind millions of specific antigens, highlighting antibodies as robust molecular scaffolds for protein engineering and drug discovery applications. Non-antibody scaffolds that also have the ability to tolerate substantial mutation and retain their folded structure can be developed into high-affinity binding proteins. The process of engineering alternative scaffolds to bind to a target of interest is similar to that performed for antibody engineering. Specifically, diverse protein libraries are constructed using degenerate oligonucleotides to randomize loops or residues that form a contiguous binding face within a parent gene. The resulting DNA library is then transformed for display on the surface of a host cell such as yeast, bacteriophage, *Escherichia coli,* or mammalian cells. Cell-free display systems involving functional mRNA, DNA, and ribosomal machinery can also be used. The displayed library of protein variants is screened to identify high-affinity

binders against the soluble target protein using a variety of methods including flow cytometric sorting or panning. A more detailed review of these strategies can be found in Chapters 5 and 7.

6.3 Non-antibody Scaffolds with Homogenous Secondary Structure

Many alternative scaffolds contain homogenous secondary structure that helps stabilize the protein. Among the first scaffolds to be characterized were those inspired by the β-sheet sandwich that makes up the antigen-binding domains of antibodies. The β-sheet sandwich in antibodies is connected by flexible CDR loops, which can accommodate a large number of mutations. Other alternative scaffolds contain mostly α-helical secondary structure. This section will discuss examples of scaffolds in both categories.

6.3.1 Scaffolds Comprised of β-Sheets

6.3.1.1 Monobodies

Monobodies are derived from the human fibronectin type III (FN3) protein. Fibronectin is an essential glycoprotein that binds to many extracellular matrix components, including collagen and integrins, and mediates cell–cell interactions. Its unique ability to bind proteins and sugars suggested it might be an ideal candidate for an alternative scaffold. Full-length fibronectin is a high molecular weight protein that consists of two nearly identical ~250 kDa subunits linked together by a disulfide bond pair. The protein consists of Type I–III domains, each of which is involved with a certain subset of binding interactions. In 1998, Koide and colleagues published results of the first monobody, generated from the 10th repeating domain of FN3, which they engineered to bind ubiquitin using a phage display system [33]. The structure of the molecule pays homage to the antibody-binding domain by taking advantage of a β-sheet sandwich. However, instead of the nine β-strands that make up an antibody V_H binding region, the FN3 domain has only three β-strands oriented in a similar manner (Figure 6.1a). The three connecting β-sheet loops can be mutated to create a naïve library for screening or for affinity maturation against a target of interest [33].

The 10th FN3 domain is only 92 amino acid residues long and has a molecular weight of ~10 kDa [34]. In addition, the 10th FN3 domain is monomeric and does not contain any disulfide linkages. This simple structure makes monobody engineering compatible with almost any screening method, including phage display, peptide–ribonucleic acid fusion, and the yeast two-hybrid system [35]. Moreover, monobodies can be recombinantly expressed in bacteria at high yield and have been shown to have high thermal stability, with melting temperatures over 80 °C [36]. Since monobodies can be produced in the cytoplasm of almost any cell, the proteins can be encoded into intracellular inhibitors. As an example, after

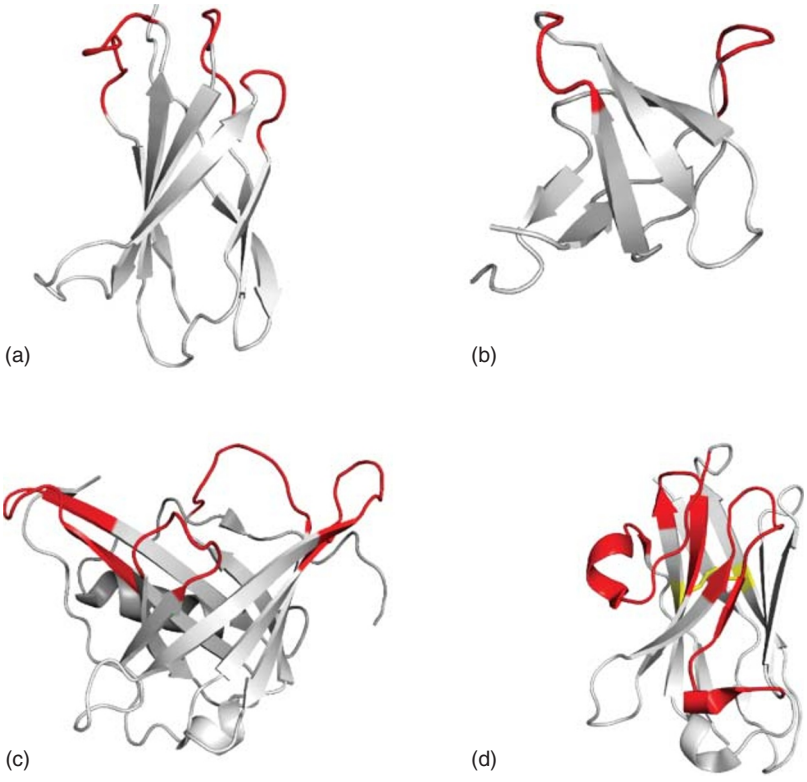

Figure 6.1 Non-antibody scaffolds with β-sheet secondary structure. Engineered variable domains are highlighted in red. (a) Monobody/Adnectin/FN3 domain (PDB: 1FNF); (b) Fynomer/SH3 Domain (PDB ID: 1M27); (c) Anticalin/Lipocalin (PDB: 2HZQ); (d) Nanobody/VHH Domain (PDB: 4KRL).

transfection with an expression vector encoding for a monobody inhibitor of the SHP2 SH2 domain, cells were able to produce this protein which inhibited the BCR–ABL signaling network [37].

Other researchers involved in the development of FN3-based scaffolds include Lipovšek (Adnexus, BMS), who made seminal contributions to demonstrating the utility of mutating the loop and framework regions of the FN3 scaffold [5]. O'Neil and colleagues (Janssen) developed a platform based on the tenascin-C protein, with added mutations to increase stability. A library created from this scaffold with randomized loop regions and portions of the β-strands was shown to be useful for isolating high-affinity binders called Centyrins [36, 38]. Wittrup and colleagues used yeast display to search an expanded sequence space of FN3 domain mutants that included longer loops. In this study, single digit picomolar binders were isolated without compromising the scaffold stability [39]. The Koide lab recently created an alternate FN3 library with diversified regions in framework domains in addition to the original β-strand loop regions.

Monobodies from this library (called side binders) were shown to have a concave binding surface that maximized monobody–target surface area and improved the original fibronectin scaffold [4]. Use of the monobody scaffold and its variants has resulted in engineered binders to EGFR [6], Abelson (Abl) kinase SH2 domain [40], IL-23 [41], and many other therapeutic targets.

Monobodies became a commercial entity for therapeutics development upon the founding of Adnexus in 2002. In 2007, Bristol-Myers Squibb (BMS) acquired Adnexus and helped continue the advancement of adnectin-based therapeutics. The acquired company's flagship oncology drug Pegdinetanib (Angiocept®), is a selective antagonist for vascular endothelial growth factor receptor 2 (VEGFR2) that is currently in Phase II clinical trials. BMS has also started developing a biologic that could help lower low-density lipoprotein (LDL) cholesterol levels in patients with cardiovascular disease. Their lead molecule, BMS-962476, is an adnectin that binds to proprotein convertase subtilisin kexin-9 (PCSK9), a protein secreted by the liver and is responsible for regulating LDL levels [42].

6.3.1.2 SH3 Domains/Fynomers

The Src-homology 3 (SH3) domain is a binding motif found in proteins involved with signal transduction, subcellular localization, organization of the cytoskeleton, or internalization of membrane receptors [43]. The SH3 domain of the enzyme *Fyn,* a protein from the Src family of tyrosine kinases, comprises five antiparallel β-sheets that are connected by two flexible loops (Figure 6.1b) [44]. *Fyn* sequences are completely conserved between human, mouse, rat, and monkey. Wild-type SH3 domains are known to bind proline-rich regions that make up a PXXP core-binding motif. Mutagenesis of the two flexible loops enables binders to be generated against a variety proteins that do not necessarily contain a proline-rich region [7]. The utility of these domains as alternative scaffolds was first demonstrated by Neri and colleagues. In this work, the *Fyn* SH3 domain was mutated to generate a library of over a billion variants, which was displayed on phage and screened to isolate a "Fynomer" that binds with low nanomolar affinity to the extra-domain B (EDB) of fibronectin [7]. A similar method was used to generate a low picomolar binder to the IL-17A cytokine [8].

The Fyn SH3 domain does not contain disulfide bonds, which facilitates expression in bacterial systems. Additionally, these scaffolds are naturally monomeric and are also quite stable, with melting temperatures of ~70 °C [7]. The conservation of Fyn sequences across species is proposed to lower the chance for immunogenicity [45]. Hence, their stability and biocompatibility make Fynomers advantageous for therapeutic development. The main commercial player in the Fynomer space is Covagen, located in Zurich, Switzerland. The company is developing FynomAbs, which are fusions of Fynomer binding proteins to full-length antibodies. These fusions can result in bi- or tri-specificity to distinct binding partners. Covagen's lead molecule is a bispecific anti-TNF/IL-17A FynomAb (COVA322), which is being developed to treat rheumatoid arthritis and other inflammatory diseases. Additional FynomAbs, including an HER2-targeting bispecific [46], are in early phases of the company's pipeline [47]. In 2014,

Covagen was acquired by Cilag GmbH, an affiliate of the Janssen Pharmaceutical Companies of Johnson and Johnson.

6.3.1.3 Lipocalins/Anticalins

Lipocalins are a diverse group of proteins that are found in vertebrates, plants, and bacteria. Twelve different lipocalins have been identified in the human body alone [48]. Most of the proteins in the lipocalin family are thought to have a role in the transport or storage of small molecules and vitamins. Although the structure of each lipocalin is distinct, almost all of the proteins in this family have a conserved β-barrel core. There are eight antiparallel β-sheets that wrap around a central axis (Figure 6.1c). The loops that connect these β-sheets make up a variable binding interface. The broader open end of the protein has four loops that can be mutagenized for antigen-specific binding. The other side of the β-barrel consists of four additional closed loops that are away from the binding site. Another interesting structural component of lipocalin proteins is the presence of a single α-helix that is packed against one side of the β-barrel. Although ubiquitous in the lipocalin family, this α-helix has no obvious role in ligand specificity or binding. Thus, lipocalins are considered to be scaffolds with primarily β-sheet secondary structure.

The first binding protein made from a lipocalin scaffold was derived from the bilin-binding protein (BBP) from *Pieris brassicae,* a type of butterfly. Skerra and colleagues mutagenized a 16-residue region at the center of the BBP binding site and screened the library using bacterial phage display. Using this strategy, variants that bound to fluorescein with less than 1 μM affinity were generated [9]. Engineered anticalins that bind to the oncology target MET have also been developed as positron emission tomography (PET) imaging agents used to study tumor biodistribution [49]. Additionally, an engineered lipocalin was recently reported that binds to prostate-specific membrane antigen with 500 pM affinity [10].

Lipocalin proteins have a molecular weight of ~20 kDa. While there is one conserved disulfide linkage in the human lipocalin family, it is not necessary for overall stability and ligand binding [50]. Thus, these proteins can be efficiently produced in bacterial expression systems. Additionally, many lipocalins lack glycosylation, which helps minimize batch-to-batch variation and simplifies bacterial expression. Nicknamed "anticalins," lipocalin commercial development is being led by Pieris Pharmaceuticals, located in Freising, Germany. Pieris has collaborated with other companies including Zydus Cadila and Stelis Biopharma to develop anticalin-derived therapeutics. The company has a variety of candidate drugs, most in preclinical development or Phase 1 trials, with applications ranging from anemia to oncology [11, 51].

6.3.1.4 Nanobodies/VHH Domains

Full-length human antibodies consist of two heavy chains that are each paired with a smaller light chain. A joint antigen-binding surface is formed from variable regions of both the heavy and light chain. Although the heavy chain variable region has some affinity to the antigen by itself, the juxtaposition of both chains increases antigen affinity significantly, up to 250-fold [52]. The antibodies of other

organisms, however, have been found to comprise different structures. Hamers and colleagues were the first to report camel antibodies that are devoid of light chains [12]. This discovery sparked decades of single antibody domain engineering that has culminated in the "nanobody."

A nanobody consists of a single variable domain derived from a heavy chain antibody (HCAb). While human heavy chains interact hydrophobically with their paired light chain, HCAbs contain structural mutations that prevent these interactions. Four hydrophobic framework residues that typically facilitate interchain interactions are substituted for more hydrophilic or smaller residues, allowing the heavy chain to remain stable in an aqueous environment. These mutations (V42F, G49E, L50R, W52G) are located in the second framework region (FR2) and are conserved across soluble VHH domains [53]. Nanobodies are structurally similar to the variable domain of a human heavy chain (hence the alternate name "VHH Domain"). Their protein backbone consists of two β-sheets with three variable loops and a single disulfide bond (Figure 6.1d). However, since nanobodies comprise only a single domain, their size is an order of magnitude smaller than a full-length antibody, with a molecular weight of only ~15 kDa.

Nanobodies have many distinct advantages over full-length monoclonal antibodies or antibody fragments. First, protein production can be carried out in bacterial and fungal hosts, which allows for relatively less expensive large-scale production. As an example, expression in *Saccharomyces cerevisiae* has yielded protein concentrations up to 100 mg/L in shake-flask cultures [54]. Second, nanobodies have markedly increased chemical and thermal stability in comparison to other antibody fragments such as scFvs and Fabs [55]. In one study, two VHH domains with specificity for human lysozyme and the NmcA β-lactamase were found to have denaturation midpoints (C_m) of 2.3–3.3 M in urea and >6 M in guanidium chloride, values that are significantly higher than those observed for scFv fragments [56]. Although the melting temperature of nanobodies is similar to those of full-length antibodies and other antibody fragments ($T_m \sim 60–80\,^{\circ}C$), protein folding of VHH domains has been shown to be reversible at temperatures up to 90 °C [56–58]. Because of their stability in extreme conditions such as high temperatures and in the presence of chaotropic agents [56], nanobodies have been constructed to survive in harsh environments such as the gut [55]. Third, nanobodies can be evolved to bind targets not accessible to monoclonal antibodies. In particular, the CDR3 loop of a nanobody is two amino acids longer than that of a conventional human antibody and can therefore more easily insert into the active site of an enzyme or create a unique binding interface for a protein partner. For example, a camel VHH domain against lysozyme relies on deep CDR3 loop insertion into the enzyme's active site for binding [59]. This lengthy CDR3 region can be leveraged to create naïve libraries for the selection of high-affinity binders. The nanobody scaffold has been used to engineer binders to many therapeutic targets including interleukin 6 receptor (IL-6R) [14], tumor necrosis factor (TNF-α) [60], and von Willebrand factor [61].

Nanobodies can be developed by immunizing an organism that naturally creates HCAbs and creating a DNA library from the collected antibody repertoire. Since the organism has already evolved high-affinity binders for the target *in vivo,*

screening a library of $10^6 - 10^7$ HCAbs can result in the isolation of nanomolar binders [12, 13]. Additionally, naïve libraries have been constructed from llamas without antigen immunization and screened using ribosome display. In contrast to *in vivo* maturation, however, these isolated nanobodies only exhibited micromolar affinity for their target [62]. Nanobody engineering and development is currently being commercialized by Ablynx, a Belgian company. Ablynx has at least five nanobody-derived drugs in its pipeline, including therapeutics for oncology, rheumatoid arthritis, and psoriasis. Their first potential product launch is expected in 2018 [63]. Other companies such as TeneoBio and Crescendo Biologics have also entered the space and are developing human heavy chain variable domains as therapeutics.

6.3.2 Scaffolds Comprised of α-Helices

6.3.2.1 DARPins

Designed ankyrin repeat proteins (DARPins) consist of multiple ankyrin molecules connected to form a complete binding interface. The ankyrin domain is ubiquitous; it is known to mediate protein–protein interactions in almost all examined phyla [64]. In fact, there are more than 3000 known proteins with ankyrin repeats (ARs), underscoring their importance to biological function [65]. The proteins formed from these stacked repeats have an innate ability to bind and interact with a wide variety of natural molecules, highlighting their promise as a protein engineering scaffold.

The structure of an individual AR consists of a β-turn followed by two antiparallel α-helices. A final β-turn caps off the AR and connects to the next AR domain. The binding surface is constructed from the first β-turn and connecting α-helix from each repeat (Figure 6.2a). Most AR proteins have between four and six individual domains; however, there are exceptions, such as the human ankyrinR protein which consists of 12 repeats [66]. Since each ankyrin domain is only about 3.5 kDa in molecular weight, the entire protein scaffold is relatively small, ranging from 14 to 21 kDa depending on the number of repeats it contains [67]. The capacity to create a large binding surface with variable molecular length makes this repeat protein useful for constructing diverse libraries.

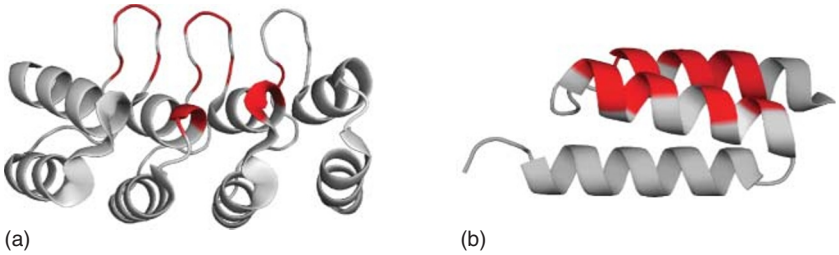

Figure 6.2 Non-antibody scaffolds with α-helix secondary structure. Engineered variable domains are highlighted in red. (a) DARPin/Ankyrin repeats (PDB ID: 3HG0), (b) Affibody (PDB: 1LP1).

Plückthun and colleagues first demonstrated the utility of the ankyrin repeat as a scaffold for protein engineering. Their scaffold consisted of a repeated modular ankyrin domain flanked by an N-terminal cap ankyrin domain and a C-terminal cap domain (Figure 6.2a). A library was created by designing the modular repeat domain to contain seven variable residues out of a total of 33 amino acids [68]. In a subsequent study, a DARPin library was screened using ribosome display to isolate single digit nanomolar binders to the maltose binding protein (MBP) and mitogen-activated protein kinase (MAPK) [15]. In addition to this proof-of-concept work, DARPins have also been engineered against HER2 [17], epithelial cell adhesion molecule (EpCAM) [69], and VEGF-A [70]. Recently, the DARPin scaffold has been modified to include an additional loop region between helical repeats, creating a large, continuous binding surface. These LoopDARPins have similar stability as original DARPins and can be engineered via ribosomal display to isolate low nanomolar to picomolar binders [16]. DARPins have also been utilized for therapeutic delivery as drug conjugates [71] and small interfering RNA (siRNA) transporters [72].

Ankyrin repeats have several unique advantages. First, they lack disulfide bonds, which facilitates large-scale production. DARPin proteins can be expressed and purified from *E. coli* with yields up to 100 mg/L in some cases [67]. Second, DARPin libraries can be created with a variable number of ARs, allowing the construct size to be tailored more directly to the target. Libraries containing an N-terminal capping domain, 2–4 variable repeats, and a C-terminal capping domain have been created and screened for high-affinity binders [15, 67]. In contrast, most other scaffolds are limited by a fixed size, which can limit identification of an optimal binder. Third, many DARPins exhibit high thermal stability due to their helical core, with melting temperatures of >100 °C [73]. Fourth, their small size and high target binding affinity make DARPins ideal for *in vivo* molecular imaging applications [17].

DARPins are under commercial development by Molecular Partners in Zurich, Switzerland. The company is currently developing and advancing a number of DARPin-based drugs for indications in oncology, ophthalmology, and immunology. Two of their drugs, Abicair and a multispecific concatenated anti-VEGF/PDGF DARPin fusion, are being developed for macular disease in collaboration with Allergan [74]. Two other lead molecules are aimed at cancer therapy and are proprietary: MP0250, which antagonizes the cancer targets VEGF and HGF, and MP0274, which has anti-HER2 activity [75].

6.3.2.2 **Affibodies**

Affibodies were first derived from the immunoglobulin binding receptor known as staphylococcal protein A (SPA) [18]. This protein, known colloquially as "Protein A," is commonly used for protein purification and affinity chromatography. Protein A has a unique ability to bind Fc portions of immunoglobulins from a number of different species. The protein itself is composed of five homologous Fc-binding domains that are each around 60 amino acids long. The second domain in the protein, domain B, was engineered to minimize sequence repeats in order

to simplify the addition of mutations. The resulting domain was termed protein Z and is the basis of the affibody scaffold today [76].

Nygren and colleagues were the first to demonstrate the affibody technology. In their work, a library of Z domain molecules was displayed on phage and screened to isolate binders to Taq DNA polymerase, human insulin, and human apolipoprotein with micromolar affinities [18]. Affinity maturation of affibodies using a variety of display technologies, including *E. coli* display, can improve binding, resulting in nanomolar and picomolar binders [77]. Protein Z and its related affibodies are made up of 58 amino acids, which form a characteristic structure comprised of three α-helices. Based on the co-complex of the SPA domain B and its IgG binding partner, 13 individual residues were deemed "variable" and are mutated during library creation [18] (Figure 6.2b). In contrast to antibodies and the other scaffolds presented earlier, most of these variable residues are part of the secondary structure and do not exist solely in loop regions. As might be expected, certain mutations of the variable residues can disrupt the three-helix bundle and are accompanied by a loss in thermal stability. However, other mutations make minimal changes to the structure and allow the α-helix bundle to stay intact, conferring relatively high thermal stability to the scaffold ($T_m \sim 70\,^{\circ}C$) [78]. Despite significant secondary structure in each affibody, these proteins are remarkably compact and have a molecular weight of only 6–7 kDa.

Similar to other alternative scaffolds, the small size of the affibody offers clear advantages over antibodies for applications including molecular imaging and tumor penetration. Additionally, affibodies do not contain native cysteine residues, allowing large-scale bacterial production. Because of their small size and rapid folding properties, affibodies have been produced by chemical synthesis, which allows the introduction of moieties for conjugation [79, 80]. Chemical handles have also been introduced by incorporating a cysteine residue at the affibody termini [19, 81]. Affibodies also have the unique property of being modular, which allows for unique combinations and fusions. Notable examples include bispecific affibody molecules constructed from two distinct affibodies targeting a respiratory syncytial virus (RSV) surface protein and Taq DNA polymerase, respectively [82], a trispecific affibody for HER2, HER3, and albumin to combine tumor targeting with serum half-life extension [83], and binding pairs that function as fluorescence resonance energy transfer (FRET) sensors [84]. Affibody molecules can also be fused to drugs to facilitate targeted therapy. For example, Capala and colleagues created Affitoxin, a HER2-targeted affibody fused to *Pseudomonas* exotoxin [85].

Recent affibody development has resulted in improvements such as a second-generation scaffold with mutations in the framework region that increase thermal and chemical stability [78] and a novel ability to bind FcRn in a pH-dependent manner [86]. Continuing advancements have resulted in affibodies targeting HER2 [87], HER3 [88] TNF-α [20], CD28 [89], and amyloid β-peptide [90]. The scaffold technology was commercialized by a company bearing the namesake Affibody, located in Stockholm, Sweden. Affibody is developing molecules for cancer diagnostics, inflammation, autoimmune disease, and a number of other therapeutic areas [91].

6.4
Non-antibody Scaffolds with Mixed Secondary Structure

6.4.1
Disulfide-Rich Scaffolds

6.4.1.1 A-domain Binders (Avimers)

Avimers are small protein scaffolds that are based on the A-domain motif. A-domains consist of alternating β-α-β secondary structure in an orientation known as the "Rossmann fold" [92]. The A-domain itself is only 35 amino acids in length and has a molecular weight of about 4 kDa. Additionally, A-domains contain six cysteine residues that form three stabilizing disulfide bonds [21]. These bonds hold different strands of the A-domain together producing a repeated "U"-type fold (Figure 6.3a). There are more than 200 A-domains that have been identified in human proteins, including high-density lipoprotein (HDL) and very low density lipoprotein receptor (VLDLR) [93]. The A-domain structure contains roughly 12 conserved residues that are necessary for structural integrity and about 25 variable residues that can be mutated for target binding [21].

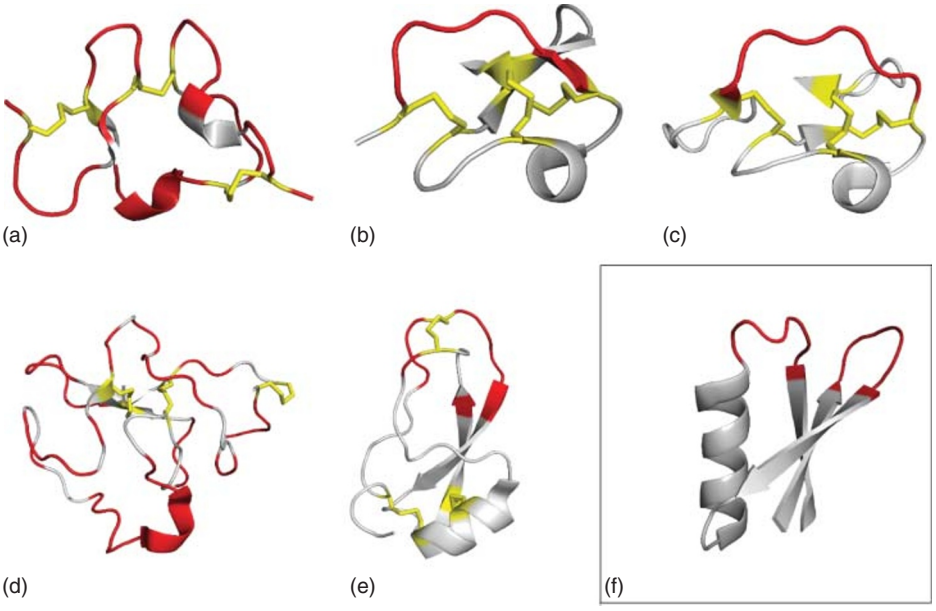

Figure 6.3 Non-antibody scaffolds with mixed secondary structure. Engineered variable domains are highlighted in red. Disulfide linkages are highlighted in yellow and represented as sticks. The GP2 protein in F is isolated because it does not contain disulfide linkages. (a) Avimer/A-domain binders (PDB ID: 1AJJ), (b) EETI-II knottin (PDB ID: 2IT7), (c) MCoTI-II cyclotide (PDB ID: 4GUX), (d) Kringle domain (PDB ID: 1I5K), (e) Kunitz domain (PDB ID: 1AAP), (f) GP2 (PDB ID: 2WNM).

Stemmer and colleagues were the first to demonstrate the potential of the Avimer technology within a company called Avidia, Inc. This work demonstrated that Avimers can be engineered for target binding and can be produced in the cytoplasm of *E. coli* since the three characteristic disulfide bonds were purported to form rapidly after cell lysis [21]. A distinct benefit of Avimers is that they can be connected and engineered sequentially to create binders that interact with a target through multiple epitopes. The name "Avimer" comes from "avidity multimer," which is a reference to the significant boost in affinity achieved from these tandem binding domains. In 2006, Amgen purchased Avidia and took over Avimer development. One of the more advanced Avimer projects was a binder that inhibited the proinflammatory cytokine IL-6 (AMG220); however, this was discontinued in clinical trials for Crohn's disease for unstated reasons. Amgen recently published results demonstrating the agonistic effects of an engineered bispecific Avimer that mimics the functionality of FGF1 [94].

6.4.1.2 Cyclotides/Cystine Knot Peptides

Cystine knot miniproteins, also known as knottins, are 30–50 amino acid peptides comprised of antiparallel β-strands connected by a disulfide-bonded "knotted" core that confers high thermal, chemical, and proteolytic stability (Figure 6.3b,c) [95]. In particular, knottins have been shown to retain their three-dimensional structure after boiling or incubation in acid, base, and serum [96, 97], which has attracted great interest in using these proteins as potential drug scaffolds. Cyclotides are a closely related structural family whose backbone is cyclized through an N- to C-terminal peptide bond [98]. Polypeptides discovered to contain cysteine-knot motifs number in the thousands, are found in a wide variety of plants, insects, animals, and fungi, and have diverse functions such as ion channel blockade, protease inhibition, and antimicrobial activity [99].

Almost all protein therapeutics developed to date have to be administered through the blood since they are not stable enough to survive the harsh conditions of the stomach and the gastrointestinal (GI) tract. Ironwood Pharmaceuticals has taken advantage of the remarkable stability of knotted peptides to develop an orally delivered, natural, guanylate cyclase-C agonist called linaclotide (Linzess®), which is approved by the FDA for treating chronic constipation and irritable bowel syndrome [100]. Despite their small size, some natural knottin peptides have extremely high affinity for their targets, for example, single-digit picomolar affinity in the case of Ziconitide (Azur Pharma), an ion channel inhibitor that is FDA approved for treating chronic pain [101]. These examples have bolstered enthusiasm for developing knottins as alternative scaffolds for an expanded range of therapeutic and diagnostic applications.

Kolmar and colleagues first demonstrated the utility of cysteine knot peptides for protein engineering applications by grafting functional loop epitopes into the *Ecballium elaterium* trypsin inhibitor II (EETI-II; Figure 6.3b), which is a 28-amino-acid plant serine protease inhibitor, using rational methods or through *E. coli* display [102]. Since then, engineered variants based on EETI-II or other knottins have been generated, including human agouti-related protein (AgRP),

a regulatory neuropeptide, or Agatoxin, an ion channel inhibitor from spiders, to bind to thrombopoietin, CTLA-4, and a variety of integrin receptors [95–99]. Craik and colleagues have led work in the use of cyclotides as protein engineering scaffolds. As an example, fragments from extracellular matrix proteins and growth factors known to promote angiogenesis were grafted into the loop regions of the *Momordica cochinchinensis* trypsin inhibitor-II (MCoTI-II; Figure 6.3c) [103] a 34-amino-acid cyclotide. Compared to the corresponding linear peptides, cyclic grafted peptides were significantly more stable in human serum and induced angiogenic processes in cell assays. Several groups have used MCoTI-I and MCoTI-II as molecular scaffolds to engineer binders against matriptase-1, β-tryptase, leukocyte elastase, and $\alpha_V\beta_6$ integrin [103–105]. In other work, Camarero and colleagues engineered MCoTI-based targeting agents against CXCR4, angiotensin (1–7), and the intracellular targets Hdm2 and HdmX [25, 106]. Daugherty and colleagues screened bacterial display libraries to identify kalata B1 variants that bound cancer targets neuropilin-1 and 2 with nanomolar affinities and inhibited cell migration. The cyclotide kalata B1 knottin can resist denaturation in boiling solutions of 6M guanidine hydrochloride, 8M urea, and solutions containing a range of active proteases [107].

Because of their small size (~3.5 kDa) and high stability, knottin peptides are ideal *in vivo* molecular imaging agents [108]. In 2007, James Olson and his team developed "tumor paint" a Cy5.5-labeled chlorotoxin knottin from scorpion venom that could illuminate many tumor types, including brain tumors [109]. A variant termed BLZ-100 is being developed by Blaze Bioscience as a tool for image-guided tumor resection. Additionally, engineered variants of EETI-II and MCoTI that bind to tumor-associated integrin receptors have also been developed as multimodal molecular imaging probes [110], and in some cases also demonstrated the ability to penetrate the blood–tumor barrier for targeting of intracranial tumors such as medulloblastoma [111]. Knottins and cyclotides can be produced by microbial expression [110, 112] or using chemical synthesis, which allows facile introduction of unnatural amino acids as chemical handles for conjugation [113]. While some knottins like EETI-II adopt their native fold readily, others are more challenging to produce as a number of different species can be formed from undesired disulfide pairings (e.g., a protein that contains three disulfide bonds can potentially form 15 different isomers). Cyclotides also require an additional step to cyclize the N- and C-termini, which can make production more difficult. Protagonist Therapeutics is currently pursuing the development of disulfide-rich engineered peptides for oral delivery in diseases including inflammatory bowel disease, irritable bowel syndrome, and Crohn's disease. BioNTech AG in Mainz, Germany, is also pursuing the development of engineered knottin peptides for tumor imaging and drug delivery applications.

6.4.1.3 Kringle Domain

Another protein motif that has been utilized as a binding scaffold is the kringle domain (KD). There are 39 KDs known to be present in humans, each of which consists of 70–80 amino acids. KDs are modular and can appear between 1 and 10 times in a single protein. Most proteins containing KDs are present in blood

plasma and function as cofactors, proteases, growth factors, or anti-angiogenic agents [112, 113]. The structure of the KD family members is well conserved and contains a rigid core composed of two short antiparallel β-sheets connected by three distinct disulfide bonds, making the KD compact and stable. The cysteine residues that mediate disulfide bonds are highly conserved across all kringle motifs. As shown in Figure 6.3d, orientation of the β-sheets and disulfide bonds creates seven distinct loops that are surface-exposed. About 40 of the 70–80 residues in the KD are located in one of these extruding loops and are variable among KDs in different proteins [22]. KDs are believed to play a major role as a binding mediator with other proteins, lipids, or small molecules. The ubiquity of this protein motif thus suggested that it would be a useful scaffold for protein engineering.

The Kim lab at Ajou University first demonstrated the utility of the KD scaffold. In this work, a yeast-displayed library was created based on the second KD (KD2) of the human plasminogen protein, and screened to identify single-digit nanomolar agonists against the human death receptors 4 and 5 (DR4, DR5), or antagonists against human TNF-α [22]. Further development led to the grafting together of individual loops to create bispecific KD proteins that have low nanomolar affinity to both death receptors 4 and 5 [114]. While the presence of disulfide bonds confers increased thermal and proteolytic stability, it may also make large-scale production more difficult as described above. However, the presence of seven variable loops within a small-sized framework (~15 kDa) presents an opportunity to create diverse libraries for protein engineering applications.

6.4.1.4 Kunitz Domain

The Kunitz domain is a common protein motif found in many natural protease inhibitors. Examples of such Kunitz domain inhibitors include bovine pancreatic trypsin inhibitor (BPTI), human pancreatic secretory trypsin inhibitor (PSTI), and the periplasmic *E. coli* protease inhibitor ecotin [115]. Kunitz domain inhibitors primarily target serine proteases, which are widespread in biology and are responsible for fundamental cellular functions such as the activation of proteins from their inactive "pro" state. Kunitz domains have also been found as ion channel blockers in insect toxins [116]. The structure of the Kunitz domain is comprised of mixed α and β framework (Figure 6.3e). A single α-helix is connected to two antiparallel β-sheets, which are connected to a small α-helix at the C-terminus. The loops that connect the secondary structure together form variable regions that can be mutated for target binding. Kunitz domains are typically around 60 amino acids in length and are stabilized by three disulfide bridges. The six cysteine residues that comprise the disulfide bonds are well conserved across almost all observed Kunitz domains. Protein inhibitors that contain Kunitz domains operate by forming noncovalent interactions with the enzyme in or around the active site to block function [116]. In the enzyme inhibitor space, the Kunitz domain has an advantage over other non-antibody scaffolds because of its inherent ability to function as a protease inhibitor. Moreover, the compact structure mediated by disulfide linkages makes Kunitz domain proteins proteolytically stable [117]. The presence of disulfide bonds, however, potentially make the production more challenging as described previously for other disulfide-linked scaffolds.

The Kunitz domain is one of the few non-antibody scaffolds that has advanced to the stage of FDA approval. Dyax used phage display technology to create and screen a library of variants based on the first Kunitz domain of the human tissue factor pathway inhibitor (LACI-D1), and isolated an inhibitor of plasma kallikrein with a binding affinity of about 10 pM [26]. In 2009, Dyax received FDA approval for its kallikrein antagonist (now known as Ecallantide or DX-88) to treat the main mediator of symptoms associated with hereditary angioedema. Other Kunitz domain engineering successes have been achieved, such as the generation of human tissue factor VIIa from Genentech [118], but none has yet progressed as far as Ecallantide.

6.4.2 Mixed Secondary Structure without Disulfides

6.4.2.1 T7 Phage Gene 2 Protein

Most non-antibody scaffolds have been identified from nature through observation of their ability to bind a multitude of targets. However, because of the rise in popularity of antibody mimetics, researchers have also begun to use computational tools to find potential scaffolds. Hackel and colleagues searched through the PDB database for single domains of 40–65 amino acids that were rich in β-sheet content and had two solvent exposed loops, but were devoid of disulfide bonds. The lead protein domain according to their ranking system was the T7 phage gene 2 protein (GP2) [27].

GP2 is an *E. coli* RNA polymerase inhibitor that also helps package the DNA of the T7 bacteriophage [119]. The native protein, which is 64 amino acids in length, was modified to create a protein engineering scaffold. The N- and C-termini were genetically removed to minimize size, and a framework mutation was inserted to increase stability. The final GP2 scaffold is thus only 45 amino acids long with a molecular weight of 5.2 kDa. The scaffold consists of a single α-helix that is connected to three antiparallel β-sheets. There are two loops available for diversification: one between the helix and the first β-sheet, and the other between the second and third β-sheets (Figure 6.3f). The Hackel lab showed that the loop regions of the GP2 protein could be randomized and screened using yeast surface display to identify variants that bound to three model proteins: lysozyme, immunoglobulin G, and EGFR. The observed binding affinities were in the nanomolar to picomolar range, demonstrating that, while GP2 is a small scaffold, it has adequate surface area to achieve high-affinity target binding [27]. An additional advantage of GP2 as a scaffold is its lack of disulfide bonds, which facilitates large-scale production in microbial systems. Additionally, the lack of framework cysteine residues creates an opportunity to introduce thiol groups for site-specific chemical conjugation. Engineered GP2 proteins also demonstrate high chemical and thermal stability ($T_m = 65–80\,^{\circ}C$). The GP2 scaffold has not yet been commercialized or evaluated in therapeutic applications; however, it is a promising recent addition to the repertoire of alternative scaffolds.

6.5 Conclusions and Considerations

6.5.1 General Advantages of Alternative Scaffolds

Non-antibody scaffolds are growing in popularity and usage because of their important advantages over standard antibodies. First and foremost, the small size of scaffolds such as DARPins, affibodies, and knottins has been shown to be ideal for maximizing signal to noise in *in vivo* molecular imaging applications [68, 82, 111], and has been proposed to offer enhanced diffusion through dense extracellular space in deep-tissue applications [30]. Second, many non-antibody scaffolds do not contain any cysteine residues, which can increase the ease of production and allow the addition of a free thiol at the N- or C-terminus for site-specific conjugation of chemical moieties. Some scaffolds, including affibodies and DARPins among others, have been leveraged to form drug conjugates and imaging probes using this strategy [68, 70, 77, 83]. Other scaffolds, including affibodies and knottins, have been produced by chemical synthesis and incorporate non-natural amino acids as chemical handles for conjugation [81, 113]. Third, most alternative scaffolds possess high thermal, chemical, and proteolytic stability, making them preferable over antibodies for applications that involve exposure to harsh experimental conditions or environments. The consequence of being small and compact is rapid serum half-life due to renal clearance. Small alternative scaffolds must therefore bind their targets with high affinity to increase their likelihood of localizing to the target within the short residence time in the body [120]. Despite their smaller size, scaffolds such as DARPins, affibodies, and GP2 possess contiguous surface areas for target binding that mirror or exceed the interaction surface area of an antibody–antigen binding site, which is around 600 $Å^2$ [26, 119]. Thus, many of the scaffolds discussed in this chapter can be affinity-matured to reach impressive binding affinities in the picomolar range. Lastly, the small molecular weight of alternative scaffolds permits higher injection concentrations on a per molar basis. This can reduce the need for multiple injections which can be critical for certain applications such as ocular delivery.

6.5.2 Scaffold Modifications to Improve Pharmacological Properties

In this chapter we have highlighted desirable attributes and potential limitations of alternative scaffolds for those involved in the field of protein therapeutics. It is important to note, however, that additional modifications can be made to alternative scaffolds to further improve their therapeutic qualities. First, as discussed earlier, in order to effectively compete with bivalent antibodies, these mimetics must be able to bind their target with high affinity. Just like antibodies, alternative scaffolds can take advantage of higher avidity via multimerization to bolster their binding interactions. Concatenated Avimers, for example, utilize the avidity effect

by binding to multiple epitopes on a given target protein. Monobodies have been fused to a pentamerization domain to create a five-pronged binder with significantly improved kinetics [121]. Knottins have been multimerized using chemical cross-linking or genetic fusion to an Fc domain [101, 111, 122]. Additionally, bivalent affibodies or DARPins with improved affinities over monomeric alternatives have been created against targets such as HER2 [68, 121]. Second, in addition to affinity, the amount of time that a molecule stays functional in the body can be a determining factor for drug potency. One strategy is to improve serum half-life by increasing the protein molecular weight above the threshold of renal clearance [123]. Fusion to an antibody Fc domain effectively increases the half-life by slowing renal clearance and by taking advantage of native FcRn binding, a process that recycles the protein back into the serum to escape endosomal degradation [123, 124]. Since Fc domains naturally dimerize, the fusion of an alternative scaffold to an Fc also results in increased avidity through multivalent effects. Alternatively, conjugating the scaffold molecules to albumin (~60 kDa) can also increase the half-life by preventing rapid renal clearance and taking advantage of FcRn binding and recycling [125]. Strategies to conjugate an albumin-binding peptide to the scaffold can result in similar effects [126]. Another important strategy for extending the half-life is the covalent attachment of polyethylene glycol (PEG) [127] or genetic fusion of polypeptide-based alternatives [126, 127]. The addition of polymer coatings can increase molecular volume and retard kidney clearance, as well as potentially help reduce immunogenicity. Third, in addition to improvements in affinity and half-life, engineered scaffold proteins can also be fused in creative and novel ways to facilitate dual action, or multispecificity. Scaffolds such as affibodies, DARPins, and monobodies are highly modular and can be readily combined to create multifunctional proteins. Examples of these applications are numerous and have been reviewed elsewhere [24, 128] and in Chapters 7 and 8.

6.5.3 Concluding Thoughts

While many of the alternative scaffolds presented here have been shown to bind a range of targets, certain scaffolds may be preferred for particular applications over others. For example, intracellular targets and targets with a cleft or pocket might be best accessed by scaffolds with extended loops instead of those that have a flat binding surface. GI disease targets or those in other hostile environments such as wounds are best approached using scaffolds with high protease stability. Targets that require ultrahigh binding affinity or are involved in complex biochemical signaling events with redundant pathways may be best modulated by multifunctional or multivalent alternative scaffolds as discussed in this chapter. Because of their unique advantages, an increasing number of alternative scaffold-based therapeutics are being pursued each year. At the time of writing, there are at least 38 antibody mimetic drugs in development, and the list is growing [129]. Most of them are designed for cancer therapy, and are still in the preclinical phase; however, some of these drug candidates are in clinical trials, and a select few have

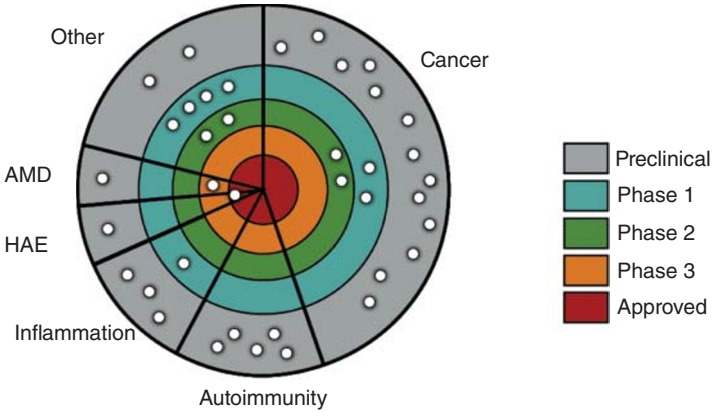

Figure 6.4 Drugs derived from non-antibody scaffolds in clinical development. Each slice corresponds to a clinical target. Individual colors delineate phase of development. White circles represent a single drug in a certain phase of clinical development. AMD: Age-related macular degeneration, HAE: Hereditary angioedema. "Other" includes cachexia, pain, anemia, IBD, plaque psoriasis, and acute respiratory distress syndrome.

reached the FDA approval stage (Figure 6.4, Table 6.1: clinical trial candidates in bold/italics). Thus, although the antibody is still the biologic workhorse of the pharmaceutical industry, alternative scaffolds are proving themselves viable contenders for therapeutic development.

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7
Protein Engineering: Methods and Applications

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7.1
Introduction

Proteins are an increasingly important class of therapeutic drug because of their exquisite specificity and high affinity, which enables them to target and modulate a variety of disease pathways. The first therapeutic protein, insulin, was purified from bovine and porcine pancreas and used for the treatment of diabetes mellitus [1]. Since then, the development of recombinant DNA and other molecular biology methodologies has facilitated the large-scale production of therapeutic proteins such as antibodies, enzymes, hormones, and interleukins. Consequently, these proteins have played a significant role in the treatment of cancers, immune disorders, infections, and other diseases.

A major challenge has been the development of next-generation protein therapeutics with enhanced efficacy, greater safety profiles, or improved delivery. Over the last two decades, the development of new protein engineering techniques to mimic Darwinian evolution, together with *in silico* based approaches using computational design, has revolutionized this class of drugs. In this chapter we will provide examples of different protein engineering techniques to elucidate structure–function relationships. These will be illustrated with case studies describing the optimization of therapeutic proteins for specificity and affinity.

Monoclonal antibodies (mAbs) represent the largest growing class of therapeutic proteins, with over 50 mAbs currently on the market [2]. They are differentiated from other serum proteins because of their comparatively long half-life as a consequence of binding to the neonatal Fc receptor (FcRn). We will describe how protein engineering has been used to modulate immunoglobulin G (IgG) serum half-life either through Fc engineering or via pH-dependent antigen binding. A

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further advantage of antibodies is the strong relationship between efficacy and the effector function. Approaches to augment effector function to improve antibody efficacy, as well as methods to eliminate antibody effector function with the aim of reducing IgG mediated toxicity, will also be described.

This review aims to illustrate how protein engineering can unlock the huge therapeutic potential of this important class of drugs.

7.2 General Approaches for Protein Optimization

7.2.1 Directed Evolution

Directed evolution is a method to mimic Darwinian evolution in the laboratory. It generally does not require structural information, but rather relies on the generation of a library of protein mutants that can be screened for desired properties. Mutations can be introduced either individually or in combination using a variety of techniques described in the following sections. Several platforms have been developed that enable high-throughput screening of large libraries of protein mutants tethered to phage, bacteria, yeast, mRNA, or ribosomes. Alternatively, functional screens of individually expressed clones can be used to select mutants with desired phenotypes. A key aspect of all these platforms is that genotype is linked to phenotype and therefore DNA encoding the desired mutations can be recovered for amplification, sequence analysis, or further mutagenesis.

7.2.1.1 Point Mutagenesis

Random mutagenesis is an approach that introduces point mutations in an unbiased manner throughout the whole gene and provides a useful tool to optimize proteins in the absence of a structure–function relationship. The most common method to introduce random mutations into a DNA sequence is by error-prone polymerase chain reaction (epPCR) [3]. Low-fidelity PCR conditions are achieved by modulating components of the PCR mix, such as increasing magnesium concentrations, supplementing with manganese, or using dNTP analogs [4]. This increases the mutation rates of DNA polymerase to $10^4 - 10^3$ per replicated base [5]. The number of mutations in the protein sequence can be further enhanced by increasing the number of PCR cycles, since the number of mutations increases with each round of PCR amplification. An alternative method to introduce random point mutations throughout a gene is through the use of an *Escherichia coli* (*E. coli*) mutator strain, such as mutS, mutT, and mutD [6–9], to propagate the DNA. These strains are defective in DNA repair and are therefore capable of introducing random mutations at a very high frequency compared to normal *E. coli* cells [6].

Random mutagenesis is a very useful approach to identify amino acid positions associated with function, activity or biochemistry within the protein of interest. It is also able to identify "long-distance" key residues that contribute indirectly to the catalytic activity of enzymes or to the recognition properties of antibodies

[10, 11]. Such key positions can then be explored more exhaustively through the construction of a library by site-directed mutagenesis and subsequent screening for a variety of phenotypes, such as improved affinity [12].

7.2.1.2 Amino Acid "Scanning"

Scanning mutagenesis is a widely used method to systematically and comprehensively substitute amino acids within a protein with the aim of understanding the structure–function relationship. For example, alanine scanning mutagenesis [13], in which single alanine mutations are introduced at every residue within a protein domain, provides a detailed map of a protein binding interface. Amino acids responsible for the functional contribution to binding can subsequently be modified to optimize desired properties such as activity or affinity. However, this can be a laborious process since many mutant proteins must be produced and assessed separately. Alternatives to this approach include parsimonious mutagenesis [14–17], which is an efficient method of mutational scanning to bias mutagenesis toward the parent sequence or to sterically or chemically conservative changes. Using this approach, libraries of mutants can be created for the selection of clones with improved characteristics such as affinity [18, 19], specificity, or stability. A variation of this technique termed look-through mutagenesis (LTM) has also proved to be a successful approach to "amino acid" scan a protein in a rapid manner. The strategy requires the construction of a library in which each "wild-type" residue is individually substituted by one of nine amino acids that are representative of the major chemical functionalities provided by the 20 natural amino acids. This enables the generation of a comprehensive energetic map of a protein and the identification of mutations responsible for conveying a beneficial property such as affinity [20].

7.2.1.3 Block Mutagenesis

Although point mutagenesis can identify individual amino acids, resulting in improved activity, the simultaneous saturation mutagenesis of multiple adjacent residues, also termed block mutagenesis, can rapidly access combinations of mutations that may exhibit synergism. Target residues are generally chosen on the basis of structural, functional, and/or sequence-derived information and randomized such that any of the 20 common amino acids might be incorporated at that position.

The conventional approach to achieving saturation mutagenesis employs synthetic DNA oligonucleotides containing degenerate codons (e.g., NNS or NNK) at positions corresponding to the targeted residues. In these codons, N represents an equal mixture of the nucleotides A, C, G, and T, whereas S is a mixture of only G and C and K is a mixture of just G and T. While technically straightforward, the resulting focused libraries contain amino acid biases since each of the possible amino acids will not necessarily be represented equally in the oligonucleotide mix. For example, NNS mutagenesis results in leucine, serine, and arginine being three times more likely to be encoded than histidine. To circumvent this limitation, nondegenerate alternatives have been developed, which add whole codons (rather than single bases) during oligonucleotide synthesis [21]. "Small

intelligent libraries" designed by the program DC-analyzer [22] and the "22c trick" [23] have also been described, which enable effective saturation of a small number of codons. However, it is impractical to use these approaches for saturation mutagenesis of more than three consecutive codons because of the large number of primers required. To randomize six amino acids using "22c trick" would require >700 primers and >4000 primers to make a "small intelligent library" [24]. More recently, Ashraf *et al.* have described ProxiMAX randomization, which uses DNA cassettes for saturation mutagenesis of up to 11 contiguous codons without degeneracy or bias [24].

The resulting library size is also a major consideration when deciding how many contiguous amino acids to mutate. For example, mutating six positions to any of the 20 common amino acids via the commonly used NNS or NNK codon mutagenesis strategy generates a diversity of 1×10^9, which should be covered in a phage library with a size of 5×10^9 transformants, based upon a 99% confidence value in the Poisson distribution [25]. The limitations of bacterial transformation efficiency restrict the maximum library sizes for phage display to between 10^9 and 10^{11} [26].

7.2.2 Rational Design

Rational design relies on the structural analysis of proteins to identify regions for introducing mutations to improve desirable traits such as affinity, stability, specificity, and enzyme activity. This approach has been widely used in recent years because of the increased number of protein structures solved by X-ray crystallography, NMR, and cryo-electron microscopy. Rational design offers an advantage over random methods since it enables greater exploration of sequence space, resulting in more focused, and more in-depth, mutagenesis that concentrates on key residues.

Rational design approaches have been successfully applied in the generation of therapeutic enzymes with new or improved activities. This has been accomplished by modeling the enzyme backbone in a single fixed state and constraining the reactive side chains to identify mutations that stabilize the transition state, thereby lowering the activation barrier and accelerating catalysis [27–31]. By applying this methodology, several putative therapeutic enzymes have been created. For example, Gordon *et al.* [28] engineered the endopeptidase kumamolisin-As from the bacterium *Alicyclobacillus sendaiensisto* for improved proteolytic activity and specificity toward the immunogenic portions of gluten peptides for the potential treatment of Coeliac disease. The engineered enzyme (designated KumaMax) displayed a 116-fold greater proteolytic activity for a model gluten tetrapeptide than the native scaffold enzyme and improved substrate specificity toward immunogenic portions of gluten peptides. Computational design approaches have advanced such that it is now possible to introduce catalytic properties into previously inert scaffolds. However, further improvement of these computational

approaches is still required since the kinetic performance of *de novo* enzymes generally falls short of their naturally occurring counterparts [27, 30, 31].

Another application of computational design is to predict aggregation-prone regions of proteins. This is relevant for the development of biotherapeutics since protein aggregation involving reversible self-association is an increasingly recognized problem that influences both shelf-life and efficacy [32–34]. Trout and coworkers have developed a structure-based computational tool called spatial aggregation propensity (SAP) [35–38], which allows the identification of aggregation-prone regions in mAbs by quantifying surface-exposed hydrophobic residues. This tool has been applied to constant regions of human IgG molecules [35] as well as antibody fragments, such as Fab or Fc [39]. More recently, this measure has been used to identify and engineer out aggregation-prone regions in the Fab domain of a therapeutic antibody, bevacizumab, resulting in a fourfold reduction in monomer loss without compromising binding to the target, namely vascular endothelial growth factor (VEGF)-A [40].

Rational design can also be applied to engineer therapeutic antibodies for higher affinity or improved specificity [41–43], which are described in Section 7.3.

7.3 Engineering for Affinity and Specificity

7.3.1 Affinity

The affinity of a therapeutic protein for its target defines its efficacy and influences the dose required to obtain the desired effectiveness *in vivo*. *In vitro* engineering methods combined with display technologies, such as phage, yeast, and ribosome display, have been widely used to improve the affinity of therapeutic proteins, and in particular antibodies. By applying these techniques, it is possible to overcome the limitations of *in vivo* affinity maturation, which is restricted to ~100 pM by the physiological mechanism of B-cell activation [44, 45]. There are multiple options for diversifying variable (V) genes for antibody affinity maturation, and here we highlight some key examples.

7.3.1.1 Improving Affinity by Directed Evolution

Antibody chain shuffling is a simple method for optimizing antibodies. It mimics the process of *in vivo* affinity maturation whereby the heavy (V_H) and light (V_L) chains are exchanged following the initial immune response, a process known as "repertoire shift" [46]. This can be easily replicated in the laboratory using standard molecular biology techniques to replace the V_H or V_L gene with a variable gene repertoire (chain shuffling) [47]. The approach has been used successfully for the affinity maturation of a large number of antibodies to a wide range of targets, such as HIV-1 gp120 [48], c-erbB-2 [18], and the chemokines

monocyte chemo-attractant protein-1 (MCP-1) [49] and eotaxin-1 [50]. Affinity improvements of up to 15-fold have been readily achieved.

An alternative method to optimize the affinity of antibodies is by random mutagenesis. Using this approach, mutations can be introduced anywhere within the antibody variable genes. The technique was first applied by Hawkins *et al.* to create a library for phage display. Iterative rounds of selection were subsequently performed using decreasing concentrations of antigen in solution to select antibodies with a higher affinity [51]. Random mutagenesis has also been successfully used to create libraries for other surface display platforms such as ribosome [52], bacteria [53], and yeast [54], achieving affinity increases of 200-fold [55]. A random approach is typically used to identify frequently mutated "hotspot" positions, which can be further randomized using other mutagenesis strategies to achieve additional gains in affinity [56].

Targeted mutagenesis offers an advantage over random mutagenesis, as it enables mutations to be directed to the antibody complementarity-determining regions (CDRs), which are known to be responsible for antigen binding. Li *et al.* used targeted mutagenesis to affinity-mature a natural antibody against the gH glycoprotein of human cytomegalovirus (CMV) and enhance its neutralization potency. Using Fab phage display libraries, site-directed mutagenesis was employed to vary the CDRs by one amino acid at a time to scan for mutations that improve binding affinity. Hotspot mutations were identified at two sites within the VHCDR2 and recombined to produce an antibody with high affinity to the CMV gH/gL glycoprotein complex ($K_D < 10$ pM). The resulting antibody displayed increased neutralization potency without an apparent increase in off-target binding [57]. Interestingly, a number of the individual mutations introduced into the VHCDR2 created a glycosylation site that impaired binding of antibodies expressed in mammalian cells. This limit imposed by glycosylation on affinity maturation may be a relatively common situation *in vivo,* and can potentially be overcome by *in vitro* mutagenesis which can sample a greater number of codons.

Block mutagenesis of heavy and light chain CDR loops via NNS or NNK mutagenesis has been employed by multiple laboratories to improve the affinity of antibodies. This is an attractive approach since it can rapidly access combinations of mutations that may exhibit synergism. Votsmeier *et al.* [58] used NNK mutagenesis to create a library in which two neighboring residues within the CDRs of the anti-TNF antibody, adalimumab, were fully diversified. Mutations resulting in improved affinity were recombined to generate a lead with ~500-fold improvement in K_D, resulting in femtomolar binding. VHCDR3 is often targeted by this method, as it has been shown to be an area that is particularly amenable to optimization to increase affinity by >1000-fold [50, 59–65].

Alternatives to this saturation approach include methods to bias the mutagenesis toward the parent sequence or to sterically or chemically conservative changes using parsimonious mutagenesis [15–17]. Targeting the naturally occurring

hotspots seen in *in vivo* somatic hypermutation (SHM) has also proved to be a successful approach [66]. Tenfold improvements in potency were achieved when an anti-CD22 antibody was mutated at specific positions in its CDRs [67]. More recently, Lim *et al.* have described a novel approach using the chicken B-cell line DT40, which constitutively hypermutates its immunoglobulin (Ig) loci [68]. This cell line has been adapted to enable the surface display and selection of human scFvs that are targeted by SHM and has been successfully used to refine the affinity and specificity of scFv isolated by other methods.

Targeted mutagenesis approaches can also be used to improve the affinity of other therapeutic proteins. For example, the affinity of IL2 for the IL-2Rβ receptor was improved from 280 to 1.2 nM to create an IL-2 superkine [69] for the potential treatment of a variety of immune disorders ranging from AIDS to cancer [70]. This was achieved by creating an error-prone PCR library of IL-2, which was displayed on the surface of yeast. The library was selected to identify IL-2 mutants with improved binding to the IL-2R β and γc complex, which resulted in the selection of a predominant IL-2 variant containing an L85V mutation. Targeted mutagenesis of amino acids that were structurally in close proximity to L85V identified mutants, which further improved the affinity of IL-2 for IL-2Rβ.

7.3.1.2 **Improving Affinity by Rational Design**

In silico approaches for affinity maturation using computational design have become more widely used because of the increased number of available protein structures. In the first example, Clark *et al.* enhanced the affinity of a therapeutic antibody targeting the integrin VLA1 [71]. Structure-based computational design identified a combination of four mutations (light chain S28Q/N52E and heavy chain T50V/K64E), which led to an antibody with a K_D of 850 pM, representing an eightfold improvement in affinity. Barderas *et al.* demonstrated that computational methods can still be applied even when the structure of the antibody is not known. Their approach was to create homology models of the anti-gastrin scFv (TA4) followed by docking of the experimentally determined epitope of gastrin in to the binding sites to identify residues for mutagenesis through phage display. This led to a 454-fold improvement in affinity over the parent antibody [72]. An iterative computational design method that focuses on electrostatic binding contributions and single mutants was used by Lippow *et al.* By combining multiple designed mutations, they engineered a 10-fold affinity improvement (to 52 pM) into the anti-epidermal growth factor receptor drug (Erbitux) and a 140-fold improvement in affinity to 30 pM for the anti-lysozyme model antibody D44.1 [42]. In all of these studies, the optimized antibodies incorporated multiple mutations in their primary sequences. Kiyoshi *et al.* demonstrated that a single mutation carrying a positive charge in a CDR could be responsible for a 4.6-fold improvement in affinity to an already high affinity (4.6 pM) anti-MCP-1 antibody, 11K2. This was identified from a virtual library of mutants, and resulted in the formation of new electrostatic interactions with MCP-1 [73].

7.3.2 Specificity

The mutagenesis techniques described to improve antibody affinity have also been used to modify the specificity of therapeutic proteins. The main differences lie in the design of an appropriate selection and screening strategy to identify those variants that display the desired specificity. Here we provide examples of both broadening and reducing the specificity of therapeutic proteins.

7.3.2.1 Improving Species Cross-Reactivity

Specificity engineering is often used to improve the binding of human therapeutic proteins, particularly antibodies, to nonhuman species to facilitate the assessment of pharmacology and toxicity in animal models [74, 75]. However, achieving such cross-reactivity can be very challenging, particularly if the human target displays little similarity to the orthologous protein from another species. This can affect the choice of preclinical animal models and can result in extended clinical studies if cross-reactivity is not achieved [74].

Werther *et al.* improved the species cross-reactivity of an anti-human lymphocyte function associated antigen-1 (LFA-1) antibody for binding to the rhesus orthologue by 200- to 400-fold. This was achieved through randomization of four amino acids within the VHCDR2, which were identified via alanine scanning [74]. As an alternative approach, Farady *et al.* [75] used a computational design strategy to identify antibody CDR residues that could be mutated to improve the species cross-reactivity of an antibody-based inhibitor, E2, of the cancer-associated serine protease MT-SP1. Although E2 is a potent inhibitor of MT-SP1, with a $K_I = 12$ pM, it displays 300-fold less potent inhibition of the mouse orthologue (epithin), even though the human and mouse versions of the enzyme differ only by three residues within the binding epitope. Eight mutations predicted to improve inhibition of the mouse version of the enzyme were selected using the protein local optimization program (PLOP), which measures the change in binding free energy upon mutation. These mutations were confirmed by experimental testing, revealing that the substitution T98R in VHCDR3 improved binding to the mouse orthologue by 14-fold, resulting in a K_I of 340 pM. The improved affinity was valuable when exploring the role of MT-SP1 in mouse models of cancer.

7.3.2.2 Reducing Cross-Reactivity to Other Proteins

The specificity of therapeutic proteins is also extremely important to minimize off-target effects and improve efficacy. Webster *et al.* used a directed evolution approach to improve the catalytic activity and selectivity of neprilysin, a protease that cleaves a range of physiological peptide substrates, including amyloid beta (Aβ), a key pathological component of Alzheimer's disease. One-hundred and thirty-four amino acids lining the active site were identified from the crystal structure [76] and a yeast display library of variants generated by site-directed mutagenesis at each of these positions. The resulting neprilysin variants were subsequently screened for their ability to degrade the pathological peptides

Aβ1–40, Aβ1–42, and a mutant that displayed increased catalytic efficiency on Aβ1–40 and Aβ1–42, and up to 3000-fold reduced cleavage of a range of off-target peptides. The improved selectivity offers the potential of more efficient degradation of Aβ *in vivo*, making this an attractive molecule for the potential treatment of Alzheimer's disease [77].

T-cell receptors (TCRs) are increasingly being explored as therapeutics since they specifically recognize peptide antigens bound by major histocompatibility (MHC) proteins (pMHCs) and initiate T-cell killing of these targeted cells. TCRs typically display relatively weak affinities toward pMHC (~1–300 μM), which correlates to some extent with *in vivo* potency [78], limiting their therapeutic potential. Previous *in vitro* engineering efforts have yielded significant improvements in TCR affinity (up to 1 000 000-fold) [79]; however, a concern during affinity maturation is the maintenance of peptide specificity. Pierce *et al.* have used computational design to simultaneously alter the affinity and specificity of a therapeutic TCR, DMF5, which recognizes the nonameric and decameric peptide epitopes from the MART-1 melanoma antigen presented by the class I MHC HLA-A2. This was achieved by crystallizing the DMF5 TCR in complex with both the MART-1 nonameric epitope (AAGIGILTV; referred to as AAG) and the anchor-modified decameric epitope (ELAGIGILTV; referred to as ELA), both bound to HLA-A2 [80]. Twelve computationally designed mutations were subsequently chosen for experimental testing based on the predicted TCR–pMHC affinity. This identified DMF5 YW, a variant containing two mutations (αD26Y and βL98W) with affinity enhancements of 400-fold toward ELA/HLA-A (from 9.5 μM to 24 nM) and 30-fold toward AAG/HLA-A2. However, no binding was detectable toward unrelated peptides presented by HLA-A2, demonstrating that their approach avoided peptide-independent off-target effects [81].

7.3.2.3 Increasing Cross-Reactivity to Other Proteins

The ability to broaden antigen specificity is advantageous for the development of antibodies against pathogens. In the case of botulism, which is caused by botulinum neurotoxin (BoNT), the existence of multiple subtypes, which differ from each other by up to 35% at the amino acid level, requires antibodies with broad cross-reactivity for diagnosis and treatment [82]. Starting with an scFv that binds the BoNT/A1 subtype with high affinity (136 pM) and the BoNT/A2 subtype with low affinity (109 nM), Garcia-Rodriguez *et al.* [83] increased the cross-reactivity for BoNT subtypes A1 and A2 through mutagenesis of multiple antigen binding loops (VLCDR1 and VH CDRs 1, 2 and 3), which were selected based on solvent accessibility. Antibody gene diversity libraries were created using parsimonious mutagenesis [16, 18] and expressed on yeast. A dual selection strategy on BoNT subtypes A1 and A2 was then employed to select yeast displaying scFv with the highest affinity binding to both BoNT/A subtypes. Using this approach, they increased the affinity of scFv for BoNT/A2 1250-fold, to 87 pM, while maintaining high-affinity binding to BoNT/A1 (115 pM).

Mutagenesis techniques have also been used to broaden strain cross-reactivity of HIV-1 neutralizing antibodies. Early library-based engineering efforts

to improve HIV-1 antibodies involved b12, one of the first HIV-1 broadly neutralizing antibodies (bNAbs). The affinity of b12 was enhanced by nearly 400-fold by selecting gp120 binding from libraries of phage displayed mutants in CDRs [84]. These studies demonstrated that increasing affinity through *in vitro* evolution could also increase the breadth of binding to different HIV strains [85]. In a further example, a random mutagenesis library of the broadly cross-reactive HIV-1 neutralizing scFv, X5, was subjected to sequential rounds of selection on non-homologous HIV-1 envelope glycoproteins (Envs), an approach termed sequential antigen panning. This led to the identification of m9, which exhibited significantly higher neutralization activity and was able to inhibit a broader range of HIV-1 primary isolates compared to scFv X5 [86].

Perhaps the most significant example of engineering cross-reactivity involved the evolution of trastuzumab, an anti-human epidermal growth factor receptor 2 (HER2) antibody, to recognize an unrelated protein, VEGF. Trastuzumab is known to interact with HER2 mainly via the heavy chain CDRs [87, 88]. Bostrom *et al.* therefore chose to randomize a subset of solvent-exposed amino acids within the light chain CDRs to create the antibody bH1, which conferred binding to VEGF while maintaining the original specificity for HER2 [89]. Structural and functional studies confirmed that the amino acid interactions between bH1 and the two unrelated proteins were energetically distinct, but there was extensive overlap between the antibody surface areas contacting the two antigens.

Engineering the specificity of other therapeutic proteins can be more challenging since, unlike antibodies where antigen binding occurs through the CDR loops of the heavy and light chains, additional structure–function information is often required. CTLA4-Ig is an Fc fusion protein containing the extracellular domain of cytotoxic T-lymphocyte associated Ag-4 (CTLA4), a receptor known to deliver a negative signal to T cells by blocking the CD80 and CD86 ligands from binding to CD28. CTLA-4-Ig has an affinity bias toward CD80 over CD86 (K_D values of 0.29 and 3.6 nM, respectively) and is an approved agent for the treatment of rheumatoid arthritis (abatacept) [90]. However, blockade of CD80 or CD86 can have divergent effects on disease outcome depending on the stage of disease or the tissues involved [91]. Xu *et al.* employed a high-throughput protein engineering method called point-by-point (P × P) mutagenesis to identify CTLA4-Ig variants with altered binding affinity to CD80 and CD86 with the potential for improved safety and efficacy in particular disease settings. Using the published X-ray crystal structures of the CTLA-4/CD80 and CTLA-4/CD86 complexes [92, 93] they identified three regions, ^{26}PGKATEVR^{33}, ^{51}TYMMGNELTFLDD^{63}, and ^{93}KVELMYPPPYYL^{104}, responsible for ligand binding and therefore suitable for the introduction of amino acid substitutions. A CTLA4-Ig library comprising all possible single amino acid substitutions in these domains was generated using defined codons to allow equal representation of the amino acid variants. The library containing 594 variants was then expressed on the surface of mammalian cells and sorted by flow cytometry for binding to CD80 and CD86. The frequency of each mutant in each subpopulation was determined by massively parallel pyrosequencing and used to generate an affinity ranking of the entire library.

Affinity improvements (fold K_D) of up to 6.1-fold (K93Q) for CD80 and 4.4-fold (A29H) for CD86 were observed by surface plasmon resonance (SPR) [94].

7.4 Optimizing IgG Serum Half-Life

7.4.1 Fc Engineering

In order to standardize the description of all Fc variants described hereafter, we have used the EU numbering system throughout [95].

7.4.1.1 Introduction and General Considerations

Two features differentiate IgG molecules from other serum proteins: first, their ability to be actively transferred across the placenta from mother to the fetus or neonate [96, 97], and, second, a comparatively long serum half-life, which is a direct consequence of their binding to a gamma globulin receptor via the Fc portion [98–100]. More precisely, these properties are mediated by binding of IgG Fc to the neonatal Fc receptor (FcRn) [101–103]. As such, Fc engineering to strengthen or weaken the corresponding interaction constitutes a well-established strategy to modulate IgG serum half-life. In particular, human IgGs engineered for increased binding to human FcRn exhibit significantly longer serum half-life in human and nonhuman primates when compared with their unmutated counterparts. This has direct application to the therapeutic antibody field, as increased serum persistence of therapeutic IgGs could allow a decrease of their administration frequency or dosing requirements while maintaining the overall efficacy. Various areas can potentially benefit from such engineering efforts, including oncology, auto-immunity, inflammation, and infectious diseases.

7.4.1.2 FcRn Controls IgG Recycling

FcRn is central to regulating IgG circulating levels [101–103]. It comprises β2-microglobulin and a membrane-anchored α-chain related to the α-chain of the class I MHC complex [104–106]. FcRn is expressed in endothelial cells throughout the adult body [107, 108] and acts as a salvage receptor for IgGs in mammals. Once pinocytosed and bound to FcRn, IgG molecules are transported across, or recycled within, endothelial cells and rescued from a lysosomal degradative pathway (Figure 7.1) [109]. This rescue process occurs mostly within the endothelial cells of small vessels and capillaries [108] and accounts for the comparatively long serum half-lives of IgGs, ranging from ~7 to 21 days in humans [110].

A remarkable feature of the IgG–FcRn interaction is found in its pH dependence. More precisely, IgG binding to FcRn is strongest at acidic pH and lowest to nondetectable around neutral pH [111–113]. This property is critical to IgG recycling from within acidic endosomes back to the general circulation, thereby prolonging their serum half-life (Figure 7.1) [101, 114]. Until recently, the molecular

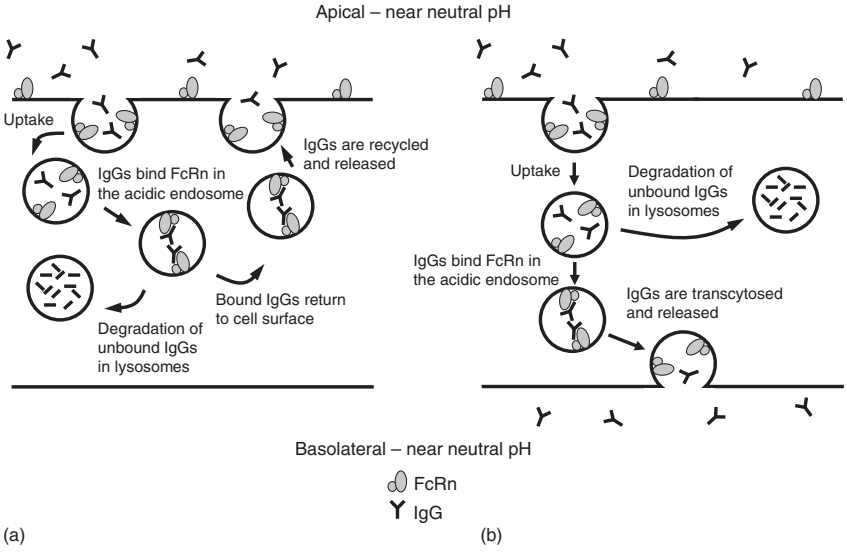

Figure 7.1 Cross section of an FcRn-expressing endothelial cell showing (a) IgG recycling and (b) IgG transcytosis. After pinocytosis, IgG molecules enter acidic endosomes, bind to FcRn, and are rescued from a lysosomal degradation pathway. IgG-containing vesicles then cycle back or migrate to the cell surface where the IgG molecules are released.

mechanism responsible for this pH dependence was widely believed to rely on the direct titration of two histidine residues on both Fc (H310/H433) [112, 115] and FcRn (H250/H251) [112]. However, the recent first three-dimensional structure between a human Fc and human FcRn has allowed a more accurate structural definition and revealed Fc H310 as the most significant, if not sole direct contributor to pH-dependent binding [116].

7.4.1.3 **Decreasing IgG Binding to FcRn**

Numerous mutagenesis studies have provided evidence that decreasing the affinity of IgGs for FcRn at acidic pH typically results in a concomitant decrease of their serum half-life. In particular, mouse IgG1 Fc-hinge fragments containing the I253A, H310A, H435A, or H436A substitution see their binding affinity to murine FcRn at pH 6.0 reduced by over fourfold. Such mutated fragments exhibit ~2.5- to 7-fold reduction in serum half-life in mice when compared with their unmutated counterpart [117, 118]. Similar results were observed when the H435A substitution was introduced into the Fc portion of a humanized IgG1. In this situation, no binding of the modified antibody to murine FcRn could be seen at pH 6.0, whereas its serum half-life in mice was reduced by ~10-fold [97]. When the I253A, H310A, and H435A mutations were separately introduced into a human IgG1 Fc-hinge fragment, the resulting affinities to mouse FcRn at acidic pH were reduced by ~5-, 14- and 13-fold, respectively [119]. This loss resulted in ~2.5-, 3-, and 3-fold reduction, respectively, in the fragments serum half-life in mice

[119]. Finally, a humanized IgG1 containing the I253A mutation was described, whose binding to human FcRn was substantially reduced [120]. Its serum half-life in transgenic mice expressing human, but not mouse, FcRn was reduced by up to sixfold when compared with the same unmutated IgG1.

Such so-called knock-out mutations constituted the first examples of Fc engineering to modulate IgG serum half-life, as they required little, if any, structural knowledge of the IgG–FcRn interaction. Recently though, the first three-dimensional structure of a human Fc/human FcRn complex [116] has shed new light on these historical data. In particular, the side chain of Fc H310 was found to be positioned in such a way that it makes a strong hydrogen bond with FcRn E115, the elimination of which is expected to significantly alter the formation of the corresponding complex. Similarly, Fc I253 was found to be an important component of a large hydrophobic patch at the human IgG/FcRn interface, the disruption of which likely disrupts the complex. Finally, although Fc H435 is known to play a critical role in the human IgG/human FcRn interaction [121], Oganesyan *et al.* found that this residue is not involved in any significant intermolecular interaction with human FcRn; therefore, the dramatic effects seen on human IgG–FcRn complex formation upon mutating this residue are likely caused by indirect effects, such as local conformational rearrangements.

Short-lived molecules generated using such a strategy have important applications in the diagnostic field where the patient's overall exposure to the radioactive moiety of imaging compounds should be minimized. For instance, ^{124}I-labeled anti-CEA single chain Fv (scFv) fragments fused to a human Fc moiety engineered for decreased binding to FcRn at positions I253, H435, and/or H310 exhibited quick tumor localization and resulted in clear images in xenografted mice [122]. The H310A/H435Q double mutant, which showed the most rapid serum clearance rate, was also the most efficient in terms of localizing to the tumor and providing high-quality images. Similarly, another iodinated anti-HER2 scFv fragment fused to a human Fc/H310A/H435Q also showed improved tumor targeting and reduced kidney uptake in mice [123]. Finally, antibodies or antibody fragments engineered for fast serum clearance could also be desirable to reduce the overall toxicity of therapeutics such as immunotoxins and radioconjugates [124].

7.4.1.4 Increasing IgG Binding to FcRn

Correlation between Improved IgG–FcRn Affinity and Serum Half-life The development of efficient phage library construction and selection methods, as well as the availability of relevant structural information on rat and human FcRn [105, 106, 125], provided the tools to identify Fc mutants exhibiting increased binding to FcRn (Figure 7.2a). The earliest results were obtained using murine molecules. Among these, a mouse IgG1 Fc-hinge fragment was randomly mutated and selected for increased binding to mouse FcRn [126]. This led to the identification of a triple mutant, which exhibited significant increases in both its pH-dependent binding to murine FcRn at pH 6.0 (~3.5-fold) and serum half-life in mice (~1.6-fold) when compared with the wild-type fragment. As knowledge about

human FcRn grew and recombinant material became available, studies shifted to the human system. In particular, an alanine scanning approach targeting solvent-exposed residues on a human IgG1 Fc led to the identification of over a dozen single variants, the most improved of which (N434A) exhibited ~3.5-fold increase in its binding to human FcRn at pH 6.0 [121]. When some of these beneficial mutations were combined, further increases in human IgG1 binding to human FcRn were attained. In particular, ~12-fold affinity improvement at pH 6.0 was seen for a human IgG1 containing the T307A/E380A/N434A substitutions [121]. All variants retained good pH sensitivity, namely no or very poor binding to FcRn at or near neutral pH. When dosed in transgenic mice expressing human FcRn, the N434A and T307A/E380A/N434A mutants exhibited ~2–3-fold increase in their serum half-life when compared with the same unmutated antibody [120]. Therefore, these early studies demonstrated that a broad correlation exists between strengthening the IgG–FcRn interaction and improving IgG serum half-life.

Beyond the IgG–FcRn Affinity Component A more thorough understanding of the necessary requirements for such engineering campaigns to be successful was achieved when a combination of rational design and phage-display-based library screening identified several humanized IgG1 Fc mutants whose binding to human and murine FcRn at pH 6.0 was improved by up to 60- and 30-fold, respectively [127]. These authors showed that Fc mutants whose affinity to murine FcRn was increased at both acidic and neutral pH were adversely affected in terms of their serum persistence in mice. Therefore, increased IgG binding to FcRn at neutral pH offset the benefit of enhanced binding at pH 6.0. It is likely that such IgG mutants are sequestered throughout the body in FcRn-containing tissues and not efficiently released into the general circulation. A similar observation was made when a human IgG1 containing the T307A/E380A/N434A mutations and whose binding affinity toward murine FcRn was increased at both pH 6.0 and neutral pH exhibited a serum half-life in mice similar to that of the unmutated molecule [128]. Therefore, the pH dependence, which is a hallmark of the IgG–FcRn interaction, must be maintained. In addition, an affinity threshold at neutral pH exists, which governs the pharmacokinetics (PK) outcomes of affinity-improved Fc variants. Once this binding threshold is satisfied (i.e., when IgG variants exhibit a K_D to FcRn at neutral pH of $< \sim 10$ nM), serum clearance decreases with increased FcRn binding at pH 6.0 [129]. IgG variants exhibiting an affinity to FcRn at neutral pH beyond this threshold see the benefits of increased binding at pH 6.0 offset.

A certain threshold in the IgG–FcRn binding affinity at acidic pH may also need to be overcome, as a triple mutant of a mouse IgG1 Fc-hinge fragment (T252A/T254S/T256A) exhibiting an approximately twofold increase in binding to murine FcRn at pH 6.0 and no binding at pH 7.4 failed to show improved serum half-life in mice [126].

Finally, other parameters may also be at play. For instance, humanized IgG1s containing the P257I/N434H, D376V/N434H or P257I/Q311I mutations exhibited >50-fold pH-dependent increase in their binding affinity to cynomolgus monkey FcRn, yet did not see their serum half-life improved in the same host

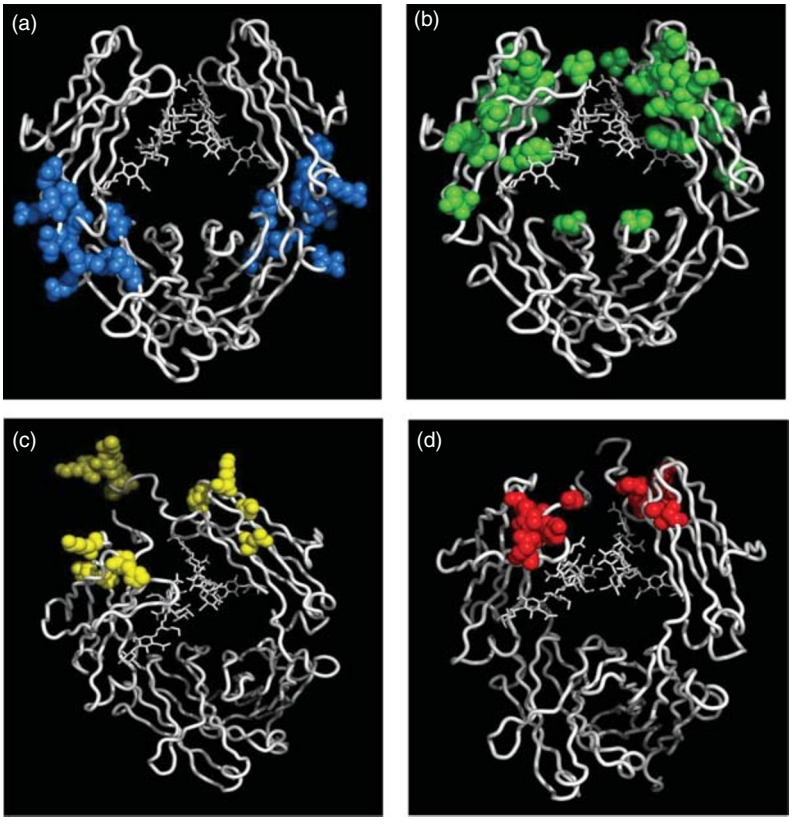

Figure 7.2 Model of an Fc fragment derived from a human IgG1, based upon the X-ray structure corresponding to protein database ID number 1HZH [214]. Colored residues correspond to the various positions where single or multiple substitutions were shown to improve the affinity of the (a) human IgG/FcRn, (b) human IgG/CD16A, (c) human IgG/C1q, and (d) human IgG/CD32A interaction. Carbohydrates are shown as sticks.

[130]. These results suggested that the dissociation rate (k_{off}) plays a major role in the recycling process since the enhanced affinities of these mutants to cynomolgus monkey FcRn were predominantly driven by an increase in the corresponding association rates (k_{on}). The same conclusion was achieved when a human IgG1 containing the T250Q/M428L mutations was dosed in cynomolgus monkeys and did not exhibit improved PKs properties despite its 40-fold pH dependent, k_{on}-driven affinity increase to cynomolgus FcRn [131]. Conversely, the same mutated human IgG1 exhibited a serum clearance ~2-fold slower than the unmodified antibody in mice, in accordance with its 500-fold pH dependent, k_{off}-driven affinity increase to mouse FcRn.

The YTE Mutations One particular set of Fc mutations identified using a library-based phage display approach is worth noting owing to its extensive

characterization and validation: M252Y/S254T/T256E ("YTE") [132]. Binding of YTE-modified humanized IgGs to both human and cynomolgus monkey FcRn improved by ~10-fold at pH 6.0 while a strong pH dependence of binding is maintained. This resulted in a nearly fourfold increase in their serum half-life in cynomolgus monkeys [132].

The three-dimensional structure of the complex between Fc-YTE, human FcRn, and human serum albumin was recently solved at 3.8 Å resolution [116]. This has shed light on the molecular mechanisms by which the YTE mutations lead to improved affinity with FcRn. It was found that YTE results in the creation of two salt bridges between Fc-E256 and β2-microglobulin Q2 and one new hydrogen bond between Fc-T254 and FcRn E133. Interestingly, Fc-Y252 contributes significantly to the increased binding affinity between Fc-YTE and human FcRn in its own right [127]. However, it is not an actual part of the human Fc/FcRn interface, and thus plays this beneficial role in an indirect, yet-to-be determined, fashion.

Finally, a study in healthy adult volunteers was designed to evaluate the PKs and safety profile of a YTE-modified humanized IgG1 (motavizumab-YTE; [133]). This constituted the first Fc-modified, PK-improved monoclonal antibody to be studied in humans. The clearance of motavizumab-YTE was significantly lower (71–86%) and its half-life was two- to fourfold longer than with motavizumab. Safety and incidence of anti-drug antibodies were comparable between groups. Motavizumab-YTE was well tolerated and exhibited an extended half-life of up to 100 days, providing the definite proof that Fc engineering for increasing IgG serum half-life in human constitutes a valid approach.

Fc Engineering to Improve Serum Half-Life: Reaching a Plateau Fc engineering efforts to improve the affinity between IgG and FcRn have now been carried out successfully by many groups. These seem to point to an upper limit in terms of achievable increase in serum half-life. For instance, human IgG2 mutants with increased affinity to human FcRn have been generated using a focused library-based approach, including M428L and T250Q/M428L [134]. These variants showed an increase in binding to human FcRn at pH 6.0 of 7- and 28-fold, respectively, and no binding to the receptor at near-neutral pH. Significantly improved PK properties were observed, with serum half-lives ~2-fold longer in rhesus monkeys than the wild-type form of the same human IgG2. T250Q/M428L was also introduced into a human IgG1, which resulted in a ~30- and 40-fold pH-dependent increase in IgG binding to human and rhesus monkey FcRn, respectively [135]. This led to 2.5-fold extension in serum half-life in rhesus monkeys when compared with the unmutated parent molecule. Using a library-based approach, Borrok *et al.* [129] have also described an extensive series of improved human Fc mutants. These exhibited increased serum half-life in cynomolgus monkeys by up to nearly twofold. Finally, as described in "The YTE Mutations" section, the YTE mutations result in ~4-fold and 2–4-fold increase in human IgG1 serum half-life in cynomolgus monkeys and humans, respectively.

Although early molecular modeling and rational design-based procedures have been described [136], only recently have they progressed to the point of identifying *bona fide* beneficial Fc mutations [137]. In particular, a series of Fc variants exhibiting greater affinity for human FcRn were generated and introduced in the context of a humanized anti-VEGF IgG1 antibody. These molecules displayed ~3–20-fold greater affinity to human FcRn at pH 6.0. One of these mutations (M428L/N434S) resulted in an increase of this antibody's binding affinity to human FcRn and serum half-life in cynomolgus monkeys of ~11- and 3.2-fold, respectively.

Thus, it emerges from a plethora of approaches and studies in human and non-human primates that a nearly fourfold increase in serum half-life constitutes the upper limit of what can be achieved by engineering the IgG/FcRn pathway.

7.4.1.5 **The Next Steps**

Great progress was seen recently in the area of FcRn-mediated IgG serum half-life extension. This included (i) validation of the concept in humans using the most advanced set of mutations (YTE), (ii) detailed understanding of the molecular mechanisms underpinning the improved PK properties, and (iii) a better appreciation of the multiple requirements that a given Fc mutant must fulfill to show improved serum half-life. Such efforts promise to deliver a novel class of medicines and will provide valuable additions to the fields of antibody therapy and diagnosis. In addition to allowing a decrease of therapeutic IgG administration frequency or dosing requirements, improved efficacy may also be seen. The latter point is illustrated by the description of anti-EGFR and anti-VEGF IgG variants enhanced for increased serum half-life whose antitumor activity in a mouse model was also improved [137]. However, because of their prolonged retention in serum and increase in overall exposure, the potential toxicity of such enhanced molecules will need to be closely monitored on a case-by-case basis.

FcRn is also involved in the transport of IgG molecules across the epithelium of various tissues such as placenta [97], neonatal or adult intestine [138], kidney [139], lung [140], yolk sac [141] and mammary gland [142]. Therefore, the possibility exists that further Fc engineering or characterization of existing Fc variants will lead to the generation of therapeutic antibodies with optimized distribution to or across specific tissues. This has implications in neonatal medicine in cases where therapeutic antibodies need to be efficiently transferred to the fetus. Finally, if more efficient IgG transcytosis from the intestine or lungs to the serum can be achieved, more convenient IgG routes of administration could also be developed (such as oral- or inhalation-based, respectively).

Fc engineering approaches can also be used to generate molecules exhibiting high-affinity, pH-*independent* binding to FcRn. These have the ability to outcompete endogenous IgGs for binding to the receptor, which results in increased clearance of the host's own antibodies from the serum [143]. Such Fc-engineered IgGs could be useful to reduce IgG levels in antibody-mediated diseases or enhance the clearance of imaging antibodies to increase contrast [144].

7.4.2
pH-Dependent Antigen Binding to Improve Serum Half-life

Antibodies targeting membrane-bound antigens with a high rate of synthesis, such as IL-6R, EGFR, and CD40, are rapidly eliminated from the plasma by antigen-mediated clearance. Even with a high binding affinity, a conventional antibody is able to bind to its target antigen only once during its lifetime in plasma before being internalized into the cell and trafficked to the sorting endosome and then the lysosome, where the antibody–antigen complex are degraded by proteolysis (Figure 7.3a). Consequently, a high or frequent dose of antibody is required to neutralize the antigen, thus compromising its therapeutic utility.

A high antibody dose is also required to neutralize soluble molecules produced at high concentrations in plasma, such as IgE and C5, to achieve clinical efficacy [145, 146]. In this situation, the half-life of an IgG antibody is significantly longer than that of the target antigen. Consequently, binding an antigen to an antibody results in the accumulation of antibody–antigen complexes in plasma, prolonging the half-life of the antigen (Figure 7.3b) [147–152].

These difficulties motivated the design of pH-dependent antigen-binding antibodies, also referred to as recycling antibodies, which take advantage of the pH differences between plasma (pH 7.4) and endosome (pH 5.8). *In vivo,* a pH-dependent binding antibody binds to its antigen in plasma and, following endocytosis, dissociates from the antigen in the acidic endosome. The antigen is subsequently degraded by proteolysis in the lysosome, and the antibody is recycled back to the cell surface via FcRn, enabling it to bind to another antigen (Figure 7.3c,d). By repeating this cycle, the antibody can bind antigen an infinite number of times and its efficacy is not limited by dose.

A number of protein engineering approaches have been described to incorporate pH sensitivity into antibodies through the introduction of histidine residues into the CDRs [153–155]. Histidine is chosen because it has a pK_a of ~5.5–6.5 and therefore alterations of electrostatic interactions that are induced upon histidine protonation at lower pH values can lead to decreased binding affinity to its target antigen. A common approach to identify mutations that introduce pH sensitivity into antibodies is through histidine scanning of the CDRs. This has been successfully applied to antibodies against interleukin 6 (IL6), interleukin 6-receptor (IL6-R), and proprotein convertase subtilisin kexin type 9 (PCSK9) [153–155]. By combining mutations, antibodies were generated that retained high-affinity target binding at pH 7.4 and displayed decreased binding at acidic pH (pH 4.5–6.0). PK analyses of the recycling IL-6R and PCSK9 antibodies demonstrated enhanced antigen clearance, which may enable less frequent or lower antibody dosing [153, 154].

Murtaugh *et al.* hypothesized that the extent of pH sensitivity over a modest reduction in pH, for example, from pH 7.4 to 6.0, depends on the number of histidines as well as the magnitude of the pK_a changes. To test this hypothesis, they created a combinatorial histidine library of an anti-RNase A VHH (K_D ~20 nM) in which all 22 VHH interface positions sample both histidine and the

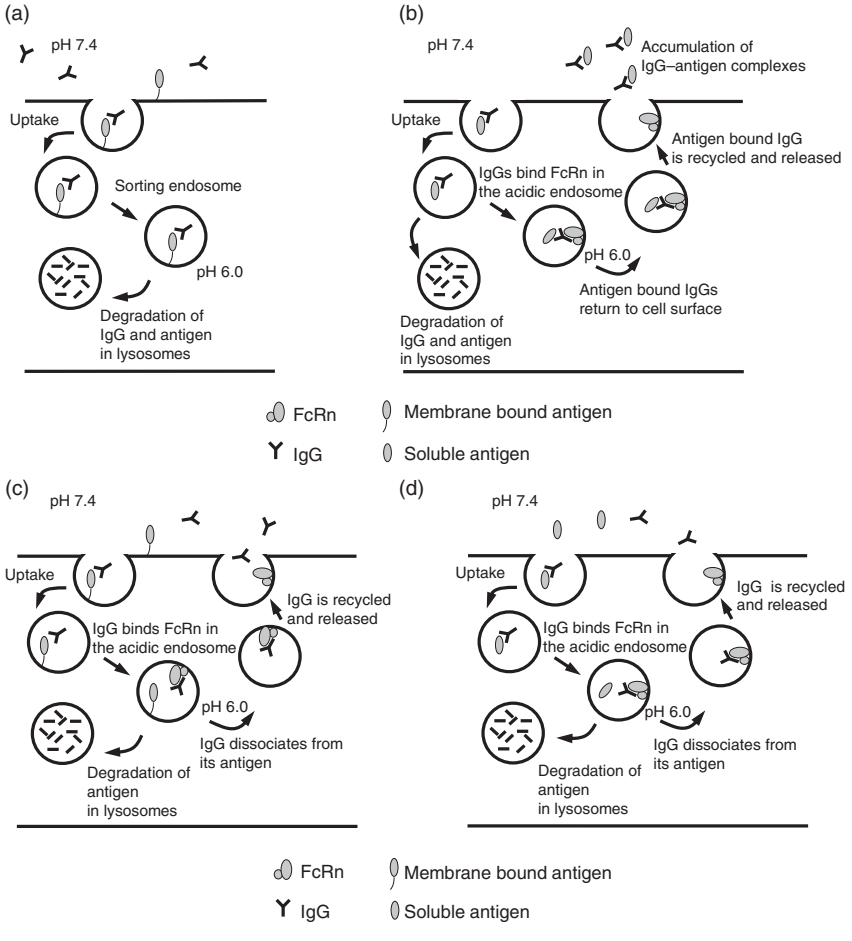

Figure 7.3 Cross section of a cell showing the fate of (a) a conventional antibody binding to a membrane-bound antigen and (b) binding to a soluble antigen. In both situations, the antibody–antigen complex is degraded via proteolysis in the lysosome. A pH-dependent antigen binding antibody will dissociate from its antigen, either membrane-bound (c) or soluble (d), in the acidic endosome and is recycled back to the plasma via FcRn, enabling it to bind to another antigen.

original wild-type residue. *In vitro* screening of the VHH library (4×10^6 unique histidine/wild-type combinations) identified pH-sensitive variants possessing a minimum of two and up to a maximum of five histidines within the binding interface. These clones displayed reduced affinity ($K_D = 10\,\text{mM}$) with minor decreases in pH while retaining near-wild-type affinity at physiological pH [156]. Interestingly, nearly one-third of VHH clones had two histidines located adjacent to each other in either sequence or space.

Although several examples of pH-dependent binding antibodies have been reported in the literature, there are a number of potential challenges when

generating such antibodies for a therapeutic application. First, the introduction of histidines into the CDRs of the antibody may abrogate or reduce antigen binding at pH 7.4 [155], thus compromising therapeutic efficacy. However, the binding affinity at neutral pH can subsequently be improved by the affinity maturation process, which is commonly applied to generate a potent therapeutic antibody [42, 157]. Second, the extent of pH dependence required to dissociate antigens in acidic endosomes and improve serum half-life *in vivo* is unclear. An anti-PCSK9 antibody with only a 2.6 larger K_D and 12-fold larger k_d at pH 6.0 than at pH 7.4 demonstrated a prolonged half-life in cynomolgus monkeys [154]. It is therefore reasonable to assume that it is the absolute k_d value in acidic pH rather than the relative pH dependence (the ratio of K_D at pH 7.4 to that at pH 6) that is important to achieve sufficient dissociation of the antigen.

7.5 Engineering IgG Effector Function

7.5.1 Introduction and General Considerations

A strong relationship exists between the efficacy of some therapeutic antibodies and their effector functions. This is particularly true in the case of antibody-dependent cell-mediated cytotoxicity (ADCC), a major contributor to the mechanism of action of various therapeutic IgGs, and in particular of those directed against CD20 [158–161]. Although the importance of complement-dependent cytotoxicity (CDC) is less well validated clinically, some clues also point to its relevance, such as in the case of the anti-CD52 antibody alemtuzumab [162]. Therefore, engineering strategies to augment effector functions are of great interest to improve the efficacy of antibody therapy. Conversely, the elimination of antibody effector functions could be relevant to decrease IgG-mediated toxicity, as shown for the CDC activity of the anti-CD3 monoclonal antibody OKT3 [163]. Such efforts are central to the next wave of therapeutic IgGs and hold great promises to expand their development. Results of a plethora of ongoing human studies with ADCC-enhanced molecules such as obinutuzumab (anti-CD20), ocrelizumab (anti-CD20), ocaratuzumab (anti-CD20), mogamulizumab (anti-CCR4), MOR208 (anti-CD19), MDX-1342 (anti-CD19), MEDI551 (anti-CD19), Xmab2513 (anti-CD30), MDX1401 (anti-CD30), PF-04605412 (anti-α5b1), and benralizumab (anti-IL5Rα) are likely to lead to a better understanding of the impact of engineering effector functions and provide new clues for designing the next generation of therapeutic antibodies.

7.5.2 Molecular Basis of ADCC and CDC Activity

ADCC and CDC are triggered by the binding of various effector molecules to the Fc portion of IgGs. Much knowledge was accumulated while deciphering the

molecular basis for these interactions. As will be seen in the following sections, such a detailed understanding was crucial to engineer human IgG Fc fragments in an effort to modulate their effector functions.

7.5.2.1 **ADCC**

Important antibody-mediated effector functions such as ADCC and phagocytosis are triggered by the interaction of IgG Fc with human Fc gamma receptors (FcγR) [164]. Support of these functions is widely attributed to three activating receptors, namely CD16A (FcγRIIIA; low-affinity receptor expressed on neutrophils, NK cells, and macrophages), CD64 (FcγRI; high-affinity receptor expressed on monocytes, macrophages, and dendritic cells), and CD32A (FcγRIIA; low-affinity receptor expressed on monocytes, macrophages, and neutrophils), and to one inhibitory receptor, CD32B (FcγRIIB; low-affinity receptor expressed on B cells, dendritic cells, monocytes, and macrophages). Allelic variations exist, particularly for CD32A (e.g., H/R131) and CD16A (e.g., V/F158), which result in various IgG Fc binding characteristics and clinical outcomes. The anti-CD20 antibody rituximab, whose mechanism of B-cell depletion strongly relies on ADCC [165], constitutes a good example. Here, patients expressing the high-affinity CD16A/V158 allotype show improved clinical benefits compared with patients expressing the low-affinity CD16A/F158 allotype.

In particular, ADCC is initiated by the concurrent binding of an IgG to its target cell and of its Fc portion to CD16A on the surface of immune effector cells. This triggers the activation of the effector cells and the secretion of various substances such as lytic enzymes, ultimately leading to the destruction of the target cell. The various human IgG isotypes differ in their natural ability to trigger ADCC, with IgG1 > IgG3 > IgG4 > IgG2 [166, 167]. The three-dimensional structure of the complex between a human IgG1 Fc and CD16A [168] revealed the importance of IgG's lower hinge region (L235–S239) and of P329. Mutagenesis studies further confirmed the importance of Fc L234 and L235 in the formation of the Fc/CD16A complex [169].

7.5.2.2 **CDC**

CDC is triggered by the binding of the first component of complement activation C1q to the Fc portion of IgG molecules. This eventually results in pore formation on the surface of target cells, followed by cell death. Natural differences exist in the ability of the various human IgG isotypes to bind C1q and trigger CDC, with the following rank order: IgG3 > IgG1 > IgG2 > IgG4 [170, 171].

Various mutational studies have identified Fc "hotspots" for C1q binding. In particular, the upper, middle, and lower hinge positions 216–225, 226–230, and 234–235, respectively, were shown to have a strong modulating effect on human IgG Fc binding to C1q [163, 169, 172]. Likewise, the IgG1 C_H2 domain and particularly positions 265, 270, 322, 326, 333, 329, and 331 are major determinants for human IgG binding to C1q [166, 167, 173–175]. Finally, the presence of carbohydrates at position N297 is crucial for complement activation [176].

7.5.3 Enhancing ADCC Activity

Various strategies have been used to increase the binding affinity between human IgG and CD16A. Such approaches can be divided into two broad categories, namely protein engineering and glycoengineering.

7.5.3.1 Protein Engineering

Much effort was aimed at the identification of IgG Fc mutants exhibiting improved binding to CD16A (Figure 7.2b). In particular, Lazar *et al.* [177] generated one particular set of substitutions (S239D/A330L/I332E) using a combination of library-based and computational modeling-based approaches. S239D/A330L/I332E typically results in a ∼10- to 100-fold increase in both human IgG1 binding to F/V158 allotypes of human CD16A and ADCC activity [132, 177]. Interestingly, the three-dimensional structure of a human IgG1 Fc fragment containing the S239D/A330L/I332E substitutions shed some light on the nature of the molecular mechanisms at play [178]. More particularly, the enhanced interaction between Fc/S239D/A330L/I332E and CD16A seems to rely on an enhanced "openness" of the Fc region as well as on the introduction of additional hydrophobic contacts, hydrogen bonds, and/or electrostatic interactions at the corresponding interface. Stavenhagen *et al.* [179] screened a randomized Fc library using yeast surface display and also reported a series of beneficial human IgG1 Fc mutations. The most effective of those resulted in increased human IgG1 binding to both F/V158 allotypes of CD16A and ADCC activity by ∼10- and 100-fold, respectively. Other beneficial mutations, though exhibiting a less dramatic effect on CD16A binding and ADCC activity, have also been identified. Among these, Shields *et al.* [121] used alanine scanning and reported a series of single and combinatorial mutants, resulting in ∼20–30% increase in human IgG1 binding to CD16 and ADCC activity.

7.5.3.2 Glycoengineering

Human Fc fragments contain two N-linked glycosylation sites at position N297. The corresponding carbohydrate is of the complex biantennary type with an N-acetylglucosamine $(GlcNac)_1$-$GlcNac_2$-Mannose (Man) core, a core α1,6 fucose (Fuc) attached to GlcNac ,and two Man-GlcNac-Gal-Sialic acid (Sia) arms attached to the bisecting $GlcNac_2$ [180]. Multiple points of intervention in this carbohydrate chain exist to optimize ADCC activity.

By far the best improvements in ADCC activity are achieved by engineering human IgGs for lack of core Fuc. This results in ∼10- to 100-fold increase in both human IgG1 binding to human CD16A and ADCC activity in an F/V158 allotype-independent manner [181–184]. Such approaches typically make use of expression cell lines deficient in their ability to add the Fuc moiety. These lines can be naturally deficient (e.g., Lec13) [181] or specifically engineered, most particularly by knocking out the gene encoding α1,6-fucosyltransferase [185]. Interestingly, the structural analysis of an afucosylated human IgG1 Fc fragment

bound to CD16A suggested that the molecular mechanisms responsible for ADCC enhancement only involved subtle conformational changes. In particular, an increase in flexibility of Fc Y296 resulted in more favorable contacts with CD16A K125 and D126 [186].

Another approach consists in expressing human IgG1 in host cells expressing acetyl-glycosaminyltransferase-III. This results in the addition of a bisecting GlcNac to the core $GlcNac_2$ and leads to ~10-fold increase in human IgG1 ADCC activity [187, 188]. It has been suggested that this effect stems from the inability of such modified molecules to serve as a substrate for α-1,6-fucosyltransferase [189], effectively resulting in their a fucosylation.

Alternate nonmammalian expression systems, such as glycoengineered yeast [190] and moss [191], have been described to produce human IgG1 molecules exhibiting human N-glycosylation structures and lacking core fucosylation. Though resulting in human IgG1s exhibiting significant (>10-fold) increase in ADCC activity, such approaches require delicate engineering and elimination of several plant or yeast enzymes to preclude the formation of nonmammalian carbohydrate structures.

7.5.4
Enhancing CDC Activity

Although the beneficial impact of CDC is not yet fully validated in a clinical setting, several clues point to its potential importance. In particular, CDC is thought to be an important factor in the activity of the anti-CD20 antibody rituximab [192] and of the anti-CD52 antibody alemtuzumab [162]. Furthermore, the greater *in vitro* activity on chronic lymphocytic leukemia (CLL) cells of the anti-CD20 antibody ofatumumab compared with that of rituximab was also suggested to be correlated to its potent ability to trigger CDC [193]. Finally, mice lacking C1q exhibit poor response to anti-CD20 antibody therapy [194]. Therefore, several Fc engineering approaches have aimed at increasing this property (Figure 7.2c).

During the course of a C1q/IgG1 epitope mapping effort, Idusogie *et al.* [175] described Fc mutations leading to a few-fold increase in C1q binding (~2–3-fold) and IgG1 CDC (~2-fold) activity. In a separate study aiming at elucidating the role of human IgG1 hinge region in C1q binding, Dall'Acqua *et al.* [172] generated upper hinge mutations with significant beneficial effects on C1q binding and CDC activity. In particular, the corresponding human IgG1 variants exhibited >20% increase in C1q binding and approximately fivefold increase in CDC activity. Substitutions at Fc positions 267, 268, and 324 were also reported [195] which, when combined, resulted in increases in human IgG1 binding to C1q and CDC activity up to ~50- and ~7-fold, respectively.

Other approaches have been described that mixed IgG1 and IgG3 sequences based on the natural ability of human IgG3 to elicit strong CDC activity [170]. In particular, a variant containing the C_H1 and hinge portion each from IgG1 and the Fc from IgG3 exhibited dramatically enhanced CDC activity exceeding that of wild-type levels [196]. Also worth noting – but difficult to apply for therapeutic

use due to potential production- or toxicity-related issues – is the dramatic increases in CDC activity (~200-fold) that can be achieved by increasing avidity for C1q via forced Fc dimerization [197].

7.5.5 Reducing ADCC and CDC Activity

Specific circumstances exist where neither ADCC nor CDC activity is desired. These include cases where such activity may lead to IgG-mediated toxicity [163] or is not part of the therapeutic IgG's mechanism of action, as seen with so-called benign blockers whose activity is solely antagonistic [198]. As such, several Fc engineering campaigns were carried out in the effort to eliminate effector functions.

Initial efforts have taken advantage of the natural inability of select human IgG isotypes (IgG2/IgG4) to elicit effector functions, a result of their Fcγ receptors and C1q binding properties, or lack thereof [199, 200]. Such a strategy has been widely applied to various therapeutic antibodies, including panitumumab (anti-EGFR; IgG2), denosumab (anti-RANKL; IgG2), nivolumab (anti-PD1; IgG4), pembrolizumab (anti-PD1; IgG4), natalizumab (anti-a4 integrin; IgG4), gemtuzumab (anti-CD33 calicheamicin; IgG4), and eculizumab (anti-complement protein C5; IgG2/4). Given the natural tendency of IgG4 antibodies to form half-antibodies [201], it is often necessary to "stabilize" the molecule by introducing the S228P hinge mutation [202].

One particular set of Fc substitutions, L234F/L235E/P331S (referred to hereafter as "TM"), results in a profound decrease in the binding activity of human IgG1 molecules to human C1q, CD64, CD32A, and CD16A [203]. In order to understand the mechanisms by which TM negatively affects these interactions, the X-ray structure of a human IgG1 Fc fragment containing TM was solved [203]. Interestingly, the broad-ranging effects of TM on IgG binding to effector molecules could be explained in terms of the localized loss of only a few interactions at the mutation sites and did not involve major structural rearrangements in the Fc. These TM mutations are now included in the Fc portion of the anti-IFNAR1antibody anifrolumab [204, 205].

Other approaches have also been described, though they are yet to be validated in the clinic. In an effort to define the role of human IgG1 hinge region in CDC activity, Dall'Acqua *et al.* [172] have described several human IgG1 middle-hinge substitutions, resulting in impaired C1q binding (>30% decrease) and CDC (>10-fold) and no significant effect on CD16A binding. These authors also generated middle-hinge modifications with impaired ability to bind CD16A (>20% decrease) and C1q (>30% decrease) as well as trigger ADCC and CDC (>10-fold reduction). These included substitutions, deletions, and insertions. In addition to providing molecules with the desired properties, this effort allowed defining the relationship between various characteristics of the middle and upper hinge such as the length, flexibility, and biochemical properties, and the corresponding effector functions.

In a separate effort, An *et al.* [198] generated a human IgG2 containing key amino acid changes derived from human IgG4. This resulted in a near-elimination of complement (C1q) and Fcγ receptor (CD32A, CD32B, CD16A and CD64) binding. Likewise, L234A/L235A mutations in a humanized IgG1 version of the anti-CD3 antibody OKT3 resulted in ∼100-fold reduction of IgG binding to CD64 and CD32 and no detectable binding to C1q [163]. Finally, C1q and FcgR receptor binding can also be nearly abolished if IgG1 Fc carbohydrates are removed by mutagenesis at the Fc N-linked glycosylation site [176].

7.5.6
Modulating IgG Effector Functions Beyond CD16A and C1q

Engineering human IgGs to generate molecules with improved or reduced ADCC or CDC has been a very successful endeavor, whose benefits in a clinical setting should soon be revealed. Preclinical data in transgenic mice expressing human CD16A look promising in terms of preventing tumor growth [206–208]. Moreover, in addition to the ADCC/CD16A and CDC/C1q pathways, other IgG effector functions and Fc receptors could constitute a fertile ground for further engineering.

In particular, strategies to enhance human IgG1 binding to CD32A have been described (Figure 7.2d). CD32A engagement is crucial for the IgG-mediated phagocytosis of tumor cells by macrophages (antibody-dependent cell-mediated phagocytosis, ADCP). Such an endeavor is complicated given the 93% sequence identity between CD32A and the inhibitory receptor CD32B. In an attempt to increase human IgG ADCP activity, Jung *et al.* [209] used a human Fc randomized library approach coupled with bacterial IgG display and generated aglycosylated Fc domains containing multiple mutations. These resulted in up to ∼160-fold increase in human IgG1 binding to CD32A with high selectivity against CD32B, as well as ∼75% increase in human IgG1-mediated tumor cell phagocytosis. Using both a library- and computational-based approach, Richards *et al.* [210] also described Fc mutations G236A/I332E, resulting in ∼70-fold increase in human IgG1 binding to CD32A, 15-fold improvement in FcγRIIa/FcγRIIb ratio, and significantly enhanced phagocytosis ability.

Engagement of CD64 by human IgG1 is thought to trigger ADCC and phagocytosis by monocytes and macrophages, respectively [211]. Much work remains to engineer the corresponding interaction, as no mutation have been described to date that results in enhanced human IgG binding to CD64. However, the recent three-dimensional structures of the complex between human Fc and CD64 [212, 213] have shed some new light on how the two partners interact at a molecular level. In particular, the critical structural and functional role played by the second subdomain of CD64, the major energetic contribution of Fc "LLGG" motif at positions 234–237, the so-called "lock-and-key" mechanism played by L235, and the strictly indirect role played by Fc carbohydrates were revealed. Such structural insights may provide new engineering clues to modulate this interaction.

7.6 Conclusion

We have attempted here to show multiple ways that protein engineering methods can be applied to modulate IgG (and other therapeutic proteins) binding characteristics and functional activity. Such an ability to tailor affinity, specificity, stability, serum half-life, and effector functions will undoubtedly lead to a combination of several improved properties into one therapeutic modality. This is expected to deliver further clinical benefits. The corresponding techniques will, of course, continue to evolve. In particular, as both computing power and our knowledge about basic protein structure increase, *in silico* based approaches will play a larger role in engineering therapeutic proteins for a given function.

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8
Bispecifics

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8.1
Introduction: Continuing Evolution of Antibody-based Therapeutics

Antibodies are naturally multifunctional molecules that link adaptive and innate immunity through interactions with specific targets via variable domains and a number of effector molecules via the Fc region [1]. The exquisite target specificity of an antibody is determined by the complementarity-determining regions (CDRs) within variable domains [2]. Functional flexibility in physically recruiting extracellular effector molecules of the immune system is through interactions between the Fc region and soluble factors (e.g., complement components) and receptors (e.g., FcγRs and pattern recognition receptor, PRR) expressed on the cell surface [1]. The Fc region also interacts with intracellular molecules, such as the neonatal Fc receptor (FcRn) [3]. Interactions with FcRn play a critical role in extending serum circulation half-life [3]. Although most naturally occurring monoclonal antibodies (mAbs) are monospecific for their target binding, a rare population of mAbs are reported to bind two or more targets. The dual specificity in such mAbs is an inherent function of natural CDRs and cannot be predicted.

Monospecific mAb-based therapeutics have demonstrated remarkable clinical impact in the past two decades, with more than 40 mAbs approved by the US Food and Drug Administration (FDA) [4]. However, clinical experiences have identified limitations of current mAbs and provided directions for future improvement. Current therapeutic mAbs have not exploited the full potential of their natural architecture and ability to mobilize innate and adapt immune systems to develop therapeutics with even greater impact. Strategies to develop next-generation antibodies can be broadly grouped into two categories: (i) Optimizing existing interaction sites in an antibody. For instance, modulating the interactions with antigens for optimal paratope/specificity, affinity/avidity, potency; expanding antibody target classes (e.g., G-protein coupled receptor (GPCR), glycan, lipids, major histocompatibility (MHC)/peptide complex, intracellular molecules, etc.); modulating

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the interactions with FcγRIIIa for antibody-dependent cell-mediated cytotoxicity (ADCC) (natural killer (NK) cells), FcγRIIa for antibody-dependent cell phagocytosis (ADCP) (macrophages) and antigen presentation (dendritic cells, DC) and with complement components for complement-dependent cytotoxicity (CDC); modulating the interaction with FcRn for placental transfer and antibody pharmacokinetic properties; modulating Fc receptor interaction (particularly, FcγRIIb) for *in vivo* efficacy of agonistic antibodies against TNFR family proteins; optimizing glycosylation of IgG molecules for interaction with PRRs; optimizing the hinge region for antigen–antibody interaction; expanding therapeutic antibodies to other immunoglobulin classes such as IgA or IgM for particular applications; using antibody fragments to improve tissue penetration and extend or shorten drug exposure when needed. (ii) Adding moieties with new functionalities to existing antibodies to enhance specificity or target delivery of therapeutic agents, for example, adding new variable domains (bi/multispecifics) to target a second disease mechanism; fusing a protein toxin for targeted delivery of a cytotoxic enzyme (immunotoxins); fusing a cytokine for targeted delivery of cytokines (immunocytokines); and attaching other effector agents such as enzymes, radionuclide, siRNA, or small molecule drugs (e.g., antibody–drug conjugates, ADC). These bispecific antibody (bsAb) fusions can also be therapeutic delivery vehicles or combined with other delivery strategies such as nanoparticles, liposomes, gene therapy and cell therapy (chimeric antigen receptor T (CAR-T), TCR-T and DC-based vaccine, etc.). Engineered bispecific or multispecific molecules can perform certain functions that are otherwise not possible with mAbs.

Although diversified, the criteria governing the development of therapeutic antibodies are generally applicable to the development of these next-generation antibody-based therapeutic proteins. At least five key requirements must be fulfilled: (i) desired functional properties, (ii) good physicochemical properties, (iii) scalable manufacturability, (iv) low immunogenicity risk, and (v) pharmacokinetic and pharmacodynamic (PK/PD) properties appropriate for the intended therapeutic application. Additional challenges to satisfy these criteria involve additional domain configuration and linker optimization. Also, complex biology is associated with these newly constructed molecules. Further complicating the issue is the fact that, when engineering one of these properties, the other properties may be altered or, in many cases, adversely affected. Therefore, a therapeutic candidate must be screened for each of the five parameters with the goal that the combination of these selected properties will result in a successful drug.

In this chapter, we will focus on engineered bispecifics, with a focus on bsAbs, immunotoxins, and immunocytokines. We will discuss the unique functions – target biology and mechanism of action (MOA) – enabled by these molecules, pairing functional moieties with targeting agents, common engineering considerations shared for such molecules to meet clinical development standards (developability), issues that may be important for one class of molecules versus the others, and clinical progress (efficacy/safety).

8.2
Enhancing Antibody Therapeutics by Addition of Functional Moieties

More than a hundred years ago, Paul Ehrlich introduced the "magic bullet" concept of treating disease by directing therapeutic agents to specific cell populations [5]. This concept established a new line of research and accelerated advancements in the field of targeted biologics. Understanding the structures and modes of action of antibodies has allowed fundamental discoveries on selected cellular processes, and the knowledge gained has been applied to create many bsAb formats for use in medicine.

Attaching an additional functional moiety to an antibody (Ab) has enhanced Ab therapies and expanded the number of therapeutic areas impacted. These formats combine multiple receptor-binding domains to act on target structures or are fusions that target the delivery of therapeutically active agents to selected cell types. In both cases, efficacy is dependent on many factors including the cell type, receptor density, antigen/epitope, affinity of the targeting agent, internalization, intracellular trafficking mechanisms, and MOA of the attached therapeutic agent.

Although the description of bispecificity of antibodies can be traced back to the work in the 1940s by Rob C. Aalberse and in the 1960s by Alfred Nisonoff [6], the definitive bsAb was described in the 1980s by Cesar Milstein and later by Michael Bevan [7, 8]. A chronicle of major events in the bsAb field can be found in a recent review by Riethmuller [9]. bsAbs target two or more distinct molecular targets by combining the specificities of multiple mAbs in a single agent. Bispecifics fall into two classes: (i) those that are biologically equivalent to the mixture of two parent antibodies, and (ii) those whose activity is dependent on the connectivity of the two targeting domains (e.g., redirected cytotoxicity or novel biology by receptor modulation). The latter bsAbs are not simply an alternative to combination therapy using two mAbs but offer benefits that are difficult to achieve using a combination of single agents.

Redirecting potent enzymatic toxins from nature using antibodies that target selected cell types, notably tumor cells, has been an active line of research for decades [10–14]. Immunotoxins are bifunctional molecules that combine the specificity of a receptor-binding moiety (e.g., antibody) and one-molecule-per-cell potency of an enzymatic toxin [15]. Immunotoxins are generated by fusing a modified form of a toxin to a receptor-binding moiety such as an antibody, an antibody fragment/mimetic, or a cytokine. The receptor-binding moiety targets receptor-positive cells, which are subsequently killed by the toxin.

Immunocytokines, similar to immunotoxins, harness the targeting specificity of Abs to deliver potent functional proteins to desired cell populations [16]. Cytokines are a large group of peptides, proteins, or glycoproteins that are secreted by specific immune cells. Unlike immunotoxins, which solely kill cells, immunocytokine fusions function as intercellular messenger molecules that evoke particular biological activities to the target cell [16]. To date, treatment with free cytokines is limited by the inability to deliver optimal concentrations to selected cell types due to dose-limiting systemic toxicity [17]. Attaching cytokines

to antibodies increases the potential to specifically deliver cytokines to disease sites at high concentrations for improved efficacy and reduce systemic exposure for better safety [16]. Immunocytokines have great therapeutic potential for the treatment of various human diseases such as viral infection, cancers, and autoimmune diseases.

8.3 Format Selection – Pairing Function and Target Biology

Many formats and applications for bsAbs, immunotoxins, and immunocytokines have been explored. Selection of the appropriate bispecific format or additional functional domain to link to an Ab depends on many factors, especially the therapeutic area and disease.

8.3.1 Selection of Bispecific Antibody Format

Recombinant bsAbs are typically engineered via different placement of antigen-binding domains. Examples include single-chain Fv (scFv), tandem scFv, diabody, tandem diabody, Fv, single domain, engineered cross-reactivity, and the combination of IgG with molecular recognition domain built upon alternative scaffolds [18, 19]. Valency of each antigen-binding domain may be different depending on the format. Some formats allow simultaneous binding to both targets, while others permit binding to one target at a time [18]. The distance and geometry of two binding domains in various formats may also be different. The PK/PD and tissue distribution/penetration/retention profile of different formats will also vary, particularly when comparing formats with and without an Fc portion where swift clearance from the circulation for small bsAb formats will be evident [18]. These features may have implications for potential applications and clinical outcomes. For example, if a format does not allow simultaneous binding, it may not be suitable for certain applications such as redirected cytotoxicity.

Over the years, the bsAb field has learned significantly from various recombinant bsAb formats. These studies have demonstrated that placement of binding domains and connecting linkers within bsAbs can impact the binding, functional/physicochemical properties, and expression.

scFv is the most widely used building block for bsAb construction. A popular bispecific format is to link an scFv to a conventional IgG at either the N- or C-terminus of the heavy or light chain via a glycine-serine linker (e.g., G4S). Linking scFv at different positions of an IgG may yield different biological activities and physicochemical properties [20, 21]. In addition, scFv is usually less stable from the lack of C_H/C_L support [22]. The instability may get even worse in a bispecific diabody format where the V_H and V_L are not physically linked by a peptide [23]. Various strategies of engineering thermostability (V_H/V_L interface residues/stable framework engineering, introducing a disulfide bond

between V_H/V_L, thermostability challenging in yeast display scFv antibody library selection, in-cell thermostability engineering using Hot-CoFi technique, etc.) have been reported to stabilize scFv in the context of a bsAb [20, 21, 24]. Fv may be more stable than scFv as both V_H and V_L are directly linked, but it may lead to steric hindrance issues that require optimization. Structurally, a Fab is inherently more stable than an scFv or Fv because C_H/C_L acts to stabilize V_H/V_L. Recently, a few new Fab-based bsAb formats have been reported with improved physicochemical properties; however, clinical viability of these formats remains to be seen [25–27].

For dual-variable-domain immunoglobulin (DVD-Ig) molecules, optimization of the antigen-binding domain combination, orientation, and the linker have been explored [28, 29]. In a recent study, we "visualized" the dynamic architecture of an optimized DVD-Ig molecule specific to interleukin-12 (IL-12) and IL-18 using single-particle electron microscopy [30]. The outer binding domain of the DVD-Ig molecule was highly mobile, and three-dimensional (3D) analysis showed that the binding of the inner antigen caused the outer domain to fold out of the plane with a major morphological change. This allows DVD-Ig molecules to bind both inner and outer antigens [30].

Fc hetero-dimeric bsAbs are an important class of the bsAb format. Heavy chain hetero-dimerization is largely based on altering the C_{H3}/C_{H3} homo-dimerization interface to hetero-dimerization by complementary amino acid size (knobs-into-holes), electrostatic interactions, or hydrophobic interactions [31, 32]. Other strategies to enable hetero-dimeric heavy chain pairing include a hybrid IgG/IgA Fc (SEEDbody) or an engineered IgG4 C_{H3} domain (DuoBody) [33, 34]. Light chain mis-paring is the major issue for the bsAb design based on this hetero-dimeric heavy chain strategy. Several approaches have been developed to alleviate this issue, such as common light chains derived from phage display libraries, transgenic mice with common light chains, κ/λ body with a common heavy chain assisted with two-step purification, or cross-mAb format with crossover C_{H1}/C_L on one arm [35–38]. Several strategies of pairing two half IgG molecules at the protein production stage have also been described [39, 40].

Another approach to avoid heavy and light chain pairing issues is diverting the single specificity of the V_H/V_L (paratope) of a regular IgG to two distinct antigen-binding sites (bi-paratope). This strategy includes 2-in-1 bsAb, bsAb reformatted from two single-domain antibodies (dual-targeting antibody, DT-IgG), and DutaMAb (engineering CDR H1/CDR L2/CDR H3 into one paratope and CDR L1/CDR H2/CDR L3 into the second paratope) formats [36, 41, 42]. A detailed list of various bsAb formats can be found in recent review articles [18, 36].

8.3.2 Immunotoxins – Toxin Selection

8.3.2.1 Single-Domain Toxins

Most single-domain toxins used in immunotoxin generation are Type I ribosome-inactivating proteins (RIPs) or ribonucleases (RNAses). Both toxin classes are

composed of an enzymatic (A) fragment that acts by site-specifically modifying a conserved eukaryotic ribosomal RNA (rRNA) structure, termed the sarcin–ricin loop. Although RIPs and RNAses act within the same rRNA loop structure, they have distinct mechanisms of action. Type I RIPs (e.g., saporin and gelonin) are rRNA N-glycosidases that recognize and depurinate a specific adenine base (A4324) [43–45]. RNAses, such as alpha-sarcin, cleave the phosphodiester bond between G4325 and A4326 [46, 47]. Either event results in conformational changes in the large ribosomal subunit, inhibiting interactions between the ribosome and elongation machinery, leading to inhibition of protein synthesis and cell death.

Single-domain toxins are attractive because of their size (~30 kDa) and absence of a receptor-binding domain. Decreased size potentially allows for improved tumor penetration, and lack of a receptor-binding domain may lead to fewer off-target effects associated with intrinsic receptor binding. However, undefined mechanisms of cytosolic delivery and absence of known motifs for translocation/intracellular routing decrease potency resulting from endosomal entrapment. Attempts to increase the cytotoxicity of a gelonin immunotoxin by incorporating a protease cleavable linker, to facilitate payload release, did not show greater potency *in vitro* [48]. Additionally, targeted cytolysins, which form pores in the cell membrane, were shown to enhance the delivery of independently targeted gelonin fusions [49]. Taken together, these results indicate that the major barrier to delivery is not release from the targeting moiety but likely escape from the endosomal compartment. This is consistent with the requirement for high concentrations of single-domain immunotoxins, compared to multidomain toxins, to demonstrate *in vivo* efficacy [50, 51]. Larger doses are not necessarily a disadvantage because the therapeutic window is large; however, it may alter the immunogenicity profile and impact downstream manufacturing of large quantities of conjugates.

8.3.2.2 **Multidomain Toxins**

Multidomain, or "AB," toxins are composed of two or more functional domains. This toxin class has evolved the ability to bind specific cell-surface receptors via a receptor-binding (B) domain and sophisticated mechanisms to shuttle their potent enzymatic (A) moieties into the cytosol of cells [52]. These additional functional domains result in more toxic proteins, compared to single domain toxins. Immunotoxins derived from multidomain toxins are typically generated by a two-step process. First, the toxin is modified to inhibit binding to native receptors, which are often ubiquitously expressed on many tissues. This is achieved by either removing the receptor-binding domain or inserting mutations to ablate native receptor recognition. The modified toxin is then conjugated to a surrogate receptor-binding moiety that redirects toxin action [10–14]. Compared to single-domain toxins, many AB toxins used in immunotoxin generation have well-characterized mechanisms of cell entry and translocation domains or trafficking motifs (e.g., KDEL) that facilitate cytosolic delivery of their enzymatic fragment.

Diphtheria toxin (DT) and pseudomonas exotoxin A (PE) are single-chain toxins composed of an enzymatic domain (A) and a receptor-binding (B) domain that is subdivided into translocation (T domain) and receptor-binding fragments [53, 54]. Before or after receptor recognition, DT and PE are proteolytically cleaved into two disulfide-linked polypeptide chains. The complex is endocytosed and trafficked to the endosomal compartment, where, under the influence of acidic pH, the T domain inserts into the membrane and mediates delivery of the enzymatic A moiety into the cytosol [55]. There, they ADP-ribosylate the eukaryotic elongation factor 2 (eEF-2), blocking protein synthesis and leading to cell death [56–58].

Although it appears to be cell-line-dependent, evidence suggests PE has two mechanisms to gain access to the cytosol: either by direct delivery from the endosomal compartment, like DT, or by retrograde trafficking and exit from the endoplasmic reticulum (ER), similar to Type II RIPs [59]. PE contains an REDL sequence, which binds to the intracellular sorting receptor KDEL [60–62].

Several modified DT and PE variants have been described and applied to immunotoxin generation to target a variety of cell-surface markers [13, 14, 63–65] (Table 8.1).

Shiga toxins and ricin are Type II RIPs composed of an enzymatic moiety (A domain), which is functionally similar to Type I RIPs, and a receptor-binding (B) domain responsible for receptor recognition. Following receptor binding, the holotoxin is internalized and undergoes retrograde transport [98]. The A fragment is released in the ER and translocated into the cytosol, where it halts protein synthesis and causes cell death by depurinating 28S rRNA [43–45].

Some of the first immunotoxins used ricin as a payload. Early versions attached the holotoxin, purified from castor beans, to full-length antibodies [99]. Next-generation ricin-based immunotoxins conjugated deglycosylated RTA (dgA). Deglycosylated RTA decreases nonspecific interactions with carbohydrate receptors and limits accumulation in the liver [100], thereby increasing the half-life and tumor localization [101, 102]. Deglycosylated RTA was initially produced by chemical deglycosylation [103, 104] but has been replaced by *Escherichia coli* expression systems [105–107]. Promising data from *in vivo* mouse experiments have led to ricin-based immunotoxins being evaluated in early stage clinical trials (Tables 8.3 and 8.4).

Shiga toxins have not been used extensively in immunotoxin generation, possibly because of the increased difficulty to express and purify the catalytic A fragment [108] compared to other toxins. Library screening approaches have been applied to discover receptor-specific variants of shiga-like toxin 1 (SLT-1), which have been shown to selectively kill human melanoma cells *in vitro* and delay tumor growth in mice [109, 110]. There is a clinical candidate currently in Phase I trial, termed MT-3724. MT-3724 is a fusion between a modified form of the SLT-1 A fragment and an anti-CD20 scFv for treatment of non-Hodgkin's lymphoma (NHL) (Tables 8.3 and 8.4).

Table 8.1 Description of DT and PE variants used for immunotoxin generation.

Toxin variant	Description	References
DT	Full-length holotoxin	[66–68]
DAB389	Used in denileukin diftitox (trade name, Ontak®)	[69–71]
DT388, DAB390, DAB486, CRM45(1–386)	DT variants lacking receptor-binding fragment. Numbers correspond to amino acid residues	[72–75]
CRM102	DT harboring P308S, S508F mutations	[76, 77]
CRM103	DT with S508F mutation	[76]
CRM107	Modified DT with L390F, S525F mutations	[76]
PE	Full-length holotoxin	[78]
PE40	Truncated 40 kDa fragment lacking the native receptor-binding domain (domain Ia)	[79–82]
PE38	Widely used modified form of PE40 harboring a deletion in domain Ib (residues 365–384)	[11, 13, 14, 82]
PE35 and PE37	Truncation of PE38, eliminating the requirement for proteolytic activation	[83–85]
PE38QQR	Modified form of PE38 with three point mutations in domain II (K590Q/K606Q/K613R) to enable conjugation via free amine groups	[86–88]
PE-LR	Modified PE38 resistant to lysosomal proteases. Almost all domain II and Ib removed, except an 11-residue sequence containing the furin protease recognition sequence	[89–91]
KDEL variants	Replacing the native C-terminal REDL motif with a KDEL sequence. Enhances binding to KDEL receptor and increases cytotoxicity	[92]
De-immunized PE variants	Removal of B- and T-cell epitopes to decrease immunogenicity	[91, 93–97]

8.3.2.3 Binary Toxins

Anthrax toxin is a binary toxin composed of three large nontoxic monomeric proteins, which form toxic complexes on the surface of cells [111, 112]. Two of the proteins – lethal factor (LF) and edema factor (EF) – are enzymatic effector proteins that covalently modify cytosolic targets [113–115]. The third protein, protective antigen (PA), is responsible for binding multiple receptors [116, 117] and delivering effector proteins into the cytosol of receptor-positive cells [111].

Following receptor binding, PA is proteolytically activated by a cell-surface furin family protease [118] and oligomerizes into a ring-shaped heptamer or octamer (termed pre-pore) [119, 120], which can bind up to three effector proteins. The resulting complexes are internalized and trafficked to the endosomal compartment, where acidic pH induces a conformational change and the

pre-pore converts into a membrane-spanning pore to actively translocate its effectors into the cytosol [111].

Multiple approaches have been developed that redirect the specificity of PA. The first approach described by Leppla and colleagues replaces the native furin activation site with a cleavage sequence recognized by proteases upregulated on cancer cells. Modified PA variants, in combination with effector molecules, specifically killed cancer cells overexpressing matrix metalloproteases [121] or urokinase plasminogen activator [122]. A second strategy alters the receptor specificity of PA and was applied to target epidermal growth factor receptor (EGFR)- and HER2-positive cancer cells [123, 124]. This was achieved by first incorporating specific mutations in domain IV [125], to ablate native receptor recognition, and fusing either epidermal growth factor (EGF) or an Affibody targeting HER2 [126] to the C-terminus of PA. A third approach was developed by engineering PA to oligomerize into specific octameric structures, where the cytotoxicity is dependent on the expression of two markers on tumor cells [127]. More recently, an anthrax-based system has been engineered to deliver antibody mimetics to inhibit Bcr-Abl and disrupt hRaf-1 signaling [128].

Retargeting anthrax toxin has yet to be explored in the clinic. Its unique characteristics and the sequence of events leading to intoxication have distinct advantages and may allow the creation of conditional immunotoxins with up to four layers of specificity by tailoring (i) protease activation, (ii) oligomerization, (iii) receptor binding, and (iv) the payload delivered.

8.3.3
Immunocytokines – Selection of Cytokine

Cytokines and related signaling molecules are major regulators of the immune system [129]. Cytokines have diverse functions depending on cell type; therefore, immunocytokine generation relies on the intended therapeutic concept and a thorough understanding of immunomodulation of the attached cytokine. Cytokines activate a variety of cell types including T cells, NK cells, and macrophages [130]. Immune cell activation promotes DC maturation, enhances NK/macrophage effector function to augment the cytolytic potential of full-length antibodies (interferons (IFNs), IL-2, IL-12, IL-15, IL-21), directly suppresses the growth of cancer cells [IFNs (α, β, γ) and IL-21], and have anti-angiogenic activity (IFNα, TNFα) [129]. No single cytokine possesses all the desired therapeutic properties; therefore immunocytokines carrying two different cytokines, termed dual-cytokine immunocytokines, may be worth considering [131]. Cytotoxic T cells and NK cells are the main immune cells required for tumor elimination [130]. A number of cytokines have shown promising antitumor activities in preclinical and clinical settings. Various antibody–cytokine fusion formats have been reviewed recently [130, 132–135].

8.3.3.1 IL-2 Family

One particular cytokine may activate different types or subtypes of immune cells and exert distinct biological functions. For instance, IL-2 has paradoxical functions in T-cell homeostasis, acting as a potent T-cell growth factor during the initiation of immune responses and has a critical function in termination of T-cell responses and maintenance of self-tolerance. IL-2 interacts with a high-affinity trimeric receptor, composed of an α-chain, a β-chain, and a γ-chain (a component shared by other cytokine receptors, including IL-15 receptor) [136, 137]. The α-chain is also expressed on T-regulatory (Treg) cells, which suppress antitumor responses [138]. Therapeutic IL-2 administration may boost antitumor T cells and NK cells, but can expand Treg cells that suppress antitumor responses [138]. It is possible that this dichotomy of functional activity may partly negate the beneficial antitumor effects of IL-2 treatment. IL-2 has been extensively evaluated for targeted delivery as an immunocytokine.

A few immunocytokines containing an IL-2 moiety, such as Hu14.18-IL2 (anti-GD2/IL-2 fusion protein), DI-Leu16-IL-2 (anti-CD20/IL-2 fusion protein), and L19-IL-2 (anti-EDB, the extracellular domain B of fibronectin/IL-2 fusion protein), have demonstrated some promising early clinical efficacy [139–141]. However, none has yet progressed to pivotal clinical trials due to various constraints in their design, in particular, the fusion of two wild-type IL-2 moieties to the antibody and retained FcγR binding of IgG-based immunocytokines. This design results in (i) high-affinity binding (picomolar affinity) to IL-2Raβγ on immune cells, compromising tumor targeting and inducing rapid systemic clearance and short half-life; (ii) high affinity for CD25 (IL-2Ra) expressed on pulmonary vascular endothelium contributing to pulmonary toxicity; and (iii) preferential activation of Tregs over immune effectors. Roche/Genentech recently described a novel class of monomeric tumor-targeted immunocytokines in which a single engineered IL-2 variant (IL2v) with abolished CD25 binding is fused to the C-terminus of an antibody with a heterodimeric Fc part [142]. FcγR and C1q binding is completely abolished by an Fc mutation. High-affinity antibodies against the carcinoembryonic antigen (CEA) (GA504, CEA-IL2v) or FAP (GA501, FAP-IL2v) were selected for tumor targeting. CEA-IL2v recognizes a membrane proximal epitope of human and binds preferentially to membrane-bound CEA, but not shed CEA. CEA- and FAP-IL2v demonstrate superior safety, PK, and tumor targeting, while lacking preferential induction of Tregs due to abolished CD25 binding, monovalency, and high-affinity tumor-targeting as compared to classical IL-2-based immunocytokines. They retain the capacity to activate and expand NK and CD8+ effector T cells through IL 2Rβγ in the periphery and the tumor microenvironment [142]. These data support their further clinical investigation for immunotherapy of cancer.

IL-15 is a proinflammatory cytokine and a member of the IL-2 family of cytokines [143]. IL-15 is also capable of stimulating the proliferation of T cells, generation and persistence of effector T cells and NK cells, and stimulation of immunoglobulin production by B lymphocytes. In contrast to IL-2, IL-15 has no effects on regulatory T cells and does not inhibit T-cell responses through

activation-induced cell death (AICD) of CD8+ effector T cells. In various preclinical studies, IL-15 has demonstrated a better safety profile compared to IL-2 with no vascular leak syndrome (VLS) [143, 144]. IL-15-based immunocytokines are being explored in preclinical studies.

Recently, IL-21 is also attracting researchers' attention for cancer immunotherapy [145]. IL-21 is produced by CD4+ T cells and NKT cells and contributes to antitumor responses by enhancing CD8+ T cell and NK cell cytotoxicity. Depending on the differentiation stage of the immune cells, IL-21 can synergize with other cytokines, especially IL-15, for immune activation [145]. Unfortunately, IL-21 also inhibits DC maturation and function and plays a major role in the differentiation of Th17 cells [145]. Other IL-2 family cytokines such as IL-7 are also being evaluated as immunocytokines [145].

8.3.3.2 Non-IL-2 Family Cytokines

In addition to IL-2 family cytokines, other cytokines such as IL-6, IL-17, IL-12, IL-10, and granulocyte-macrophage-colony stimulating factor (GM-CSF) are being explored as immunocytokines. IL-6 is mainly considered as a proinflammatory and pro-angiogenic cytokine, but its pleiotropic function is complicated by its involvement in both the prevention and promotion of lymphomagenesis depending on when and where it acts during different stages of hematogenesis [146]. IL-6 was therefore also explored as an immunocytokine [147]. IL-12 is the main driver of Th1 polarization and has emerged as one of the most potent cytokines in mediating antitumor activity in a variety of preclinical models [148]. IL-17 was found to play a role in various human inflammatory diseases and to promote tumor angiogenesis [149]. IL-17 was described to be involved in both tumor progression and tumor inhibition, in accordance with findings that inflammation is necessary for metastasis and elimination of tumor cells [150, 151]. IL-10 is produced by monocytes, macrophages, and different T-cell subtypes, but also DCs, B cells, NK cells, mast cells, neutrophils, and eosinophils, and is the major anti-inflammatory and immunosuppressive cytokine. IL-10 was evaluated as both a cytokine and immunocytokine for the treatment of chronic inflammatory and autoimmune diseases [152]. GM-CSF is a hematopoietic growth factor involved in stimulating the activity of neutrophils and macrophages [153]. GM-CSF was also explored for target delivery as immunocytokines.

8.3.3.3 Type I IFNs

Type I IFNs appear to have a unique and favorable combination of antitumor properties. IFNα can affect tumor cell function by direct inhibition of tumor growth and angiogenesis [154, 155]. In addition, these cytokines can promote the differentiation and activity of host immune cells including T cells and DCs, which may lead to IFN-induced antitumor immunity [154–156]. Early studies in mouse tumor models showed the importance of host immune mechanisms in generating long-lasting antitumor responses after treatment with IFNα/β [157, 158]. IFNα/β is being explored as a fusion to anti-CD20 mAb. An anti-human CD20-mouse

IFNα immunocytokine demonstrated *in vivo* efficacy against an established rituximab-insensitive human CD20+ murine lymphoma (38C13-huCD20) grown in syngeneic immunocompetent mice. Optimal tumor elimination required CD20 targeting [159]. No evidence of toxicity was observed. Fusion of the cytokine moiety was required, as co-administering a mixture of free IFNα and rituximab did not increase survival above controls [159]. Based on this result, an anti-human CD20-human IFNα with full-length human IgG1/κ is being developed for the treatment of B-cell lymphoma and currently in Phase I clinical trial. However, IFNα may paradoxically promote disease progression. A significant correlation was obtained between the length of survival end points and a lack of STAT3 activation by IFNα in melanoma patients, suggesting that, at least in some cases, the application of IFNα could increase the probability of disease progression via overactive STAT3 [160]. In addition, chronic IFN-I signaling is associated with hyperimmune activation and disease progression during chronic lymphocytic choriomeningitis virus (LCMV) in mice [161]. Chronic inflammation negatively impacted the number of bystander CD8+ T cells and their memory development [161]. Honjo also reported that the activation by IFNα enhanced both the induction and maintenance of PD-1 expression on T-cell receptor (TCR)-engaged primary mouse T cells through an association of IFN-responsive factor 9 (IRF9) to the IFN stimulation response element [162]. Furthermore, PD-1 expression on Ag-specific CD8+ T cells was augmented by IFNα *in vivo* [162]. Strong innate inflammatory responses promote primary T-cell activation and their differentiation into effector cells, but cause attenuated T-cell response in sustained immune reactions, at least partially through Type I IFN-mediated PD-1 transcription. Honjo further demonstrated that IFNα administration in combination with PD-1 blockade in tumor-bearing mice effectively augments the antitumor immunity [162].

8.3.3.4 Tumor Necrosis Factor (TNF) Family

The tumor necrosis factor (TNF) superfamily currently consists of 19 ligands and 29 receptors in humans, with three additional TNF superfamily receptors identified in mice [163]. TNFα and lymphotoxin-α, formerly known as TNFβ, were the first members of the TNF superfamily to be identified. Most TNF ligands are Type II transmembrane proteins with extracellular domains cleaved by specific metalloproteinases to generate soluble cytokines [163, 164]. Receptors for TNF superfamily ligands are oligomeric Type I or Type III transmembrane proteins. Several receptors for TNF superfamily ligands contain death domains (DDs) that recruit caspase-interacting proteins following ligand binding to initiate the extrinsic pathway of caspase activation [163, 164]. TNF superfamily ligands and receptors are important for normal developmental processes including apoptosis, regulation of immune cell functions, and additional cell-type-specific responses [163]. They also play a significant role in human disease including cancer and autoimmune diseases [163]. Several TNF superfamily ligands, such as TNFα, FasL, TRAIL, 41BB ligand, LIGHT, and CD40 ligand, are being explored either as cytokine – Fc fusion proteins or as immunocytokines [164].

8.4
Engineering Considerations

Early bispecific and multispecific formats relied on nonspecific chemical conjugation strategies and limited knowledge of structure and function [19]. These limitations led to sample heterogeneity, poor stability/solubility, off-target toxicities, and PK properties not suitable for drug development [19]. These technical challenges limited development, and are gradually being resolved [18]. Making an effective and safe bispecific is not straightforward; several important design and engineering criteria must be taken into consideration. One important engineering consideration not discussed here is immunogenicity, which is discussed in detail in Chapter 12.

8.4.1
Desired Properties – Selection of Tumor Antigen, Antibody, and Fusion Strategy

Careful selection of the target antigen is critical for designing multispecifics. Ideal targets are selectively and homogenously overexpressed on diseased cells compared to normal tissues, expressed only during development (e.g., CEA), or antigens in which lineage is limited to tissues considered tolerable. Importantly, antigens expressed by organs that are known to be susceptible to damage by the targeted biologic should be avoided. Surface antigens should not be shed significantly into a soluble form or detected significantly in circulation to avoid the antigen sink effect. For immunotoxins and immunocytokines, the payload must be potent enough to kill or modulate selected cell populations at concentrations achieved by antibody-mediated delivery, typically determined by the receptor levels.

For immunotoxins, the internalization of the surface target is required for toxin action. In contrast to ADCs and immunotoxins, immunocytokines do not need to be internalized for their action. If receptor binding triggers rapid internalization of immunocytokines targeting an antigen and cytokine receptor expressed on different cells (e.g., anti-EGFR-IL-2 fusion), the cytokine may not be able to engage infiltrating immune cells. Therefore, the choice of the antigen for this class of multispecific may be limited to those with low internalization rates. However, for immunocytokines targeting antigens and cytokine receptors expressed on the same cells (cis-acting mechanism), for example, CD-20 and IFNα fusion for the treatment of B cell lymphoma, it may work as long as antigen internalization is slow enough to allow engagement of the fused cytokine to its receptor on the same cells [159, 165].

Antigen targets are not required to be expressed on the cell surface but can be associated with tumor microenvironment such as the tumor stroma, tumor vasculature, or tumor debris released by dead cells. For example, fibronectin is a large glycoprotein ubiquitous in the extracellular matrix of mammalian tissues and plasma. Under tissue remodeling conditions, mechanisms of alternative splicing can lead to insertion of extensor digitorum brevis (EDB), an extra 91 amino acid

Type III homology domain, into fibronectin. EDB is undetectable in healthy individuals, but highly expressed around tumor vasculature in aggressive solid tumors [166]. High-affinity L19 antibody recognizes EDB and is applied as a targeting domain to deliver IL-12, TNF, and IFNγ. These immunocytokines are being evaluated in preclinical or clinical studies for the treatment of cancers or diseases with EDB expression near disease vasculature [167]. Similarly, the F16 antibody recognizes the extra domain A1 of tenascin and has shown selective accumulation at tumor sites and inflammatory disorders in animals and humans. An F16-based immunocytokine, F16-IL-2 (Teleukin), is currently in the clinic [168].

Bispecifics targeting two highly specific antigens with low expression or a combination of a tumor antigen and a stromal antigen may be attractive for immunocytokines and immunotoxins. A bsAb-IFNα2b immunocytokine targeting CD20 and the human leukocyte antigen-death receptor (HLA-DR) was reported to be highly specific and toxic to human lymphoma and multiple myeloma cells [169].

As mentioned previously, antibody fusions have been assembled in a wide variety of structural formats including (i) full-length immunoglobulins (Ig fusion), (ii) antigen-binding fragments such scFv, Fab, minibody, or diabody, or (iii) the Fc fragment of immunoglobulins (Fc fusion). Antibody properties such as epitope, affinity, valency, size, and format are also needed to be carefully selected. These properties are directly linked to many different aspects of a biologic, including distribution, PK/PD, etc. (discussed later). For instance, the internalization rate and the route of the complex upon target receptor engagement are largely determined by the nature of the receptor itself, whereas affinity and valency of an antibody and target epitope may significantly affect internalization. Antibodies used must be screened and characterized carefully for the desired properties, taking in account the desired format and disease indication.

First-generation antibody fusions demonstrated the benefits of cell-specific targeting, but were restricted to nonspecific chemical conjugation strategies and limited knowledge of structure and function of the antibody or fusion partner. For example, chemical conjugation approaches such as disulfide exchange [170] led to heterogeneous preparations of immunotoxins that were difficult to characterize and in some cases produced less/inactive fusions [171]. Early catalytic immunotoxin fusions often resulted in off-target toxicities against normal cells, associated with the presence of the toxin's native receptor-binding domain.

Next-generation antibody fusions applied recombinant technologies that provided several major advancements for generating bispecific formats. Targeted mutagenesis and truncation studies allowed researchers to gain understanding of structure–function relationships. The knowledge gained was applied to generate more advanced and modified formats. Fusions to truncated antibody fragments (e.g., scFv) also enabled the generation of smaller molecular weight therapeutics with increased tumor penetration. Homogeneous preparations of fusions could be produced by expression and purification as single-chain polypeptides in *E. coli* or mammalian expression systems. *In vitro* display technologies were developed to rapidly evolve high-affinity antibodies and antibody mimetics to be used as receptor-binding moieties [172, 173].

Suboptimal toxin fragments, overactive cytokines, and poor linker selection may increase or elicit unwanted toxic effects. Attenuation of a cytokine by mutagenesis may also be necessary to tone down bioactivity for safety reasons. For instance, fusion of IL-2 to the C-terminus of IgG leads to dimer formation of IL-2. The resulting IL-2 dimer exhibits higher affinity than native monomeric IL-12 to its receptor and lacks tumor targeting preference. One elegant approach to solving this problem is the "knobs-into-holes" approach. As discussed earlier, Roche/Genentech mutated IL-2 to abrogate its binding to CD25 and generated an attenuated IL-2 immunocytokine fused to only one heavy chain of "knobs-into-holes" [142]. This immunocytokine has much better tumor targeting and PK properties and is currently in Phase I trial.

Recently, *in vitro* site-specific ligation strategies have become versatile approaches to modify proteins [174–177] and have been applied to generate immunotoxins [178] and bispecifics [179]. These approaches combine some benefits of chemical conjugation and recombination technologies to enable site-specific fusion of receptor-binding moieties and fusion partners that require incapable expression systems.

After fusion, the fused functional moiety must maintain proper folding for biological activity. Different fusion strategies and orientations may significantly impact a bispecific fusion protein. Structural knowledge of cytokines and toxins is an important consideration to determine which antibody format to choose and how to fuse them. Toxins used for immunotoxin generation are typically monomeric and single polypeptide chains, while cytokines can be a single monomeric polypeptide chain (e.g., IL-2, IFNα), homodimeric (e.g., IL-10, IL-17A, IFNγ), or homotrimeric (e.g., TNFα superfamily ligands). Furthermore, some cytokines are heterodimeric (IL-12 family cytokines IL-12, IL-23, and IL-27) and are composed of two different polypeptide chains. For example, the native trimeric structure of TNF family members is essential for activity. Regular IgG fusion results in a dimeric molecule and may not be optimal. An alternative is to fuse an scFv with monomeric TNF-α, which could result in a bioactive trimeric form from noncovalent interactions between TNF-α [180]. In addition, a native monomeric cytokine may become bivalent when bound to its receptor. This may jeopardize the immunocytokine concept of using tumor antigens to drive the distribution to specifically deliver cytokines to the site of disease tissues.

Functional moieties can be joined to either the C- or N-terminus of antibodies, or to antibody mimetics using flexible peptide linkers. The length and sequence of the linker molecule need to be optimized to achieve both protein stability and bioactivity. Specific orientations may be required to maintain biological activity when fused [132, 135]. Toxin molecules are composed of multiple structured domains and arranged in different orientations. For example, an antibody that functionally replaces the receptor-binding domain of DT would be fused to the C-terminus, whereas a fusion to PE would be fused to its N-terminus. For cytokines, this depends on the impact of fusion on cytokine exposure for receptor-binding sites and needs careful optimization. More sophisticated engineering concepts

have also been reported for generating dual-cytokine immunocytokines: for instance, fusing one cytokine to the N-terminus of the heavy chain or light chain and another cytokine to the C-terminus of the heavy chain of antibodies. Alternatively, two cytokines can be arranged in tandem.

8.4.2 Pharmacokinetic/Pharmacodynamic Properties

Antibody fusions are designed to provide improved targeting as well as therapeutic index of a surrogate functional moiety, such as a toxin or cytokine. Fusion of a second functional domain may drastically alter the pharmacokinetics, pharmacodynamics, and biodistribution compared to either the antibody or the fused functional moiety. The linker separating the antibody and functional moiety must also be stable in circulation to reduce clearance and off-target toxicities.

8.4.2.1 Target-Driven Distribution

Many factors may affect the distribution and off-target toxicities of immunotoxins and immunocytokines. First-generation immunotoxins were fusions between a full-length protein toxin (holotoxin) and small subset of receptor-binding moieties such as naturally occurring ligands (e.g., cytokines) and monoclonal/polyclonal antibodies [66, 181, 182]. Full-length toxins used in early fusions demonstrated off-target toxicities to their native receptors. This led to more advanced immunotoxins that applied better understanding of toxin structure and function to decrease off-target toxicities associated with binding native receptors by mutation or deletion of receptor binding domains.

One important determining factor is the affinity of the antibody to target antigen versus the affinity of attached cytokine to its receptor. The basic principle is to use an antibody with high binding affinity to antigen to pair with a cytokine with low binding affinity to its receptor. Ideally, the K_D difference for antigen binding and cytokine receptor binding should wide enough to allow the distribution of immunocytokines be driven mainly by tumor surface antigen binding and not by cytokine receptor binding. However, most cytokines exhibit potent binding to their receptors. IL-2 binds to its receptor CD25 with picomolar affinity [183]; therefore, it is difficult to have the antibody driving antigen interactions unless an attenuated cytokine (with mutations) is fused to an antibody. The concept of the attenuated cytokine fusions discussed above is introducing mutations for decreasing the cytokine binding to their receptors, which would allow target-specific activity in antigen-positive cells but spare antigen-negative cells for potential toxicity. Another consideration is that, once delivered to tumor sites via antibody targeting, the cytokine domain(s) must be free and active to interact with receptors on the target immune or tumor cells. For instance, if the ultimate target of the cytokine is T cells, it is important to ensure that the antigen will not trigger engulfment by macrophages upon antibody binding, since this could result

in sequestration or destruction of the antibody-fused cytokines before binding to and activating the target cytokine receptors.

8.4.2.2 Half-life

Various antibody formats with size ranging from 25 kDa (scFv) to 150 kDa (IgG) have been used as targeting moieties for constructing bispecific molecules. The ability of intact antibodies and fragments to access tumor cells distant from the tumor blood supply is an important therapeutic consideration for antibody-based oncology drugs, which need to consider multiple parameters including the impact of molecular size and affinity on systemic clearance, vascular extravasation, and tumor penetration. Intact IgGs generally have limited tissue distribution, and their volume of distribution is approximately the serum volume. Wittrup modeled that the optimal size and affinity profile for tumor tissue penetration were a molecule smaller than ~12 kDa and very high affinity, or, alternatively, a molecule larger than ~75 kDa with no particular need for high affinity (i.e., low nanomolar affinity was enough) [184]. These analyses suggest that IgG-sized macromolecular constructs exhibit the most favorable balance between systemic clearance and vascular extravasation, resulting in maximum tumor uptake [184, 185]. Additionally, according to the model, molecules falling into the size range of an scFv or a Fab, that is, 25–50 kDa, were potentially the worst possible candidates for deep tumor penetration, too large for sufficiently rapid extravasation and too small to escape renal clearance, irrespective of the affinity of the molecules [184, 185].

Cytokines act through autocrine, paracrine, or endocrine mechanisms and have very short half-lives and are cleared rapidly. Cytokine action often requires high local concentrations. Once cytokines are fused to antibodies, the immunocytokine half-life is usually associated with the half-life of the antibody rather than of the cytokine. Cytokines fused to antibodies not only change the serum half-life but may also affect the local concentration. For IL-2, fusion to an IgG1 with longer half-life exhibited potent antitumor activity compared to the corresponding scFv–IL2 fusion with much shorter half-life. This suggests that increased half-life is beneficial for antitumor activity, but may increase systemic toxicity [186]. For the IFNα–CD20 immunocytokine, at low concentrations the non-targeting IFNα fusion is not biologically active, but anti-CD20 targeting IFNα immunocytokine exhibits potent IFN activity. Because anti-CD20 targeting IFNα immunocytokine engages both tumor antigen CD20 and IFN receptor at the same time, it slows down IFN receptor internalization and allows sustained IFN receptor signaling [165, 187]. The duration of cytokine receptor signaling is the key to mediate low-concentration cytokine biology, and fusing cytokines to an antibody engaging a surface antigen amplifies the signal only in antigen-positive cells. Another class of fusion – cytokine–Fc fusion – which does not belong to immunocytokines, takes advantage of the FcRn binding property of the Fc portion of IgG molecules to prolong serum half-life but lacks antigen-targeting specificity of immunoglobulins.

8.4.3 Physicochemical Properties and Manufacturability

An optimal protein therapeutic requires more than affinity and potency. Other factors such as physicochemical properties and protein expression in manufacturing are also vital to the success of a therapeutic; thus they need to be designed and assessed carefully during the discovery phase [188]. Many lessons have been learned from developing therapeutic antibodies in regard to drug-like physiochemical properties and manufacturability; these can also be applied to the development of immunocytokine- and immunotoxin-based therapeutics.

Not all cytokines or toxins can be produced well in fusion formats. It also depends where the functional moiety is attached [135]. For instance, IFNα-based immunocytokines usually have a high monomer percentage and high production yield [180], while IL-2 fusions can be produced well but will form aggregates particularly when an IL-2 dimer is used in immunocytokines [142]. IL-18 and human IFNβ usually have very poor expression when they are fused to antibodies [135]. IL-12 has to be fused at the N-terminus of an antibody to maintain its biological activity, but this could compromise antigen binding [189]. Fusing a human scFv fragment (F8) directed against the alternatively spliced ED-A domain of fibronectin to the N-terminus of murine IL-7 resulted in a fusion protein with strong tendency to aggregate, while fusion of the scFv moiety to both ends of IL-7 yielded a fusion protein of good quality [190]. Each cytokine is associated with different attributes and need to be optimized for the physicochemical properties and protein expression to select the best one for immunocytokines.

Manufacture of immunotoxins can be either straightforward or considerably more complex. Toxins derived from plants and bacteria typically express well in *E. coli* but kill many mammalian cell lines required for the production of antibodies and antibody fragments. Immunotoxins fused to smaller targeting moieties (e.g., scFv) that express well in *E. coli* can be produced easily and cheaply in large quantities as fusions. Fusing toxins to full-length antibodies (e.g., IgG) generally requires separate expression and purification strategies for each component and subsequent *in vitro* fusion. These strategies, like ADCs, lead to heterogeneous mixtures, but can typically be purified from the unconjugated antibody and free toxin.

8.4.4 Applications and Clinical Studies

8.4.4.1 Bispecific Antibodies

One limitation of bsAbs that is often debated is the fixed stoichiometry, such that each antigen-binding domain is present in a fixed ratio that cannot be titrated separately due to the physical connection between the two antigen-targeting domains. The fixed stoichiometry limitation might be alleviated to some extent through adjusting the affinity/potency of the two binding domains or through

the selection of appropriate dosing regimens. However, for target pairs with wide variation in dosing requirement and schedule, bsAb may not be a suitable approach.

Recent bispecific formats have successfully entered into early clinical development or have been approved (Table 8.2). The first bsAb, catumaxomab (Removab®, Frensenius Biotech/Trion Pharma), derived through quadroma technology, received regulatory approval by the European Medicines Agency (EMA) in 2009 for the treatment of malignant ascites [191]. Blinatumomab (BLINCYTO™, Micromet/Amgen), a first recombinant bsAb in bispecific tandem scFv format, was approved by FDA in 2015 for the treatment of relapsed or refractory B-cell precursor acute lymphoblastic leukemia (ALL) [192]. Several new formats are in clinical development for a variety of applications, as discussed in the following.

(i) *Enhancing Targeting Efficiency:* bsAbs have been explored to improve specificity by utilizing the avidity effect. This has been used to enhance tumor-targeting specificity by targeting two lower but specific tumor-associated antigens, for instance, using CD20/CD22 bsAb or CD19/CD22 bispecific-DT for cancer treatment [193, 194].

(ii) *Additive or synergistic effects:* Many bsAbs targeting distinct disease mechanisms for additive and synergistic effects are actively investigated in preclinical and clinical studies. Targeting TNF and IL-17 in rheumatoid arthritis (RA) and psoriasis for potentially improved efficacy is one example [195, 196]. When a bsAb binds to multimeric soluble cytokines, it may form large immune complexes, which may increase clearance. This could also be used for developing improved cancer immunotherapies in bispecific formats, as clinical data from combining immunotherapy drugs ipilimumab (anti-CTLA4 mAb, Yervoy) and nivolumab (anti-PD-1 mAb, Opdivo) showed significant benefit for melanoma patients [197]. However, the combination also had increased side effects, causing 36% dropout rate due to side effects in patients who got the combination, compared with 7.7% for Opdivo alone and 15% for Yervoy [197]. Given the increased toxicity, conditional activation of immune system at tumor sites may be desired. Another complex issue is that those receptors may be expressed on different immune cells, bsAbs may bring different cell types together, and the consequence of this effect is unknown.

(iii) *Redirected cytotoxicity (rCTL):* rCTL involves the recruitment of immune effector cells (T or NK cells) in proximity to tumors through one arm targeting tumor-associated antigens and the other arm targeting surface receptors on immune cells such as CD16 (FcγRIIIa), NKG2D on NK cells, and CD3 on T cells [198]. This application has been actively researched for over three decades. Initially, the concept of rCTL met with significant skepticism, particularly using BiTE constructs lacking an Fc domain. BiTE formats bring T cells in close proximity to target cells, generating an immune synapse between T cells and target cells without TCR and MHC/peptide

recognition or costimulatory signaling [199]. It is now accepted that an anti-CD3/anti-TAA bsAb can bypass the requirement of a secondary costimulatory signal to initiate cytolytic activity of T cells [199]. rCTL may also overcome resistance mechanisms for targeted mAb therapies, such as KRas and BRAF mutations [200]. Two rCTL bsAb are approved, and promising clinical results have been reported for another similar rCTL approach (ImmTAC) that applies TCRs as tumor targeting agents [201–203]. Other promising clinical results with similar therapeutic concepts to antibody-directed or TCR-directed cell therapy using CAR-T and TCR-T technology were reported recently [204]. Efficacy for this class of molecules is mainly restricted to hematological tumors. There are still many outstanding questions such as efficacy in solid tumors.

(iv) *Targeted delivery of therapeutic agents*: This involves site-specific targeting and transport of therapeutics to disease sites (e.g., joints and guts) or into immune-privilege organs (e.g., brain) or physically inaccessible sites (e.g., intracellular). A major challenge for developing drugs for brain diseases is to deliver therapeutic agents across a barrier consisting of tightly packed vascular endothelial cells called the blood–brain barrier (BBB) [205–209]. Significant progress has been made in recent years on understanding how to engineer bsAbs using a so-called molecular Trojan horse strategy across BBB, through the pioneering work by a few groups; bsAbs with one specificity against the transferrin receptor (TfR) and another specificity against a therapeutic target can hijack the TfR-mediated transcytosis mechanism to increase delivery of molecules into the brain [209, 210]. Binding affinity (on/off rate) to TfR needs to be optimized to bind TfR efficiently at the peripheral side and release bsAbs once transferred to the basolateral (brain) side [211, 212]. Another similar application of bsAb is to deliver target molecules or target cells to DCs [213–215]; the concept of this approach (DC vaccine) is for enhanced antigen presentation or cross-presentation to activate target-specific T cells for adaptive and memory responses [216–218].

(v) *Clearance of foreign molecules*: This application is particularly important for infectious diseases, as naturally occurring antibody responses to pathogen infection are polyclonal. Targeting pathogens using a mixture of polyclonal or oligoclonal antibodies, or bispecific/multispecific antibodies, has proven to be more efficient than with mAbs [219–223].

(vi) *Recruit two soluble molecules into one complex:* RG6013 is a humanized bsAb specific to Factor IXa and Factor X. In healthy subjects, these two factors are brought together by coagulation factor VIIIa [224]. In hemophilia A patients, Factor VIIIa is missing, which leads to severe bleeding. Currently, this disorder is treated with an FVIII supplement that effectively reduces bleeding complications. However, ~30% patients generated immune response against FVIII and could not continue treatment. RG6013 can replace FVIII to bring Factor IXa and Factor X together and also has longer serum half-life than FVIII ($t_{1/2} = 15$ h) [224]. RG6013 is

currently being evaluated in Phase II trials in patients with hemophilia A [225].

(vii) *Receptor targeting*: Receptors are one of the most important target classes for small-molecule drugs as well as for antibody drugs. Targeting receptors, however, could be more complex than targeting soluble ligands and cytokines. Receptors and their cognate ligands are co-evolved with ligand binding triggering defined receptor signaling patterns. However, receptors may have intrinsic signaling plasticity, where molecules other than its cognate ligand may bind the receptor and alter native receptor signalling, by recruitment of different adaptor or effector molecules, leading to distinct signaling events. Some of the most exciting observations, which reveal novel receptor biology and potential applications for antibody therapeutics, are emerging from the studies targeting two nonoverlapping epitopes on the same cell surface receptors, or paratopic bsAb, or targeting two different receptors expressed on the same cells. Table 8.2 provides a few such examples. These novel bispecific and multispecific antibodies may be easily generated "not by design" but rather "by screening."

8.4.4.2 Immunotoxins

Following the first successful *in vivo* use of an immunotoxin reported in 1981 [238], several clinical trials have been conducted over the past 30 years [13, 14, 63]. Studies in the late 1980s using fusions between mAbs and ricin A chain [107, 239] helped to define the pharmacological and toxicological barriers to be overcome. To date, only one immunotoxin (Ontak^{TM}) has received FDA approval. Immunotoxins have exceptional potency and efficient payload delivery strategies, but are hindered by side effects such as immunogenicity and vascular leak syndrome (VLS), as discussed in the following.

Immunotoxins targeting hematologic malignancies have shown the most promise. Patients with hematologic cancers are often immunosuppressed and show a lower incidence of antitoxin antibodies, which develop after several rounds of therapy [64]. Immunogenicity has been observed in 90% of patients with epithelial cell cancer after only 1 or 2 cycles of treatment [64]. Toxicities arise from targeting normal cells expressing the same receptor as cancer cells (on-target) or targeting tissues nonspecifically (off-target).

Additional information on ongoing and recently completed immunotoxin trials is provided in Tables 8.3 and 8.4.

The major dose-limiting off-target toxicity associated with immunotoxins is VLS [240, 241]. VLS is caused by weak interactions of the immunotoxin with normal endothelial cells. Endothelial cell damage leads to increased vascular permeability and is associated with edema, hypotension, and pulmonary failure [242]. VLS results from off-target delivery of an enzymatically active toxin into the cytosol of endothelial cells. Catalytically inactive mutant toxins or toxins that are not delivered to the cytosol do not cause VLS [243]. Several studies have examined the binding of immunotoxins to endothelial cells. These studies identified a small motif of three amino acids [$(x)D(y)$, where $x = L,I,G$ and

Table 8.2 Examples of Bispecifics.

Target or target pair	Observation	References
Targeting two or more distinct epitopes on the same receptor		
EGFR	bsAb- consisted of attaching an scFv of one epitope (anti-EGFR mAb 806) at the C-terminus of the V_L chain of an existing mAb (anti-EGFR mAb 225) via a G4S linker, redirected receptor trafficking to the lysosome for destruction rather than the endosome as observed with individual mAbs. This approach blocked receptor recycling and effectively eliminated the receptor from the cell surface. Trispecific antibody–fibronectin scaffold fusion proteins accelerated EGFR clustering and downregulation in culture and showed superior efficacy in a xenograft tumor model.	[36, 39, 226]
HER2	In DVD-Ig format, the placement of the trastuzumab and pertuzumab target binding domains is critical to determining the agonistic or antagonistic activities. An anti-HER2/HER2 DVD-Ig, built using trastuzumab and pertuzumab antigen-binding domains, demonstrated superior antitumor efficacy in a variety of tumor cells sensitive or resistant to trastuzumab or pertuzumab treatment. A biparatopic HER2-targeting antibody–drug conjugate enhanced toxin delivery into tumor cells and demonstrated therapeutic activity in breast cancer models representing T-DM1 eligible, resistant, and ineligible patient populations and has sufficient safety profile in non-human primates to support its translation into clinical trials	[227–229]
IGF-1R	BIIB4-5scFv, generated by fusing a stability-engineered scFv to either the C- or N-terminus of the heavy chain of an IgG molecule via (G4S)3 linker with a unique geometry, was capable of engaging all four of its binding domains simultaneously and demonstrated improved ability to reduce the growth of multiple tumor cell lines, leading to superior tumor growth inhibition over its parental mAbs *in vivo*	[20]
CCR5	The bsAbs constructed using two nonoverlapping epitopes on CCR5 with one V_H/V_L pair in disulfide stabilized scFv format connected to another antibody in IgG blocked two alternative docking sites of CCR5 tropic HIV strains and were 18- to 57-fold more potent than individual mAbs *in vitro*	[230]
CTLA-4	A bispecific CTLA-4 scFv targeting different epitopes, linked by a (KEA)*n* semi-rigid helical spacer, was reported to be an inverse agonist, although the individual scFv blocks interactions between B7 and CTLA-4 and functions as receptor antagonists	[231–233]

Table 8.2 *Continued.*

Target or target pair	Observation	References
Targeting two different receptors on the same cell		
EGFR/ IGF-1R	A stable IgG-like bsAb targeting both EGFR and IGF-1R, denoted as EI-04, was constructed with a stability-engineered scFv against IGF-1R attached to the C-terminus of an IgG against EGFR. EI-04 demonstrated enhanced *in vivo* antitumor efficacy versus parental mAbs in xenograft models and a BxPC3 model	[20]
LTR/ TRAIL-R2	An IgG-like bsAb consisting of a stability-engineered anti-LTR (lymphotoxin receptor) scFv genetically fused to the heavy chain of a full-length anti-TRAIL-R2 IgG1 demonstrated pronounced antitumor activity, an event that does not occur by simply combining the individual antibodies	[234]
EGFR/ ErbB3	MEHD7945A, a "2-in-1" bsAb, inhibits EGFR- and ERBB2/ERBB3-dependent MAPK and PI3K signaling in A431 and BxPC3 cell lines and was broadly efficacious in multiple tumor models compared with monospecific anti-HER2 antibodies	[41, 235, 236]
FcεRI/ FcγRIIb	The 9202.1/5411 "knobs-into-holes" bsAb, constructed using a mAb against FcεRIα and a mAb against FcγRIIb with shared common light chain, inhibited IgE-mediated activation of RBL, a cell line sharing characteristics with both mast cells and basophiles, transfected with human FcεRIα and FcγRIIb	[237]

$y = V,L,S$] in the A chain of ricin responsible for endothelial cell damage [244]. Subsequent studies generated mutant forms of RTA and PE by mutating putative $(x)D(y)$ motifs [245, 246] or deletion of nearly all domain II of PE [247], which dramatically reduced VLS in mice.

The humoral arm of the immune system has evolved to protect against foreign molecules. Unlike small-molecule compounds, proteins of nonmammalian origin are expected to elicit an immune response that limits the effectiveness of biologic therapeutics after repeated treatment cycles. This limitation is amplified for immunotoxins due in large part to the toxin moiety derived from plant or bacterial sources, which further increases the likelihood of eliciting an immune response after just a few doses. Humanized antibodies have been used extensively as targeting moieties for bispecifics. Adapting immunotoxins for chronic human use is complex and has not been well characterized. It remains challenging to de-immunize foreign proteins of heterologous structures and functions while retaining biological function.

Combination therapy with immunosuppressive agents has had different success rates in reducing neutralizing antibody formation against immunotoxins. Early

Table 8.3 Ongoing immunotoxin clinical trials.

Immunotoxin	Payload	Targeting moiety	Target	Condition	Clinical stage	Trial identifier
Denileukin diftitox (Ontak)	DT	IL-2	IL-2 receptors	Cutaneous T-cell lymphoma	Approved	N/A
Moxetumomab pasudotox (CAT-8015, HA22)	PE	dsFv	CD22	HCL	III	NCT01829711
				Adult ALL	I/II	NCT01891981
				Pediatric ALL	II	NCT02227108
LMB-2 (anti-tac(Fv)-PE38)	PE	Fv	CD25	HCL	II	NCT00321555
	PE + fludarabine and cyclophosphamide			Adult T-cell leukemia	II	NCT00924170
SS1P	PE + pentostatin and cyclophosphamide	dsFv	Mesothelin	Mesothelioma	I/II	NCT01362790
Resimmune (A-dmDT390-bisFv(UCHT1))	DT	2 × Fv	CD3ε	Cutaneous T-cell lymphoma	II	NCT00611208
	DT + ionizing radiation			Stage IV melanoma	I/II	NCT01888081
DT2219ARL	DT	Two scFv	CD19 and CD22	B-cell lineage leukemia or lymphoma	I/II	NCT02370160
MOC31PE	PE	Monoclonal antibody	EpCAM	Peritoneal carcinomatosis from colorectal carcinoma	I/II	NCT02219893
Combotox (mixture of HD37-dgA and RFB4-dgA)	Deglycosylated ricin A chain + cytarabine	Monoclonal antibody	CD19 and CD22	Adult ALL	I	NCT01408160
D2C7-IT (D2C7-(scdsFv)-PE38)	PE	scdsFv	EGFRwt and EGFRvIII	Glioblastoma multiforme	I	NCT02303678
MT-3724	Shiga-like toxin A	scFv	CD20	NHL	I	NCT02361346

Table 8.4 Recently completed immunotoxin clinical trials.

Immunotoxin	Payload	Targeting moiety	Target	Condition	Clinical stage	Trial identifier
MOC31PE	PE	Monoclonal antibody	EpCAM	Epithelial carcinomas	I	NCT01061645
Oportuzumab monatox (tradename Vicinium, VB4-845)	PE	scFv	EpCAM	Bladder cancer	I, II	Not listed
HuM195/rGel	Gelonin	Monoclonal antibody	CD33	AML, CML, MDS	I	NCT00038051
VB6-845	deBouganin	Fab	EpCAM	Epithelial carcinomas	I	NCT00481936
LMB-9 (B3(dsFv)-PE38)	PE	dsFv	Lewis Y	Advanced colon, breast, non-small-cell lung, bladder, pancreatic, or ovarian cancer	I	NCT00005858
BL-22 (RFB4(dsFv)-PE38, CAT-3888)	PE	dsFv	CD22	Refractory CLL, PLL, or NHL	I	NCT00126646
				HCL	I	NCT00021983
				HCL	II	NCT00074048
LMB-2 (anti-tac(Fv)-PE38)	PE	Fv	CD25	Leukemia or lymphoma	I	NCT00002765
				Melanoma, non-melanomatous skin cancer	II	NCT00295958
				Refractory leukemia or lymphoma	I	NCT00085150
				CLL	II	NCT00077922
				Cutaneous T-cell lymphomas	II	NCT00080535
SSP1 (SS1(dsFv)PE38)	PE38	dsFv	Mesothelin	Several forms of advanced solid tumor cancers	I	NCT00006981, NCT00066651
	+ Paclitaxel, carboplatin, and bevacizumab			Non-small-cell lung carcinoma (NSCLC)	I	NCT01051934
LMB-7 (B3(Fv)-PE38)	PE38	Fv	Lewis Y	Leptomeningeal metastases	I	NCT00003020

(*continued overleaf*)

Table 8.4 (*continued*)

Immunotoxin	Payload	Targeting moiety	Target	Condition	Clinical stage	Trial identifier
IMTOX-25 (RFT5-dgA)	Deglycosylated ricin A chain	Monoclonal antibody	CD25	Relapsed or refractory cutaneous T-cell NHL	II	NCT00667017
				Metastatic melanoma	II	NCT00314093
				Adult T-cell leukemia and lymphoma	II	NCT01378871
IMTOX-22 (RFB4-dgA, RFB4-SMPT-dgA)	Deglycosylated ricin A chain	Monoclonal antibody	CD22	Refractory CD22 positive B-cell lymphoma	I	NCT00001271
			IL-2	Graft-versus-host disease	I	NCT00025662
TP-38	PE38	TGF-α cytokine	EGFR	Recurrent grade 4 malignant brain tumors	II	NCT00071539
				Glioblastoma multiforme	II	NCT00104091
Cintredekin besudotox (IL-13-PE38QQR)		IL-13 cytokine	IL-13 receptors	Malignant glioma	I/II	NCT00006268
Moxetumomab pasudotox (mutated RFB4(dsFv)-PE38, CAT-8015, HA22)	PE38	dsFv	CD22	NHL and CLL	I/II	NCT01030536
				Childhood ALL or NHL	I	NCT00659425
DT2219ARL	DT	Two scFv	CD19 and CD22	B-cell lineage leukemia or lymphoma	I	NCT00889408
Ontak (denileukin diftitox, DAB389-IL-2)	DT	IL-2	IL-2 receptors	Adult T-cell leukemia	II	NCT00117845
				NHL	II	NCT00003615
scFv(FRP5)-ETA	PE	scFv	HER2	Melanoma, breast and colon cancers	I	Not listed

Abbreviations: ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; CML, chronic myelogenous leukemia; DT, diphtheria toxin; HCL, hairy cell leukemia; MDS, myelodysplastic syndrome; NHL, non-Hodgkin's lymphoma; PE, pseudomonas exotoxin A; PLL, prolymphocytic leukemia.

attempts to pretreat patients with either cyclophosphamide [248] or cyclosporine A [249] prior to RTA-based immunotoxin administration were ineffective in clinical settings. Co-treatment of CTLA4Ig and a PE-based immunotoxin blocked the production of neutralizing antibodies and extended the duration of therapy, resulting in greater exposure and increased efficacy in animal models [250]. Another study combining CTLA4Ig with anti-CD40L pretreatment significantly inhibited anti-RTA responses in mice, resulting in a 1.5-fold increase in tumor cell killing [251]. Pretreatment with rituximab did not inhibit human immune responses to LMB-1, even though patients had undetectable peripheral B-cell levels [252]. Recently, a combination of pentostatin and cyclophosphamide was shown to work synergistically to deplete T cells, spare myeloid cells, and induce immunosuppression in residual host T cells, thereby preventing rejection of bone marrow allografts [253]. This combination was effective in delaying neutralizing antibody responses to the SS1P immunotoxin in preclinical studies with mice [254] and in patients with malignant mesothelioma [255].

Several less immunogenic immunotoxins have been generated. Initial studies used mice as a surrogate model and focused on the removal of B-cell epitopes by mutagenesis [96, 97] and deletion [91, 95]. These studies were critical in demonstrating B-cell epitope identification and removal, which, in combination with structure–function studies, is a rational approach to de-immunize toxins and allow increased number of treatment cycles. More recently, similar approaches have been applied to generate immunotoxins lacking human B-cell and T-cell epitopes. Pastan and colleagues generated PE-based immunotoxins with 93% decrease in immunogenic epitopes, which maintain activity *in vivo* [93, 94, 256].

Several biotechnology and pharmaceutical companies have de-immunized other toxins from plants and bacteria to serve as platforms for next-generation immunotoxins, and more are currently under development. Angelica therapeutics is developing immunotoxins based on a de-immunized form of the diptheria toxin, termed DIDT (de-immunized diphtheria toxin). Their lead candidate, IL2-DIDT (also referred to as Angeloxin), is a fusion between DIDT and IL-2, and aims to be an advanced version of Ontak, for treatment of cutaneous T-cell lymphoma. Research Corporation Technologies has developed a de-immunized form of alpha-sarcin, termed Sarcin-DI, to be used as a next-generation toxin payload in partnership with academics and industry. Immunotoxins using a de-immunized, plant-derived toxin bouganin, termed deBouganin, are currently being developed by Viventia Biotechnologies, Inc.

8.4.4.3 **Immunocytokines**

Intratumoral or peritumoral injection of recombinant cytokines has been reported to achieve tumor eradication in animal models, highlighting the potent biological activities of cytokines and the importance of high concentrations of localized drug in the tumor microenvironment [257–261]. This may prove more challenging for treating humans; as many tumors are disseminated diseases [262]. Administration of free cytokines is often associated with severe toxic effects, including VLS and systemic inflammation due to large amounts of the

Table 8.5 Immunocytokines in the clinic.

Immunocytokine	Company	Format	Antigen	Indication	Stage
F16-IL2 (Teleukin)	Philogen	Diabody	A1 domain of Tenascin C	Breast cancer, Merkel cell carcinoma, glioblastoma AML, solid tumors	Phase Ib/II
Hu14.18-IL2 (EMD273063, APN301)	Merck KGaA	IgG	GD2	Melanoma, neuroblastoma	Phase II
L19-IL2 (Darleukin)	Philogen	Diabody	EDB of fibronectin	Melanoma, pancreatic cancer, RCC	Phase IIb
NHS-IL2LT (EMD 521873, Selectikine)	Merck KGaA	IgG	DNA	Solid tumor, NH lymphoma NSCL carcinoma	Phase I/II
BCI-IL12 (AS1409)	Antisoma/Novartis	IgG	Domain VII of fibronectin	Melanoma	Phase I/II
NHS-IL12 (hTNT3-IL-12)	Merck KGaA	IgG	DNA/histone	Various solid tumors	Phase I
L19-TNF (Fibromun)	Philiogen	scFv	EDB of fibronectin	Melanoma	Phase I/II
DI-Leu16-IL2	Alopexx Oncology	IgG	CD20	B cell NHL	Phase I
F8-IL-10 (Dekavil)	Pfizer/Philogen	scFv	EDA of fibronectin	Rheumatoid arthritis Crohn's disease; ulcerative colitis	Phase I
Anti-CD20-IFNa (IGN002)	ImmunoGen	IgG	CD20	NHL	IND
Anti-CEA-IL2v (RG-7813)	Roche/Genentech	IgG	CEA	Colorectal, head/neck, lung, non-small-cell, unspecified solid tumor	IND

proinflammatory cytokines being absorbed in the peripheral tissues en route to the tumor and its draining lymph nodes. Free cytokines also do not specifically traffic to tumor sites, thereby limiting efficacy by the lack of adequate exposure.

Currently, only a few cytokines, for example, Proleukin® (IL-2), Roferon A® (IFNα 2a), Intron A® (IFNα 2b), Inferax® (IFNβ), Actimmune® (INFγ), Leukine® (GM-CSF), and Beromun® (TNF), are approved either as anticancer drugs or adjuvants for cancer vaccines [263]. Immunocytokines harness the tumor targeting ability of mAbs to guide cytokines specifically to tumor sites where they might stimulate more optimal antitumor immune responses and avoid systemic toxicities that limit current cytokine dosing and therefore potential efficacy [130, 264, 265]. There are a few immunocytokines currently transitioning into clinical development [132, 135]. The majority of them are for the treatment of cancer, and only one is for autoimmune diseases. Table 8.5 summarizes those molecules.

8.5 Conclusion

Novel biologics with multiple functional domains are valuable research tools and promising therapeutic agents. Technologies to produce quality antibody fusions and development of innovative strategies to overcome clinical barriers are constantly improving. The key challenges aim to identify target pairs and match the structure and function for the intended biology. Given the complexity of the biological system, current progress in bsAb field, trispecific and tetraspecific antibodies, may provide new opportunities for treating diseases and may open up new therapeutic concepts. The ability to target specific cell types and deliver functional domains that act by nonoverlapping mechanisms of action, compared to other therapeutic agents, makes bispecifics and "armed antibodies" ideal candidates as single agents and combination therapies.

Disclosure of Potential Conflict of Interest

All authors are employees of AbbVie Inc. and may own AbbVie stock. AbbVie Inc. sponsored the study, contributed to the study design, participated in the collection, analysis, and interpretation of data, and in the writing, reviewing, and approval of this publication.

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9
Antibody–drug Conjugates (ADCs)

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9.1
Introduction

> In order to pursue chemotherapy successfully we must look for substances that possess a high affinity and high lethal potency in relation to the parasite but have a low toxicity in relation to the body, so it becomes possible to kill the parasites without damaging the body to any great extent. We want to hit the parasites as selectively as possible.
>
> *Paul Ehrlich 1906*

Applying Ehrlich's concept of the "magic bullet" to cancer has been a long-cherished ambition of oncologists and medicinal chemists, and antibody–drug conjugates (ADCs) offer the possibility of realizing this goal by combining the *potency* of cytotoxic agents with the *selectivity* of antibodies.

This chapter aims to set out the principles behind ADC therapy, detail the individual components of ADCs, and show how these principles and components have been utilized in ADCs approved by regulatory bodies. In the second part of the chapter, we review further developments of the first-generation payloads and the emergence of novel payloads with reference to the clinical progress of these agents.

ADCs have been recently reviewed by Peters and Brown [1], and internalization and trafficking has been covered by Ritchie *et al.* [2]. The 2011 review of Goldmacher and Kovtun provides an excellent introduction to the ADC area in general [3] (Figure 9.1).

9.2
General Mode of Action

ADCs are administered intravenously (IV) and circulate systemically until binding to their target antigen on the tumor surface. Binding to the target

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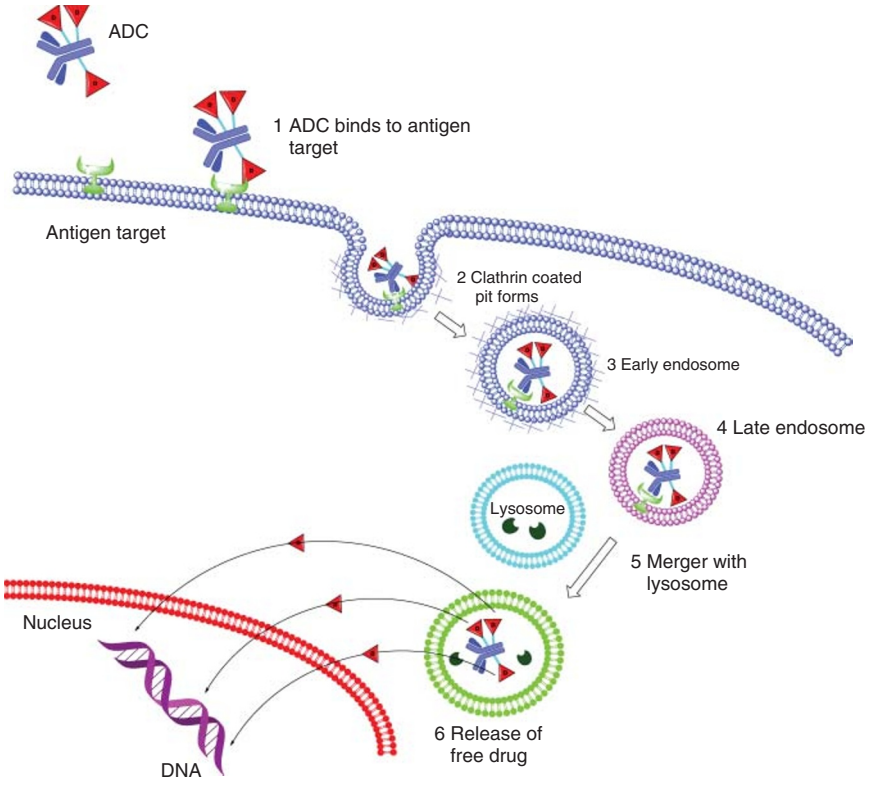


Figure 9.1 ADC mode of action (for DNA-targeting warhead).

antigen promotes the formation of a clathrin-coated pit, which deepens until the ADC–antigen complex is internalized in the target cell. Once inside the cell, the ADC–antigen complex transits through several stages of transport and endosomal vesicles before finally entering the lysosome [1].

In some cells, the endosomes contain the Fc neonatal receptor (FcRn), which can bind to the antibody component of the ADC and recycle it to the cell surface. This mechanistic pathway will reduce the amount of ADC reaching the lysosome, but it also serves to protect normal cells by preventing ADC reaching their lysosomes.

Early endosomes mature into late endosomes, becoming more acidic and forming intraluminal vesicles (ILVs) which contain the ADC–antigen complex. These ILVs ultimately deliver the ADC–antigen complex to the lysosome. Conditions in the lysosome result in the release of a cytotoxic warhead, which can attack vital components of the targeted tumor cell, leading to apoptosis [2] (Figure 9.2).

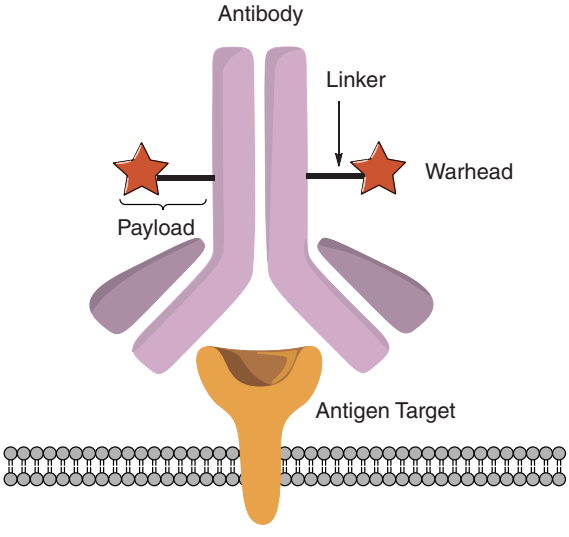


Figure 9.2 ADC components.

9.3 The Components of an Antibody–Drug Conjugate

The ADC itself can be split into three components: the antibody, the linker, and the warhead [3].

9.3.1 The Antibody

Nonhuman species can readily generate antibodies to human cancer antigens; however, these nonhuman antibodies are immunogenic in humans. For example, a single injection of a murine antibody leads to the generation of anti-mouse antibodies within 2 weeks of administration [3]. This problem can be ameliorated by replacing the nonhuman constant domains of the antibody with human domains to produce a chimeric antibody. The process can be taken a stage further by replacing the nonhuman variable domain with human to afford a humanized antibody. Fully human antibodies can be raised directly from transgenic mice (in which murine antibody encoding genes have been replaced with human) or from large recombinant libraries generated by techniques such as phage display.

Humanized and fully human antibodies have the advantage of being retained longer in circulation than their murine equivalents because of their ability to interact with FcRn, which controls the half-life of immunoglobulins (IgGs) [4]. In addition, they can provoke antibody-dependent cellular cytotoxicity (ADCC). The Fc

portion of the ADC antibody can recruit natural killer (NK) cells and neutrophils. These effector cells can employ the perforin–granzyme pathway and Fas ligands to kill the ADC-targeted tumor cell.

The nonproprietary names of chimeric antibodies contain the *tuximab* descriptor and humanized antibodies contain the *tuzumab* descriptor, while fully human antibodies are denoted by *tumumab.*

Antibodies can be divided into four isotype families, IgG1–4. IgG1 is most frequently used isotype in the ADC field but examples of IgG2 (glembatumumab)- and IgG4 (gemtuzumab and inotuzumab)-based ADCs are also known. IgG3-based ADCs are less favored because of their short half-life [5, 6].

It is possible to list the "ideal" properties of the antibody component of an ADC; however, because of the relatively early stage of development of the ADC field, the desirability some of the properties are still subject to debate.

Suggested desirable properties for the antibody component of an ADC include the following:

- High specificity for tumor antigen target
- High binding affinity
- Long circulation time
- The ability to provoke ADCC
- Tumor-suppressing modulation of antigen's biologic activity
- No loss of the above on conjugation to cytotoxic payload.

Some properties, such as specificity for tumor antigen target, are noncontroversial, whereas other seemingly desirable properties such as high binding affinity and engagement of ADCC are open to question.

At first sight, the requirement for high target affinity would appear to be a given. However, there is no proven or clinical correlation between affinity and efficacy. Indeed, given the dosing levels at which ADCs are administered intravenously, ADC concentration at the tumor site should be sufficiently high to saturate the antigen regardless of binding affinity [3]. Furthermore, high binding affinities may limit tumor penetration, with most binding taking place in the perivascular region of the tumor.

Similarly, it is assumed ADCC makes a positive contribution to the anticancer activity of ADCs. However, this may come at the expense of target localization or ADC internalization. IgG1 and 3 antibodies elicit the strongest ADCC effect, and so, conversely, this has been a factor in selecting IgG2 and 4 as antibodies for some ADCs [3].

The first generation of ADCs employed unengineered antibodies, simply attaching payloads to convenient lysine groups or cysteine residues generated by the reduction of disulfides present in the hinge region of the antibody. More recently, the trend has been employed to engineer antibodies with cysteine groups at fixed positions or non-natural amino acids that enable novel conjugation chemistries.

9.3.2
The Linker

The role of the linker is to attach the cytotoxic warhead to antibody. The linker should be robust enough to survive in the circulatory system but be capable of degradation within tumor cells to release an active cytotoxic molecule. Linkers can be split broadly into two categories: cleavable and noncleavable. Cleavable linkers contain a trigger or cleavage point that can be activated inside target cell. Noncleavable linkers lack a trigger and rely on the breakdown of the antibody itself to release an active cytotoxic species. These activation processes take place inside the lysosomes of the targeted tumor cells. Lysosomes are organelles that contain a wide variety of enzymes in order to be able to break down the range of biomolecules engulfed by the cell, including peptides, nucleic acids, carbohydrates, and lipids. The enzymes responsible for this hydrolysis require an acidic environment for optimal activity, and the pH of the lysosome is 4.5–5 pH. ADCs with acid-sensitive linkers are degraded in the lysosomal environment. ADCs with linkers containing peptide triggers can be cleaved by proteases such as cathepsin B which are present in the lysosome. The released warhead, as long as it is sufficiently hydrophobic, can cross the lysosomal membrane and continue on to engage with its cellular target. ADCs with noncleavable linkers are degraded by lysosomal proteases to leave a residual amino acid attached to the drug linker. These amino acid catabolites are ionized and incapable of crossing the lysosomal membrane without the aid of transporters such as SLC4643a [7].

9.3.3
The Cytotoxic Warhead

Ideally, the cytotoxic warhead should be highly potent, as the number of warhead molecules delivered to an individual tumor cell will be determined by the number of antigens on the surface of the target cell. The cytotoxic warhead should be synthetically accessible either by harnessing naturally occurring biosynthetic pathways or through chemical synthesis. It is important to realize that making the warhead alone is not sufficient, as the drug–linker moiety (hereafter referred to as a *payload*) also needs to be formed. Furthermore, the linker must be attached to the warhead in such a way that it does not abolish its cytotoxic activity. In the case of a noncleavable linker, the residual payload unit left after antibody degradation must still be active. For a cleavable linker, the cleavage reaction must yield a cytotoxic product. Finally, the physical properties of the warhead and payload must not be neglected: highly lipophilic moieties can be difficult to dissolve in conjugation media and adversely affect the properties of the host antibody, causing aggregation during conjugation or rapid clearance of the ADC itself after administration.

9.3.4 The Antigen

Although not part of the ADC proper, it can be useful to consider the target as a component of the ADC–antigen complex as a whole. The desirable qualities for target antigens include the following:

- Tumor selectivity
- Strong expression on tumor cell surface
- Homogenous expression over tumor
- Good penetration
- Driver of tumor progression
- Expression on cancer stem cells
- Little or no shedding
- Efficient internalization
- Efficient trafficking to lysosome
- Efficient breakdown of the ADC–antigen complex.

Ideally, the antigen would be specific to tumor tissue. However, in reality, the vast majority of antigens targeted by ADCs are simply overexpressed on tumor rather than normal tissue. For example, HER2 has been successfully targeted despite the fact that it expressed on normal tissue [3] including the heart. The location of expression is also important. Prostate-specific membrane antigen (PSMA) is expressed only on the surface of prostate tumors, but in normal tissue it is expressed inside the cell and as such is not accessible to the ADC [8].

In general, a high level of surface antigen expression is desirable, as this should maximize the amount of drug delivered into the cell. However, there is no strong correlation between antigen expression and ADC potency [3]. This is because, for each tumor cell, there is a certain amount of drug required to trigger cell death, but the cell can only die once, so delivering more drug than this threshold amount will not increase the effectiveness of the ADC. Expression levels can determine the choice of the ADC warhead; low expression of target antigen should favor the use of highly potent warheads (because, by definition, less warhead is required to kill the cell), while high expression levels would permit the use of less toxic warheads.

Homogeneous antigen expression is desirable so that the whole extent of the tumor is targeted. In practice, this is not such an important criterion for ADCs with cleavable linkers, as the released warhead should be sufficiently lipophilic to enter and kill neighboring cells even if they do not express the antigen target. Conversely, noncleavable ADCs (or ADCs with intrinsically hydrophilic warheads) would be best deployed against tumors that homogenously express antigen, as they will not be able to exploit the bystander effect.

A high degree of antigen penetrance is desirable, which means that the target is expressed across a wide range of cell lines for a particular tumor indication. For example, CD33 is found in 90–95% of acute myeloid leukemia (AML) patients [9]. High penetrance simplifies clinical trial recruitment and improves the economics of the ADC by maximizing the number of patients eligible for treatment.

The issue of penetrance highlights the utility of patient-derived xenografts (PDX) during preclinical ADC development, as they can give a better indication of clinical penetrance than cell-line-derived xenografts.

While the antigen can be thought of simply as an "address" for delivering the cytotoxic warhead, it is desirable that the antigen is actively involved in promoting tumor progression. For example, HER2 is involved in generating both growth and survival signals in breast cancer cells. Simply binding trastuzumab to the HER2 receptor can disrupt these oncogenic signaling pathways [10]. Similarly, binding of the SGN-30 antibody to CD30 causes growth arrest [11] in lymphomas. An obvious tumor resistance strategy when confronted with an ADC is to simply lose the surface antigen target. Cells that are dependent on oncogenic signaling mediated by the antigen target (so-called oncogenic addiction) cannot delete the antigen from the cell surface without compromising oncogenic growth or survival.

Cancer-stem-cell-specific antigens have potential as privileged targets in the treatment of cancer [12]. According to the cancer-stem-cell hypothesis, slowly dividing cancer stem cells can act as a reserve for the tumor. While rapidly dividing cancer cells can be killed by chemotherapy, the stem cells can remain unscathed, allowing the tumor to grow back. Actively targeting cancer stem cells with the right warheads could lead to sustained tumor regression. Potential stem cell targets include CD133, 44, and 96 [13–15] and DLL3 (see Section 9.7.2.3).

There is also interest in targeting antigens present on tissues that support the growth and spread of tumors, such as the neovasculature and stromal tissues. These tissues are not themselves cancerous and should be genomically stable, reducing the risk of mutation-mediated resistance. Targeting the neovasculature should cut off oxygen and nutrient supply to the growing tumor, while destroying stromal tissue could contribute to tumor death by reducing the level of ambient growth factors [1].

Some cell-surface antigens can be shed from tumors and circulate systemically. These circulating antigens can act as decoys, binding the ADC before it can reach the tumor target. Antigens prone to shedding should make poor targets for ADC therapy.

9.3.5
Internalization and Trafficking

Efficient internalization of the ADC–antigen complex is critical for successful implementation of the ADC strategy. Clathrin-mediated endocytosis is the best understood and predominant mechanism of receptor internalization and has been targeted as a means of ADC cell entry. Once the ADC has bound to its antigen target, a clathrin polymeric lattice together with adaptor and accessory proteins binds to the phosphatidylinositol-4,5-biphosphate plasma membrane region around the complex. Adaptor proteins such as AP2 bind to the cytoplasmic tail of the receptor through short, linear tyrosine- and dileucine-based sequences. Once the receptor has been targeted for internalization by the adaptor proteins, clathrin migrates from the cytoplasm to adaptor protein-enriched regions of the membrane, and the

subsequent polymerization of the clathrin promotes membrane displacement and the formation of a budding vesicle. These vesicles are liberated from the plasma membrane through the agency of a GTPase called dynamin. The liberated vesicles fuse together to form the early endosome (see below) [2].

While clathrin-mediated endocytosis is the best understood method of internalization, other mechanisms can also play a part in internalization. Epidermal growth factor receptor (EGFR) is typically internalized by the clathrin mediated endocytosis mechanism set out above, however upon binding to cetuximab (C225) it is internalized by micropinocytosis. This mechanism is independent of clathrin and dynamin but is dependent on actin polymerization. A feature of this mechanism is the internalization of large membrane areas, leading to highly efficient antibody-induced internalization of EGFR [16].

The example of the cetuximab/EGFR complex highlights the possibility that ADC-induced internalization may not always follow the clathrin-mediated endocytosis mechanism used by the free receptor/antigen.

Once the ADC–antigen complex has entered the cell, it must be trafficked through early and late endosomes to the lysosome where the cytotoxic warhead can be liberated. The efficiency and directionality of this process can be affected by the nature of the antigen/receptor target [2].

The endosome is a complex organelle made up of proteins and lipids which act together to regulate the intercellular trafficking of internalized proteins. Upon entering the endosome, the internalized ADC–antigen complex can follow one of two distinct pathways. It may be recycled back to the cell surface, or, alternatively, it can remain in the maturing endosome until finally being delivered to the lysosome [2].

The early endosome is mildly acidic (5.9–6.8 pH) and acts as the initial sorting station for internalized ADC–antigen complexes. The ADC–antigen complex can be recycled to the cell surface through either the long or short recycling loops of the early endosomes. Here, the nature of the antigen target can be important: for example, the oncogenic HER2 receptor is rapidly recycled from the early endosome, and as a result only 5% of trastuzumab–DM1 actually reaches the lysosome. One potential solution to this problem is to employ engineered antibodies that possess high binding affinity to their target receptor at extracellular pH but disassociate from the receptor/antigen in the more acidic endosomal environment [2].

In some cells, the endosomes contain FcRn, which can bind to the antibody component of the ADC and recycle it to the cell surface. This mechanistic pathway will reduce the amount of ADC reaching the lysosome, but it also serves to protect normal cells by preventing ADC reaching their lysosomes [2].

9.4 Assembling the ADC

The individual antibody and payload components can be prepared by standard methods and brought together via a conjugation reaction. Historically,

conjugations have been performed in a stochastic manner and lysine or cysteine residues on the antibody.

The cysteine strategy involves reducing disulfide bonds in the hinge region that connect the heavy and light chains of the antibody. The resulting cysteines are allowed to react with maleimide groups situated at the end of the linker portions of the ADC payload. In principle, eight payloads can be conjugated to a single antibody (a drug : antibody ratio, DAR, of 8) through this approach. Unfortunately, DAR-8 species are rapidly cleared and less well tolerated *in vivo,* leading to reduced average DARs of 2 or 4 being targeted during conjugations [17].

The stochastic nature of these reduced DAR conjugations means that many distinct ADCs are generated, both in terms of the site of conjugation and the number of payloads conjugated. The number of payloads follows a distribution in stochastic conjugation and, although DARs 2 and 4 species predominate, DAR 0 as well as high DAR 6 and 8 species will also be present. This distribution leads to a number of undesirable outcomes. The presence of DAR 0, or rather the unconjugated antibody, competes with genuine ADC for antigen targets on the tumor, while the high DAR species are rapidly cleared and can lead to toxic side effects. It should also be noted that the maleimide–cysteine reaction is reversible, and deconjugation of the ADC can occur through a retro-Michael reaction.

Alternatively, payloads can be conjugated to the amine side chains of lysine residues through simple amide coupling. The coupling is essentially irreversible, removing the threat of deconjugation. However, there are a large number of lysine candidates (~80) on an antibody, and in the case of Kadcyla, 10 lysines regularly take part in conjugation, leading to an average DAR 3.6, once again creating many different individual ADC species [18].

The drawbacks of both lysine- and cysteine-directed stochastic conjugation strategies have led to the development of a number of alternative strategies. Engineered antibodies offer cysteine moieties at specific sites, away from the hinge region, generated by replacing other amino acids at the genetic level. Care needs to be taken in choosing the mutation site to avoid sites that are vulnerable to premature cleavage. Alternatively, cysteine residues may be replaced with unnatural amino acids that permit cycloaddition or "click"-mediated conjugation reactions that avoid maleimide chemistry [19].

9.5 Approved ADCs: Adcetris and Kadcyla

The key features of ADCs outlined previously can be observed in practice in the two currently approved ADCs: Adcetris and Kadcyla.

9.5.1 Adcetris (Brentuximab Vedotin)

Adcetris [20] has been developed by Seattle Genetics and been approved for the treatment of relapsed Hodgkin lymphoma (HL) and relapsed or refractory

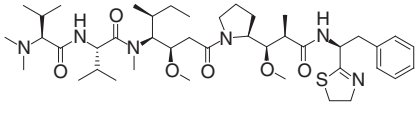
anaplastic large-cell lymphoma (sALCL) by the Food and Drug Administration (FDA) in August 2011 [21, 22] (and conditional marketing authorization by the European Medicines Agency (EMA) for the same indications in October 2012 [23]).

Adcetris targets CD30 [24], which is expressed on Reed Sternberg cells [25] that are diagnostic for HL. CD30 is also expressed on active T cells, B cells, and NK cells. CD30 is recognized by brentuximab, a chimeric antibody that combines the variable heavy and light chains of the murine anti-CD30 antibody AC10 with the constant γ1 heavy and k light chain of human IgG1.

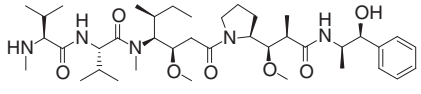
Brentuximab is attached to the vedotin warhead through a maleimidocaproic valine-citruline-PAB (*para*-aminobenzyloxy) cleavable linker [26]. The maleimido unit undergoes stochastic conjugation with cysteine residues in the hinge region to give an average DAR of 4 (the earliest versions of the ADC had a DAR of 8, but the DAR-4 version was found to be more efficacious and better tolerated [17], as discussed previously). The valine-citruline dipeptide is designed to be cleaved at its carboxyl terminus by cathepsin proteases (especially cathepsin B) present in lysosomes. The cleavage event exposes the unstable *p*-aminobenzyl unit, which undergoes self-immolation to release the vedotin warhead [26]. The vedotin warhead (monomethyl auristatin E, MMAE) is an auristatin derivative closely related to dolastatin 10 found in the sea hare *Dolabella auricularia* (Figure 9.3) [27–29].

Vedotin targets tubulin polymerization, preventing the formation of the spindle architecture required for the successful separation of mother and daughter chromosomes. Failure to separate results in mitotic catastrophe and subsequent apoptotic death of the dividing tumor cells.

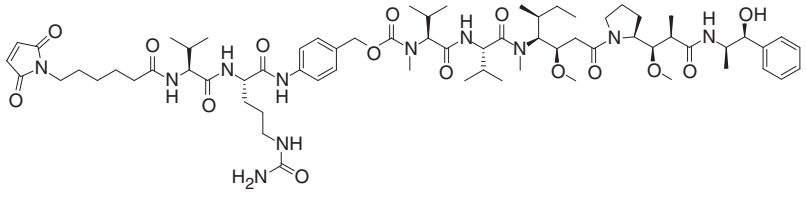
A pivotal, Phase II, single-arm, multicenter study evaluated the efficacy and safety of brentuximab vedotin in patients with relapsed or refractory HL after autologous stem-cell transplant (SCT). Patients received brentuximab vedotin at a dose of 1.8 mg/kg every 3 weeks as an outpatient IV infusion for up to 16 cycles. Patients had received prior cancer-related systemic therapies excluding autologous SCT. Seventy-one percent of patients had primary refractory disease, and



Dolastatin



MMAE



Val-Cit-PABC-MMAE (Vedotin)

Figure 9.3 Auristatin-based warheads and payloads.

42% had not responded to their most recent prior therapy. The ORR (objective response rate) was 75% (76 of 102 patients), with complete durable remissions (complete remissions, CRs) in 34% of patients ($n = 35$). The most common (≥15%) treatment-related adverse events (AEs) of any grade were peripheral sensory neuropathy, nausea, fatigue, neutropenia, and diarrhea. AEs of grade 3 or above occurring in ≥5% of patients were neutropenia, peripheral sensory neuropathy, thrombocytopenia, and anemia [30].

Systemic anaplastic large-cell lymphoma (ALCL) is an aggressive subtype of T-cell lymphoma characterized by the uniform expression of CD30. A Phase II multicenter trial was conducted to evaluate the efficacy and safety of brentuximab vedotin in patients with relapsed or refractory systemic ALCL. Patients with systemic ALCL and recurrent disease after at least one prior therapy received brentuximab vedotin 1.8 mg/kg IV every 3 weeks as an outpatient infusion. Of the 58 patients treated in the study, 50 patients (86%) achieved objective response, 33 patients (57%) achieved CR, and 17 patients (29%) achieved partial remission. The median durations of overall response and CR were 12.6 and 13.2 months, respectively. Grade 3 or 4 AEs in ≥10% of patients were neutropenia (21%), thrombocytopenia (14%), and peripheral sensory neuropathy (12%) [31].

9.5.2 Kadcyla (Ado Trastuzumab Emtansine, T-DM1)

Approximately 20% of breast tumors express high levels of HER2 receptor on their surface [18]. HER2 expression supports tumor progression and is an indicator of poor prognosis [32]. Treatment with the anti-HER2 antibody trastuzumab increases patient survival by an average of 5 months even in patients with metastatic disease [33].

Unfortunately, patients ultimately relapse through a variety of resistance mechanisms [34]. However, these mechanisms do not affect HER2 surface expression, thus allowing continued use of trastuzumab as a targeting device to deliver the cytotoxic agent emtansine. The resulting ADC has been developed by Immunogen and Roche/Genentech (Figure 9.4).

Emtansine (DM-1) [35, 36] is a macrolide antimitotic agent that acts at the rhizoxin binding site of tubulin molecules [37], interfering with tubulin polymerization and preventing spindle formation, ultimately leading to mitotic catastrophe and apoptosis in dividing tumor cells.

The antimitotic agent emtansine was conjugated to trastuzumab in a two-stage process using a noncleavable maleimide cyclohexyl carboxamide (MCC) linker. The conjugation proceeded in two stages: initially, an MCC group was conjugated to the unreduced antibody via amide coupling to lysine side chains; this was followed by the reaction of a thiol group on the DM-1 warhead with the newly installed maleimide moiety. The initial amide coupling conjugation is stochastic, so a mixture of conjugates is obtained with an average DAR of ~3.5.

The noncleavable nature of the MCC linker means that the active drug has to be released by lysosomal degradation of the trastuzumab antibody, leaving a residual

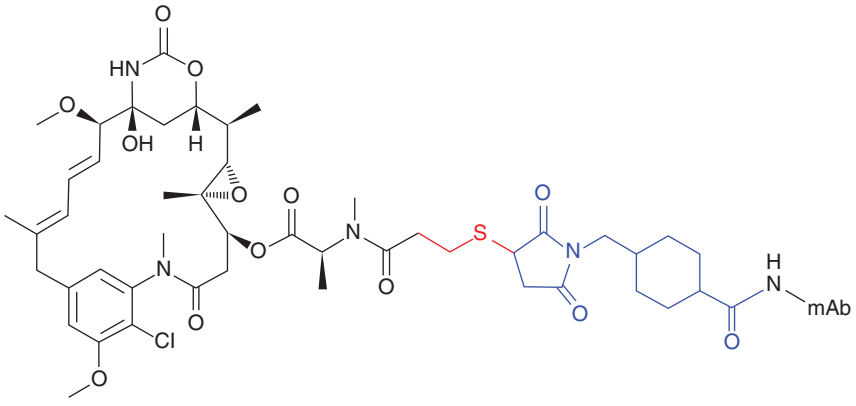


Figure 9.4 Kadcyla (where the mAb is trastuzumab).

cysteine amino acid conjugated to the emtansine payload. The derivative is less active than free maytansinoid but still highly potent; however, it cannot enter cells readily by itself due to the presence of the amino acid moiety, and, as a result, trastuzumab emtansine cannot make use of the bystander effect [38, 39].

In the United States, ado-trastuzumab emtansine was approved specifically for treatment of HER2-positive metastatic breast cancer (mBC) in patients who have been treated previously with trastuzumab and a taxane (paclitaxel or docetaxel), and who have already been treated for mBC or developed tumor recurrence within 6 months of adjuvant therapy [40].

Approval of Kadcyla was based largely on the Phase III EMILIA study [41]. A group of 991 patients were randomly assigned T-DM1 (3.6 mg/kg every 3 weeks) or a combination of lapatinib/capecitabine. The ORR for patients treated with T-DM1 was 43.6% compared to 30.8% with the lapatinib/capecitabine combination, and the median progression-free survival was 9.6 months with T-DM1 compared to 6.4 months with the combination. Furthermore, the median overall survival was 30.9 months for patients on T-DM1 therapy compared to 25.1 months for those treated with lapatinib/capecitabine. The overall rates of AEs of grade 3 or above were higher with lapatinib/capecitabine than with T-DM1 (57% vs 41%), but the incidences of thrombocytopenia and increased serum aminotransferase levels were higher with T-DM1.

Since the EMILIA study, a number of clinical trials have been conducted in an attempt to widen the scope of T-DM1 therapy. The MARRIANNE study [42] investigated the performance of T-DM1 or T-DM1 plus pertuzumab in a first-line setting compared with taxane plus trastuzumab in patients with HER2-positive, unresectable, locally advanced mBC. Unfortunately, neither T-DM1-containing treatment significantly improved progression-free survival time compared to the combination of trastuzumab and taxane [43]. Similarly, the GATSBY Phase II/III study designed to investigate the performance of T-DM1 compared to physician's choice of taxane in gastric cancer failed to meet its primary end point [44].

The TH3RESA study [45] was designed to explore the efficacy of T-DM1 in HER2-positive patients who had progressive disease after two HER2-targeted therapies (e.g., trastuzumab and lapatinib) compared to physician's choice. Six-hundred and two patients across 22 countries were recruited and randomly assigned T-DM1 or physician's choice in a 2 : 1 ratio (T-DM1 404 patients and physicians choice 198). Progression-free survival was significantly improved with trastuzumab emtansine compared to physician's choice (median 6·2 months vs 3·3 months). Interim overall survival analysis showed a trend favoring trastuzumab emtansine (but the stopping boundary was not crossed). A lower incidence of grade 3 or worse AEs was reported with trastuzumab emtansine than with physician's choice (130 events [32%] in 403 patients vs 80 events [43%] in 184 patients). Thrombocytopenia was the grade 3 or worse AE that was more common in the trastuzumab emtansince group. Based on this study, the clinicians recommended that "Trastuzumab emtansine should be considered as a new standard for patients with HER2-positive advanced breast cancer who have previously received trastuzumab and lapatinib."

9.5.3
Gemtuzumab Ozogamicin (Mylotarg)

While Adcetris and Kadcyla are the only currently approved clinical ADCs in the United States, Mylotarg has the distinction of being the first ADC to achieve regulatory approval (Figure 9.5).

Mylotarg consists of a calicheamicin warhead attached to an anti-CD33 [46] humanized IgG4 antibody through a hydrazone linker. Calicheamicin [47] is a highly potent enediyne cytotoxic agent originally found in *Micromonospora echinospora*. Unlike the auristatins and maytansinoids, calicheamicin targets

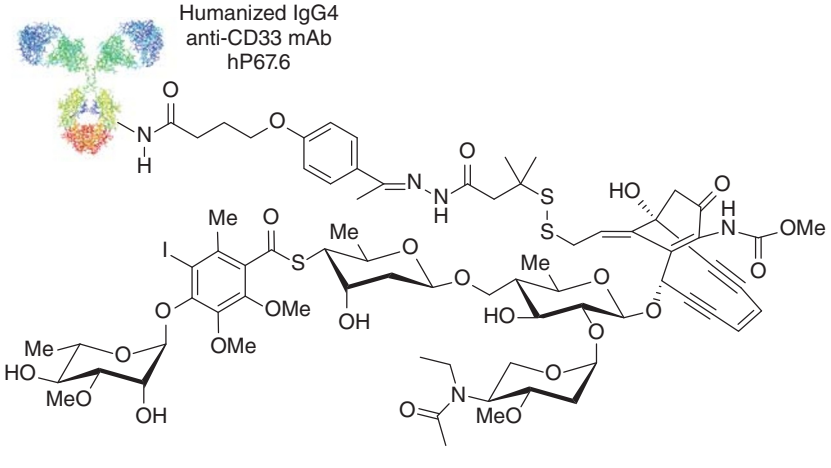


Figure 9.5 Mylotarg.

DNA and binds to the minor groove by virtue of the aryl tetrasaccharide portion of the molecule [48]. Once in the minor groove, the enediyne portion of the molecule undergoes conformational changes and performs a Bergman cyclization, producing a diradical 1,4-didehydrobenzene which abstracts protons from the sugar backbone of DNA, leading to lethal strand scission. The enediyne warhead is attached to the antibody via an acid-sensitive hydrazone linker. The linker is designed to be stable during circulation but cleaved under the low pH conditions of the lysosome. Mylotarg gained accelerated FDA approval [49, 50] for the treatment of AML in 2000, but a follow-up randomized 2004 Phase III comparative control study (required by the accelerated approval mechanism) revealed a higher fatal toxicity rate among the gemtuzumab combination therapy group compared to the standard therapy group. As a result, Pfizer voluntarily withdrew [51] the ADC in the United States in 2010 at the request of the FDA. However, Mylotarg is still an approved therapy in Japan and remains available on compassionate grounds in Europe.

Since then, withdrawal attempts have been made to mitigate safety issues by changing the dose schedule and using the ADC in combination with chemotherapeutic agents [52]. The Phase III AML19 study revealed significant improvement in overall survival compared with supportive care in elderly patients unsuited to intensive chemotherapy. A combination study with daunorubicin and cytarabine (ALFA-0701) [53] demonstrated that Mylotarg offered significantly improved event-free and relapse-free survival in adult patients at the 3-year mark compared to chemotherapy alone. These studies may lead to the reinstatement of Mylotarg as an approved therapy in the near future.

9.6 Developing the ADC Platform

The success of Adcetris and Kadcyla spurred further development of the ADC platform, both in terms of designing new warhead and payload moieties and extending targeting to new antigens in both liquid and solid tumors.

9.6.1 Vedotins

Following on the success of brentuximab vedotin, MMAE has been conjugated to a wide range of antibodies for the treatment of solid and hematological cancers. Some of the most notable vedotin ADCs that have progressed to Phase II trials are glembatumumab vedotin, lifastuzumab vedotin, indusumatatab vedotin, polatuzumab vedotin, pinatuzumab vedotin, and PSMA ADC. The efficacy and tolerability of these agents are summarized in (Table 9.1)

Table 9.1 Tabulated summary of vedotin ADCs in Phase II clinical trials.

Antibody	Sponsor	Indication/phase	Target	ORR [54, 55]	MTD [56]	DLT
Glembatumumab	Celldex Therapeutics	Breast cancer Phase II	gpNMB	12%	1.9	Neutropenia Rash
Glembatumumab	Celldex Therapeutics	Triple negative breast cancer	gpNMB	20%	1.9	Neutropenia Rash
Lifastuzumab	Genentech Inc.	Pt Res Ovarian	NaPi2b	41%	2.4	Dyspnea
Indusatumab	Millenium Pharmaceuticals		GCC		1.8	Neutropenia
Polatuzumab	Genentech Inc.	DLBCL/II + Rituximab	CD79b	56%	2.4	Nuetropenia
Pinatuzumab	Genentech Inc.	DLBCL/II + Rituximab	CD22	57%	2.4	Neutropenia
PSMA ADC	Progenics Pharmaceuticals	Castration Res Chemo naïve/II	PSMA	17%	2.5	Neutropenia
PSMA ADC	Progenics Pharmaceuticals	Castration Res/II	PSMA	7%	2.5	Neutropenia

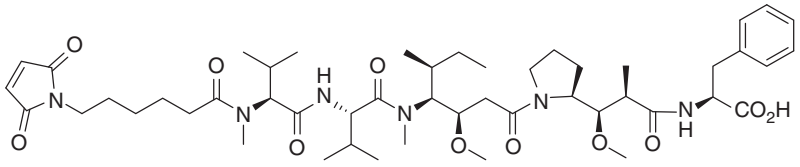


Figure 9.6 Mafodotin (MMAF payload).

9.6.2 Mafodotins

A noncleavable, hydrophilic version of the vedotin payload MMAF (mafodotin) has been developed by Seattle Genetics (Figure 9.6) [57].

The inclusion of the acid moiety and removal of the valine-citruline trigger afforded a payload that would not be able to enter a cell without attachment to an antibody. It was hoped that this would provide a safer payload, albeit at the expense of not being able to take advantage of a bystander effect. Three MMAF ADCs have undergone clinical trials: denintuzumab mafodotin, vorsetuzumab mafodotin, and depatuxizumab mafodotin.

Denintuzumab mafodotin targets CD19 in a number of leukemias and lymphomas. Two separate Phase I trials have been performed in acute lymphoblastic leukemia (ALL) [58], as well as a Phase I study covering diffuse large B-cell lymphoma (DLBCL), lymphoblastic lymphoma, and follicular lymphoma.

In the DLBCL study [59] (60 evaluable patients), 23 patients achieved an objective response, including 14 patients with CRs and 9 with partial remissions, to give an ORR of 38%. In addition, 13 patients (22%) attained stable disease (SD). Interestingly, the ORR for the subset of 25 patients who had relapsed disease was higher at 60% (including 10 patients with CRs). Among all relapsed patients, median progression-free survival was 25.1 weeks, and median overall survival was 56.7 weeks. The most common adverse effects were blurred vision (63%), dry eye (53%), fatigue, keratopathy, and photophobia (39% each). The ocular toxicities were mostly grade 1–2 and manageable with eye drops and dose modification. The maximum tolerated dose (MTD) was not reached even after escalating to 6 mg/kg every 3 weeks.

A weekly and 3-weekly dosing schedule was investigated in a Phase I ALL dose escalation study [60]. At the time of the 2015 ASH presentation, 71 patients with relapsed or refractory B-lineage acute leukemia and highly aggressive lymphoma (Burkitt leukemia/lymphoma) had been treated. The 40 patients on the weekly schedule received a dose range of 0.3–3 mg/kg and those on the 3-weekly schedule (31 patients) received a dose range of 4–6 mg/kg. An MTD of 5 mg/kg was identified for the 3-weekly schedule, whereas MTD was not reached for the weekly schedule. The cytogenic complete remission rate (CRc) [equal to the sum of the CRs, CRs with incomplete platelet recovery (CRp), and CRs with incomplete blood recovery (CRi)] [61] for the weekly schedule was 19% (four patients). In the 3-weekly (q3w) schedule, the CRc rate was similar at the 4, 5, and

6 mg/kg dose levels at 35% (8). Of the 12 patients with CRc who could be analyzed, 7 were found to have achieved minimal residual disease (MRD)-negative status (i.e., they were free of residual leukemia cells). Three of these patients stayed in remission for longer than a year, two of whom had been on continuous treatment for 19 and 22 months.

The AE profiles were similar across both dosing schedules; the most frequently reported AEs were pyrexia (54%), nausea (52%), fatigue (51%), headache (44%), chills (38%), vomiting (37%), blurred vision (35%), and anemia (34%). Ocular symptoms and corneal examination findings consistent with superficial microcystic keratopathy were observed in 40 patients (56%).

Based on the above results, the clinicians concluded that denintuzumab mafodotin was generally well tolerated and demonstrated activity in heavily pretreated adult patients with B-ALL and B-lineage highly aggressive lymphomas, including durable MRD-negative responses. The results of this trial indicated that the 3-weekly schedule, with a CRc rate of 35% in B-ALL, warranted further clinical investigation.

Vorsetuzumab mafodotin targets CD70 a tumor necrosis factor family member that is found in both solid tumors and lymphomas. A Phase I study in relapsed and refractory non-Hodgkins lymphoma (NHL) and metastatic renal cell carcinoma (RCC) yielded some objective responses but not enough to warrant further clinical development [62]. Seattle Genetics have initiated a new CD70 Phase I trial replacing mafodotin with talirine, a novel pyrrolobenzodiazepine (PBD) payload (see below).

Depatuxizumab mafodotin (AT-414) was designed to target EGFR in glioblastoma. A Phase I study [63–65] was designed with three arms to evaluate AT-414 as a single agent (Arm C), in combination with temozolomide (Arm B) and with temozolomide and radiation therapy (Arm A). With AT-414 combined with temozolomide (Arm B), one complete response and four partial responses were achieved, and with AT-414 as a stand-alone agent (Arm C) one complete response and one partial response were observed. As with denintuzumab vedotin, ocular toxicities were observed: blurred vision, the sensation of a foreign body in the eyes, and photophobia. The recommended Phase II dose (RPTD) was determined to be 1.25 mg/kg.

Based on the various clinical trial results, AbbVie will advance ABT-414 – which was granted orphan drug designation by the FDA and the EMA earlier this year – to a randomized Phase II clinical trial in patients with glioblastoma multiforme.

9.6.3 Maytansinoids

DM1 is the original warhead used in Kadcyla. When connected to a maleimido cyclohexyl carboxylic acid, it forms the emtansine payload. This payload is non-cleavable, and Immunogen went on to investigate a family of disulfide linkers in order to generate a series of payloads with differing stabilities [66]. When DM1

is joined to 4-mercaptopentanoic acid, it forms the disulfide payload mertansine, and the methyl group α to the disulfide on the carboxyl side increases the stability of the disulfide trigger (see below). The stability of the linker could be further improved by introducing a second methyl group α to the disulfide. When the dimethyl group is located on the warhead side of the disulfide, the warhead is known as DM4. Joining DM4 to 4-mercaptobutanoic acid gives rise to the ravtansine payload. Finally, the hydrophilicity of disulfide linker can be increased by including a sulfonic acid in the linker to give soravtansine [67]. All the new payloads have been used to generate experimental ADCs, which were investigated in clinical trials.

The new disulfide linkers were designed to be cleaved under reducing conditions. Studies on a panel of ADCs containing disulfide linkers with varying degrees of steric hindrance revealed that disulfide stability was proportional to the level of hindrance. Less hindered disulfide linkers were more rapidly cleaved by dithreitol *in vitro* and *in vivo* ADCs, while more hindered linkers enjoyed a longer half-life in plasma. The rate of cleavage was not affected by the location of the steric hindrance, suggesting that the cleaving agent was a small molecule such as cysteine. However, greater stability and half-life failed to correlate with *in vivo* efficacy; in fact, ADCs with linkers of intermediate stability were found to be the most efficacious. These findings support the idea that a linker should be stable in circulation but cleavable once inside the tumor cells. Highly hindered linkers would not be expected to easily release the active maytansinoid. While location of hindrance does not affect the rate of cleavage, it can influence the activity of the released maytansinoid, with hindered maytansines such as DM4 being more efficacious than unhindered DM1. The use of methyl groups on the maytansine side of the disulfide linkage to provide hindrance increases the lipophilicity of the released drug and may also enhance their ability to kill surrounding tumor cells through the bystander effect (Figure 9.7).

9.6.3.1 **Mertansines**

Three mertansine-based ADCs have undergone clinical investigation: bivatuzumab mertansine, lorvotuzumab mertansine, and cantuzumab mertansine.

Bivatuzumab mertansine [68] targets CD44v6 in head and neck squamous cell carcinoma (HNSCC) and other indications. Unfortunately, CD44v6 was also expressed on keratinocytes, leading to unacceptable skin toxicity [69].

Lorvotuzumab mertansine [70] targets CD56 and reached Phase II trials for the treatment of liquid and solid tumors including small-cell lung cancer (SCLC). However, the trial for SCLC was discontinued on the ground that it was unlikely to demonstrate sufficient improvement in progression-free survival compared to the etoposide/carboplatin standard of care [71–73].

The cantuzumab ADC [74] targets CanAg, a tumor-associated carbohydrate antigen and novel glycoform of MUC1 [75]. CanAg is highly expressed in pancreatic and colorectal tumors. Cantuzumab was originally conjugated to mertansine, which was well tolerated, and then to the more hindered ravtansine that gave an

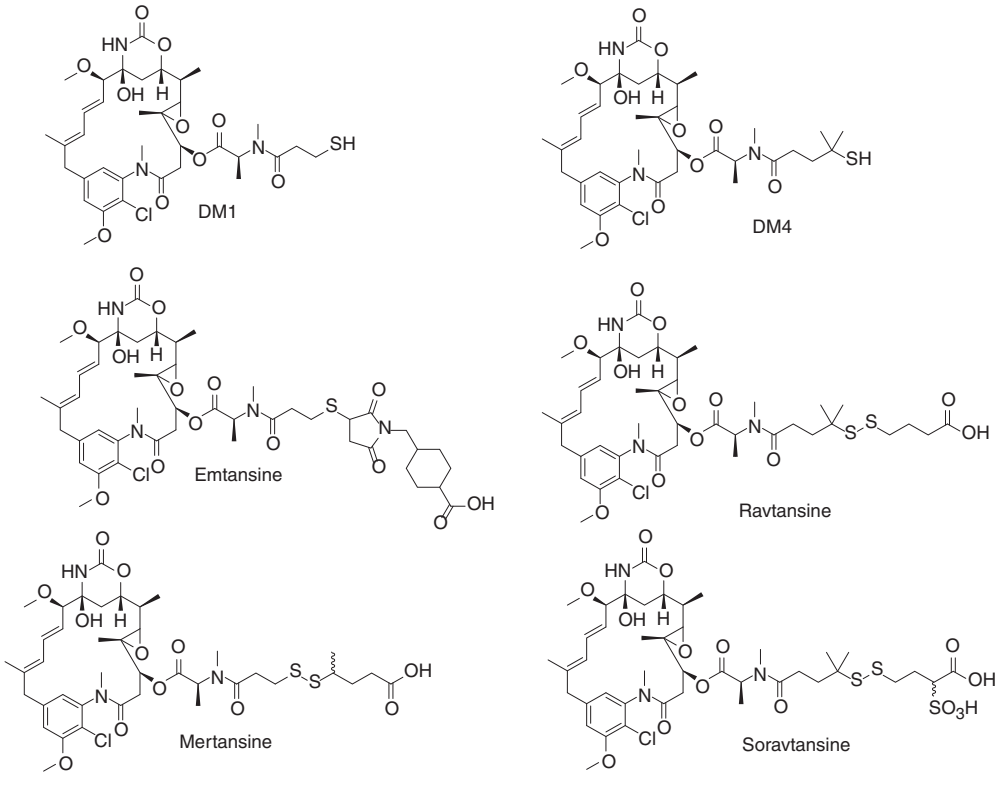


Figure 9.7 Maytansine-based payloads.

improved half-life (4.6 vs 2 days) [76]. However, Phase II clinical trial was discontinued in 2009 [77].

9.6.3.2 **Ravtansines**

Indatuximab ravtansine [78] is designed to deliver the hindered DM4 warhead to cancer cells expressing CD138 (syndecan-1). This antigen is found on many solid and liquid tumors and is one of the most specific antigens for the identification of multiple myeloma [79]. Indatuximab ravtansine has undergone Phase I/II clinical trials in breast and bladder cancer as well as for multiple myeloma. A combination Phase I study with lenalidomide and low-dose dexamethasone in multiple myeloma trial has identified an RPTD of 100 mg/m^2, with grade 1–2 diarrhea, nausea, and fatigue as the most common side effects. At the RPTD, an ORR of 83% was achieved [80].

Mesothelin [81] antigen is found on ovarian, pancreatic, and lung adenocarcinoma as well as mesothelioma. Anetumab is a fully humanized antibody that targets ravtansine- to mesothelin-positive cancers [82]. A Phase I study [83] against a range of solid tumors gave 19% partial responses and 47% SD at the MTD of 6.5 mg/kg (dose-limiting toxicity, DLT, was grade 3 AST liver enzyme increase).

For patients with mesothelioma, the partial response rose to 31% and the incidence of SD was 44%. Anetumab is currently in Phase II trial [84] as a second-line treatment for malignant pleural mesothelioma at the 6.5 mg/kg dose.

Like denintuzumab mafodotin (see above), coltuximab ravtansine targets the CD19 antigen found in many B-cell malignancies [85]. The STARLYTE [86] Phase II trial investigated coltuximab ravtansine as a single agent for relapsed and refractory DLBCL. A 44% ORR was achieved at a dose of 55 mg/m^2 with the usual physical side effects of nausea, diarrhea, and fatigue. Hematological toxicities were also reported, with 26.5% and 9.9% of patients experiencing grade 1–2 neutropenia and thrombocytopenia, respectively.

Coltuximab ravtansine has also been the subject of a Phase II trial in relapsed and refractory ALL. A total of 36 patients were treated: 19 during dose escalation and 17 during dose expansion. One dose-limiting toxicity was observed at 90 mg/m^2 (grade 3 peripheral motor neuropathy), and as a result 70 mg/m^2 was selected for the dose-expansion phase. Five patients discontinued therapy because of AEs. The most common AEs were pyrexia, diarrhea, and nausea. Of the 17 evaluable patients treated at the selected dose, 4 had a disease response (estimated ORR 25.5%). The duration of response (DOR) was 1.9 months (range 1–5.6 months). As a consequence of these results, the study was prematurely discontinued [87].

9.6.3.3 **Soravtansine**

Soravtansine combines the stabilizing dimethyl motif with a hydrophilic sulfonic acid group. Soravtansine has been conjugated to mirvetuximab, which targets folate receptor alpha (FRα) [88–90]. This antigen is highly expressed in ovarian, endometrial, and lung cancers. A Phase I trial [91] indicated an ORR of 53% with only low-grade (1 or 2) toxicities in cisplatin-resistant ovarian cancer. An RPTD of 6 mg/kg every 3 weeks was identified [92]. A Phase II (FORWARD1) [93] trial has been designed to compare mirvetuximab soravtansine against investigator's choice in adults with FRα-positive advanced epithelial ovarian cancer, primary peritoneal cancer, and primary fallopian tube cancer. A combination Ib/II study (FORWARD2) has also been designed [94].

9.6.4 **Ozogamicin**

Pfizer has developed the calicheamicin-based ADC inotuzumab ozogamicin (DAR = 2–3) for the treatment of CD22-positive leukemias and lymphomas [95]. The open-label, Phase III, INO VATE ALL clinical trial compared the ADC with physician's choice of chemotherapy (FLAG, HIDAC, or cytarabine mitoxantrone) in the treatment of relapsed and refractory ALL. Patients on the ADC arm received a total of 1.8 mg/m^2 of inotuzumab ozogamicin in a split dose schedule (0.8 mg/m^2 on day 1 of a cycle, 0.5 mg/m^2 on day 8, and a final dose of 0.5 mg/m^2 on day 15 for up to six cycles). The ADC arm achieved an ORR of 80.7%, which is more than double that achieved in the chemotherapy arm (33.3%). Of those

patients who responded to therapy, 78.4% were found to be MRD-negative. The major grade 3–4 AEs in both arms of the study were hematological cytopenias. As a result, the FDA has awarded breakthrough therapy designation to inotuzumab ozogamicin in ALL [96].

9.7 New Warheads and Payloads with a Novel Mechanism of Action

9.7.1 Govitecan

Govitecan (SN-38) is a topoisomerase 1 inhibitor and the active metabolite of the chemotherapeutic agent irinotecan. The design strategy adopted by Immunomedics was to employ moderately potent cytotoxic warheads (as opposed to highly potent agents such as calicheamicin) but with high DARs, approaching 8. In addition, a pH-sensitive carbonate linker was used to attach the warhead to the antibody. The linker also incorporates the MCC unit already used in trastuzumab emtansine, a triazole, an 8-polyethylene glycol (PEG) hydrophilic unit, and a lysine-substituted PAB unit similar to that used in Adcetris. The triazole group arises from the click reaction used to join the MCC and PEG components of the linker. The MCC group undergoes amide conjugation to lysine residues on the targeting antibody, allowing high DARs to be achieved (Figure 9.8).

Govitecan has been conjugated to a humanized anti-Trop2 antibody sacituzumab. Trop2 is found on many solid tumors including breast, lung, and pancreatic malignancies [97, 98]. A Phase II clinical trial has been reported in metastatic triple-negative breast cancer (breast tumors without HER2, estrogen, or progesterone receptors) [99]. Patients were dosed at 8 or 10 mg/kg on the 1st and 8th day of a 21-day repeated cycles. The ORR was found to be 31% with 2 complete responses and 16 partial responses. The clinical benefit rate (CBR) (which takes into account SD as well as partial and complete responses) was 53% after at least 4 months. Neutropenia was the most commonly observed grade 3 or greater AE (15% incidence). Interestingly, diarrhea, a common side effect of the parent irinotecan, was observed only in 3% of patients at the grade 3 level. The 10 mg/kg dose has been adopted for further clinical development, and the FDA has granted sacituzumab govitecan breakthrough therapy designation in triple-negative breast cancer [100]. A Phase III study is planned for 2016, and PhaseI/II trials are going on in a range of epithelial tumor indications [101, 102].

Govitecan has also been conjugated to a humanized anti-CEACAM5 to yield labetuzumab govitecan [103]. The ADC is currently in a Phase II trial in metastatic colorectal cancer, dosing at 10 mg/kg weekly or 6 mg/kg biweekly. Main toxicities appear to be G3 typhitis, G4 neutropenia, and G3 nausea/vomiting. Tumor reductions of 35% were noted in patients despite prior relapse to irinotecan-containing therapies [104–106].

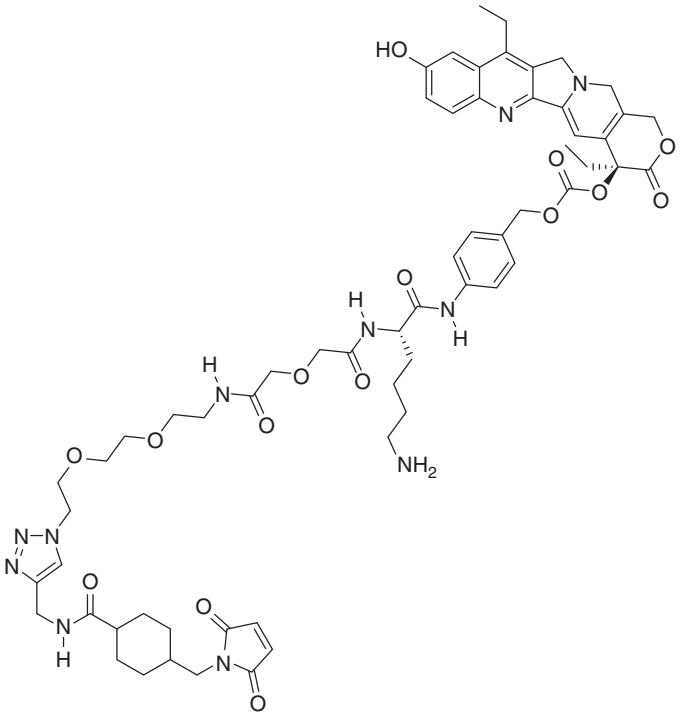


Figure 9.8 Govitecan payload.

9.7.2 Pyrrolobenzodiazepines (PBDs)

The PBD family of naturally occurring antitumor antibiotics is produced by various *Streptomyces* species [107]. Naturally occurring PBDs are monomeric in nature and exert their antitumor effects by binding to the N2 of guanine in DNA and interfering with processes such as transcription. Joining two PBD units together via an alkylene tether produces dimers that can sequence-selectively and covalently cross-link DNA. The resulting DNA adducts act as a block to replication, leading to cell cycle arrest at the G2/M boundary. Critically, the DNA–PBD adducts are nondistortive and hence difficult for many tumors to repair. As a result, PBD dimers are highly potent with *in vitro* activities in the picomolar to subpicomolar range. In addition, PBDs are not cross-resistant with platinum agents, which is important because patients in Phase I trials may well have been previously treated with platinum agents. The PBD dimers have been developed as stand-alone clinical agents by Spirogen, with SG2000 reaching Phase II clinical trial for leukemia [108].

PBD dimers are synthetically accessible, allowing linkers to be attached at both the C2 and N10 positions. Linker attachment at the C2 position, as part of a collaboration between Spirogen and Seattle Genetics, has afforded the talirine

payload. Spirogen has also developed an N10-linked payload called tesirine (Figure 9.9).

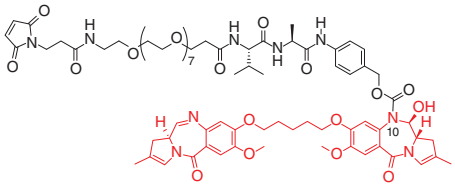
9.7.2.1 Talirine

Structure–activity relationship studies have indicated that the presence of flat aryl substituents at the C2 position of the PBD enhanced DNA binding and potency [109, 110]. Furthermore, the C2-aryl group could be used to incorporate a reactive amine group to act as a point of linker attachment. Direct coupling of an alanine-valine-caproic maleimide linker without recourse to a PAB self-immolative unit afforded the talirine payload [111, 112].

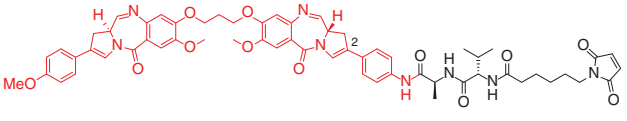
Talirine was conjugated to the anti-CD33 antibody vadastuximab [113]. This antibody was engineered to have two 239C cysteine conjugation sites (actual DAR 1.9). In preclinical *in vitro* testing, vadastuximab talirine was found to be more potent than gemtuzumab ozogamicin in both AML cell lines and primary AML cells. In subcutaneous AML xenograft studies, vadastuximab talirine exhibited antitumor activity at doses as low as 0.1 mg/kg (single injection). Moreover, antitumor activity was maintained in multidrug resistant (MDR)-positive AML models. Based on these preclinical findings, vadastuximab talirine was evaluated in a Phase I study both as monotherapy and in combination with hypomethylating agents (HMAs).

In the monotherapy study [114], 87 predominantly elderly AML patients (median age 74, range 27–89) who had either relapsed after first CR with intensive therapy or declined conventional intensive therapy were treated with doses of vadastuximab talirine ranging from 5 to 60 μg/kg. Six dose-limiting toxicities were reported in the monotherapy escalation cohorts: two grade 4 bone marrow failures (40 and 60 μg/kg), two mucositis (grade 3 at 50 μg/kg; grade 3 at fractionated 20 + 20 μg/kg), grade 3 pulmonary embolism (20 μg/kg), and grade 5 sepsis (50 μg/kg). The most common grade 3 or higher AEs reported were febrile neutropenia (69%), thrombocytopenia (29%), and anemia (23%). Increased myelosuppression was observed at doses higher than 40 μg/kg. At the recommended monotherapy dose of 40 μg/kg, 33% of patients achieved complete response or complete response with incomplete recovery (CR + CRi = ORR). Median OS in patients treated at 40 μg/kg was 10 months, with 17 patients alive at the time of abstract preparation. Across all dose levels, eight patients went on to receive an allogeneic stem cell replacement therapy.

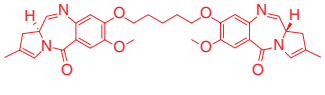
In the combination study with HMAs [115], 24 patients with a median age of 77 years (range, 66–83) were treated with azacitidine or decitabine in addition to vadastuximab talirine. Forty-two percent of patients had adverse cytogenetics (MRC), and 23 patients were treatment-naïve. A single dose level of vadastuximab talirine 10 μg/kg was administered every 4 weeks on the last day of HMA dosing. grade 3 or higher AEs reported in >20% of patients were fatigue (54%), febrile neutropenia (46%), anemia (25%), neutropenia (25%), and thrombocytopenia (21%), but no dose-limiting toxicities were observed. Fifteen of the 23 efficacy evaluable patients (65%) achieved CR (5) or CRi (10). Remissions were generally obtained after two cycles of treatment.



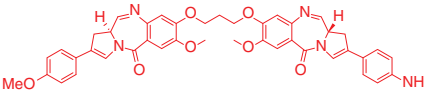
SG-3249 (Tesirine)
c Log *P* = 2.11



SGD-1910 (Talirine)
c Log *P* = 4.71



SG-3199
IC_{50} = 4.84 pM (A2780)



SG-3132
IC_{50} = 1.59 pM (A2780)

Figure 9.9 Pyrrolobenzodiazepine-based warheads.

The combination of 33A with HMA was reported to be well tolerated, active, and had no identified off-target toxicities. Activity with the combination compared favorably with historical experience with HMAs alone in this patient population. The CR + CRi rate of 65% in AML patients with poor risk factors with the observed low 60-day mortality (4%) was particularly encouraging.

In February 2016, Seattle Genetics announced initiation of a Phase I/II trial of vadastuximab talirine in combination with azacitidine in patients with previously untreated myelodysplastic syndrome (MDS) [116]. MDS covers a range of diverse bone marrow disorders in which the bone marrow does not produce sufficient healthy blood cells. In advanced MDS, CD33-expressing blasts are detectable in the bone marrow, and approximately a third of patients go on to develop AML proper.

In May 2016, Seattle Genetics announced the initiation of a pivotal Phase III clinical trial, called CASCADE, evaluating vadastuximab talirine (SGN-CD33A; 33A) in combination with azacitidine (Vidaza) or decitabine (Dacogen) in older patients with newly diagnosed AML [117].

Talirine has also been conjugated to an anti-CD70 antibody to afford SGN-CD70 [112]. CD70 is expressed in RCC and NHL but not healthy tissue. A Phase I clinical trial in both NHL and RCC was initiated in August 2014 [118].

9.7.2.2 **Tesirine**

Tesirine was designed to be less hydrophopic than talirine by removing the two C2-aryl substituents. To compensate for reduction in activity, the tether joining the two PBD units was increased from three methylenes to five. The increase in tether link improves the isohelicity of the dimer with the minor groove and generally leads to a 10-fold increase in *in vitro* activity. Removal of the C2-anilino group also removed the amine linker attachment site, and so in tesirine the linker, now including a self-immolative PAB spacer, was attached through the N10 position [119].

9.7.2.3 **Rovalpituzumab Tesirine**

Tesirine was conjugated in a stochastic fashion to the hinge region of rovalpituzumab, an anti-delta-like protein 3 (DLL3) antibody [103], as part of a collaboration between StemCentRx and Spirogen. DLL3 (a member of the Notch signaling family) is highly expressed in human neuroendocrine tumors and significantly their tumor-initiating cells. Overall, approximately two-thirds of SCLCs are DLL3-positive. DLL3 is not expressed at detectable levels in normal tissues. Rovalpituzumab tesirine was evaluated against a panel of patient-derived lung xenografts at a dose of 1 mg/kg Q4Dx3, and its efficacy was found to correlate with the level of DLL3 expression, with the highest expressing PDXs undergoing sustained tumor regression out to 150 days. Furthermore, tissue from untreated xenografts was found to have an approximately 1 : 60 ratio of tumor-initiating cells (cancer stem cells) to bulk tumor cells, while tissue from rovalpituzumab tesirine-treated xenografts was found to have a ratio of 1 : 13 000, demonstrating a dramatic reduction in the number of cancer stem cells.

In a Phase I study [120], 52 patients were treated, 34 on the q3w schedule and 18 on q6w schedule; the median age was 61 years (range 44–82). Of 38 archived tumor specimens received from enrolled patients, 23 (61%) were DLL3 biomarker positive.

Acute and chronic DLTs of thrombocytopenia and capillary leak syndrome (CLS) were observed at 0.8 and 0.4 mg/kg q3w, respectively. MTDs of 0.2 mg/kg q3w × 3 cycles and 0.3 mg/kg q6w × 2 cycles were further evaluated in expansion cohorts. The most common treatment-emergent AEs of any grade among all patients were fatigue (40%), rash (39%), nausea (29%), dyspnoea (23%), decreased appetite (21%), and vomiting (21%). Grade 3+ CLS and thrombocytopenia were seen in seven (14%) and three (6%) patients, respectively, with no reported grade 5 toxicity.

In all evaluable patients treated at the MTD without regard for DLL3 biomarker status ($n = 32$), the ORR was 22% ($n = 7$ PR) and SD 53% ($n = 17$), for a CBR of 75%. Among the 16 confirmed DLL3-positive patients treated at the MTDs, 7 patients (44%) had partial response (PR) and 8 patients (50%) achieved SD for a combined CBR of 94%. Notably, all patients with PRs who were treated at the MTD, and those having the most durable clinical benefit (up to 569 days OS), were DLL3-positive.

A Phase II TRINITY study in SCLC was announced in June 2015.

9.7.2.4 **ADCT-301**

Tesirine has also been conjugated stochastically to an anti-CD25 Humax-Tac antibody to afford the ADC ADCT-301 [121,122]. CD25 is a component of the IL2 receptor, which is expressed in many hematological malignancies including B- and T-cell lymphomas. ADCT-301 is currently in Phase I clinical trials for AML, Hodgkins lymphoma, and NHL [123]. A full list of PBD payload trials is shown below (Table 9.2).

9.7.3
Duocarmycin-Based ADCs

Duocarmycins are similar to PBDs in many ways: they are both produced by *Streptomyces* species and bind in the minor groove of DNA. Duocarmycin, however, binds preferentially to adenine bases rather than guanines (Figure 9.10) [124, 125].

Duocarmycins have been developed as payloads for ADC therapy by Syntarga and more recently by Synthon.

The *seco*-DUBA (duocarmycin hydroxybenzamide azaindole) warhead consists of a chloromethyl-substituted tricyclic benzoindole unit attached to an azaindole moiety. The 5-hydroxy group acts as a convenient anchorage for a valine citruline PAB linker. Attaching the PAB group directly to the 5-hydroxy substituent of DUBA would form a potentially unstable carbonate linkage, so a diaminoethyl cyclization unit is incorporated in the linker to allow the use of more stable carbamates. A short maleimido PEG group is then added to complete the payload. Conjugation to trastuzumab affords the ADC SYD985 [126, 127]. The ADC is designed to be activated in the lysosomes of tumor cells by cathepsin-mediated

Table 9.2 Summary of PBD ADC clinical trial.

Sponsor	Agent	Target	Indication	Study	Identifier
Seattle Genetics	Vadastuximab talirine[a]	CD33	AML	Phase I	NCT01902329
Seattle Genetics	Vadastuximab talirine[b]	CD33	AML	Phase I	NCT02326584
Seattle Genetics	Vadastuximab talirine[c]	CD33	MDS	Phase I/II	NCT02706899
Seattle Genetics	Vadastuximab talirine[d]	CD33	AML	Phase I/II	NCT02614560
Seattle Genetics	Vadastuximab talirine[d]	CD33	AML	Phase III	NCT02785900
Seattle Genetics	Vadastuximab talirine	CD70	RCC/NHL	Phase I	NCT02216890
Seattle Genetics	Vadastuximab talirine	CD19	NHL/DLBCL	Phase I	NCT02702141
StemCentRx	Rovalpituzumab tesirine	DLL3	SCLC	Phase I	NCT01901653
StemCentRx	Rovalpituzumab tesirine	DLL3	SCLC	Phase II	NCT02674568
StemCentRx	Rovalpituzumab tesirine	DLL3	Melanoma and neuroendocrine solid tumors	Phase I/II	NCT02709889
StemCentRx	Presumed PBD[e]	Unknown	CisR Ovarian	Phase I	NCT02539719
ADCT	ADCT-301(tesirine)	CD25	AML/ALL	Phase I	NCT02588092
ADCT	ADCT-301(tesirine)	CD25	HL/NHL	Phase I	NCT02432235
ADCT	ADCT-402	CD19	B-NHL	Phase I	NCT02669017
ADCT	ADCT-402	CD19	B-AML	Phase I	NCT02669264

[a]with HMA, [b]with cytarabine/daunorubicin SoC, [c]with azacytidine, [d]VT with alloSCT, [e]presumed on the basis of exclusion criteria.

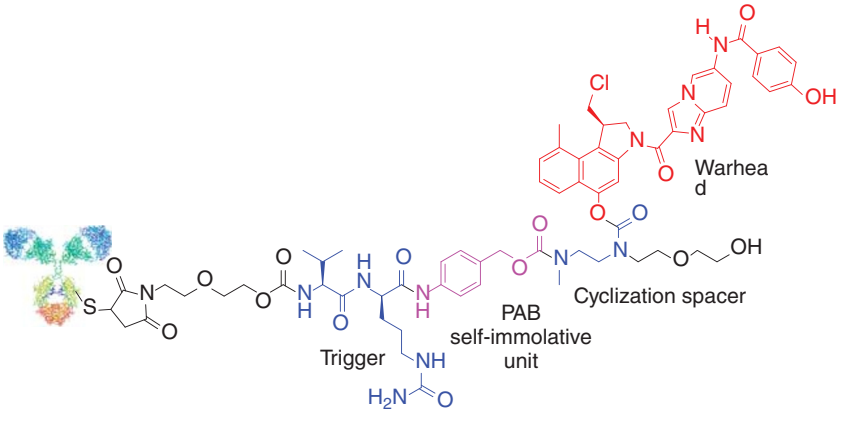


Figure 9.10 SYD985.

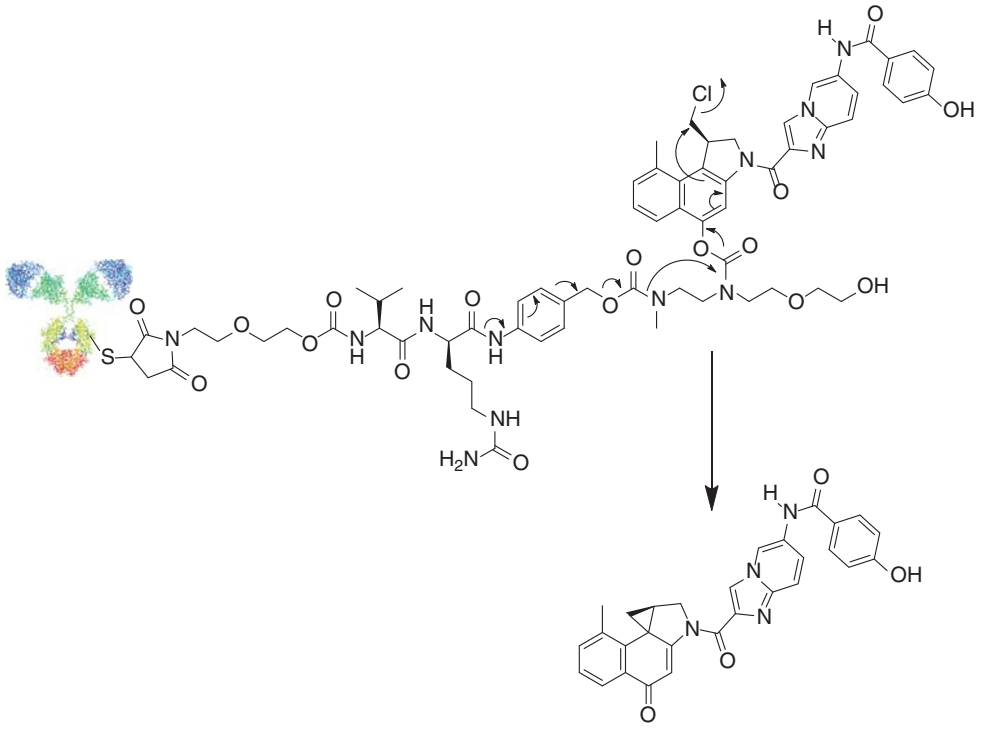
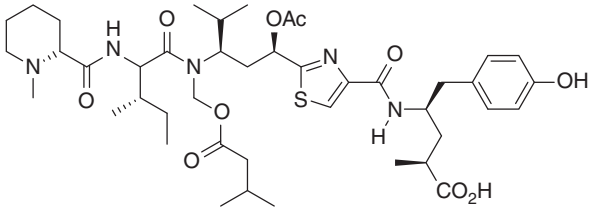


Figure 9.11 Release of duocarmycin payload from SYD985.

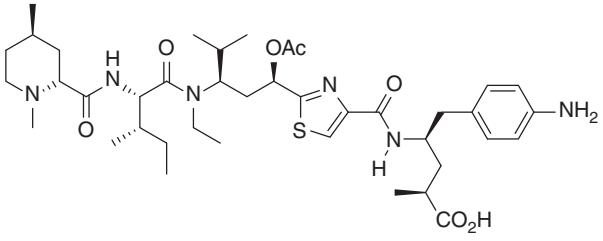
cleavage of the citruline group at its juncture with the PAB unit. This cleavage event initiates a cascade of electrons, which not only releases the DUBA warhead but ultimately activates it by allowing the formation of the highly reactive benzocyclapropaindalone species. It is the strained cyclopropyl group in the released warhead that actually reacts with adenine bases in DNA (Figure 9.11).

In vitro evaluation of SYD985 revealed that it was 3–50 fold more potent than trastuzumab-DM1 and was active both in high and low HER2-expressing cell lines. Unlike T-DM1, SYD985 was able to kill HER2-negative cells through a bystander effect, which was ascribed to the cleavable nature of the SYD985 val cit linker (the MCC linker of T-DM1 is noncleavable). SYD985 was able to inhibit the growth of low HER2-expressing PDX models at doses as low as 1 mg/kg. This may be an underestimate, as one challenge to the *in vivo* evaluation of SYD985 was the susceptibility of the linker to mouse-specific carboxylesterase (CES1c). However, the ADC was found to be stable in human plasma.

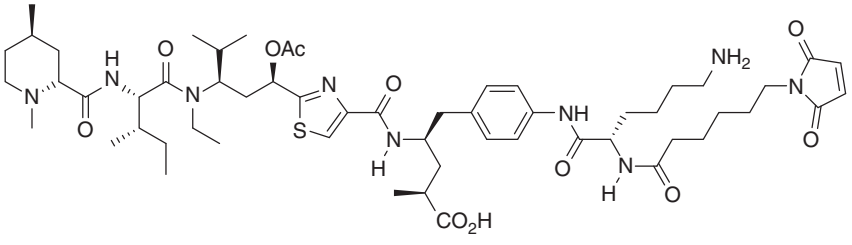
SYD985 is currently in a Phase I clinical trial against HER2-positive solid tumors. Initial results reported at the European Cancer Conference in Vienna (September 27, 2015) revealed partial responses in HER2-therapy-resistant patients. Although the MTD had not been reached at that time, a dose of 2.4 mg/kg appeared to be well tolerated [128].



Tubulysin A



AZ13599185 (tubulysin warhead)



AZ13601508 (tubulysin payload)

Figure 9.12 Tubulysin-based warheads and payloads.

9.7.4 Tubulysins

The tubulysins were originally isolated from strains of the myxobacteria *Archangium gephyra* and *Angiococcus disciformis* and found to possess potent cytotoxic activity. Their mechanism of action involves the depolymerization of microtubules, leading to cytoskeletal collapse (Figure 9.12) [129].

9.7.4.1 MEDI4276

Herceptin and Kadcyla have had a transformative effect on the treatment of breast cancer. However, it is important to note that only 20–30% of patients express levels of HER2 high enough to make them eligible for treatment with these agents. Many patients (~40%) whose tumors express higher than normal levels of HER2 remain ineligible for Herceptin or Kadcyla therapy. In addition, it is a sad fact that patients eventually develop resistance against both Herceptin and Kadcyla [130].

The development of new warheads and linkers with the aim of improving ADC therapy has been a recurring theme of this chapter, but it is important to realize that antibody engineering (beyond the development of site specific antibodies) can play a critical role in developing ADC.

MEDI4276 consists of the tubulysin payload (discussed in the previous section) conjugated to an engineered biparatopic anti-HER2 antibody. MEDI4276 was designed by MedImmune to take into account learnings arising from the preclinical and clinical development of Herceptin and Kadcyla.

HER2 differs from other receptor tyrosine kinases by not having a natural ligand. As a result, HER2 is largely recycled following spontaneous endocytosis. This behavior has important consequences for the intracellular processing of DM-1. Rather than being trafficked efficiently to the lysosome for degradation and release of the maytansinoid catabolite, the majority of the T-DM1 returns to the cell surface. The small fraction of the T-DM1 reaching the lysosome could explain its lack of activity in tumors expressing lower levels of HER2. Increasing lysosomal trafficking of HER2-targeting ADC could potentially allow the treatment of patients with lower HER2 expression levels. In addition, a number of potentially clinically relevant modes of resistance to T-DM1 have been identified. T-DM1-resistant cells lines have significantly lower levels of HER2 expression compared to their parental counterparts. Cell lines such as BT474 (whose proliferation is addicted to HER2 signaling) upregulate multidrug-resistance pumps, and, finally, overexpression of heregulins has been observed to lead to trastuzumab resistance.

The monoclonal antibody 39S is a fully humanized antibody capable of blocking HER2/HER3 receptor phosphorylation in heregulin-treated cancer cell. Furthermore, the antibody could act synergistically with trastuzumab to inhibit tumor cell proliferation. Critically, SN38 could still bind to HER2 that was already bound to trastuzumab, indicating that binding was occurring to a distinct epitope. By using variable domain sequences of 39S and trastuzumab, a single-chain, variable fragment from trastuzumab could be joined to the N-terminus of heavy chain of 39S to form a biparatopic antibody. This antibody was capable of binding simultaneously to four HER2 receptors (with the trastuzumab-derived unit binding to domain IV of the HER2 receptor and the SN38 portion to domain II). Cross-linking the HER2 receptors led to the formation of complex antibody–HER2 mesh works as large as 1700 kDa (compared to 200–300 kDa complexes observed with trastuzumab). Critically, these complexes were rapidly internalized and trafficked to the lysosome where they were efficiently degraded.

This rapid internalization and trafficking of the biparatopic antibody indicated that it would act as an effective vehicle for warhead delivery in the context of ADC therapy. Two cysteine sites (S239C or S442C) were engineered in each heavy chain of the biparatopic antibody (Fc region) to allow AZ13599185 to be conjugated via maleimide chemistry to give DAR of 4. A further mutation, L234F, was introduced in the Fc region and, together with the S239C mutation, this reduced the level of FcγR binding. By reducing FcγR binding, it was thought that nonspecific FcγR-mediated uptake of ADC by healthy tissue could be minimized, leading to reduction in off-target toxicities such as thrombocytopenia.

In vitro cytotoxicity assays across a range of human tumor cell lines with a range of HER2 expression levels demonstrated that activity correlated with HER2 expression, but also that MEDI4276 was ~10-fold more potent than T-DM1. Furthermore, MEDI4276 was active in cell lines that were intrinsically resistant to T-DM1 (JIMT-1). Cell lines that had very low levels of HER2 expression (about >6000 antigens per cell) were unaffected by both T-DM1 and MEDI4276.

In vivo studies with the ST225 PDX model confirmed the increased potency of MEDI4276 compared to T-DM1 observed during *in vitro* studies. At a dose of 3 mg/kg administered every week for 4 weeks, complete tumor regressions were observed and all the animals remained tumor-free for 120 days (in contrast, treatment with DM-1 at the same dose and schedule resulted in tumor stasis, with tumor regrowth on cessation of treatment). In addition, MEDI4276 maintained activity in *in vivo* studies in T-DM1 intrinsically resistant (JIMT-1) and acquired resistance (NCI-N87) models. While 20–30% of breast cancer patients are classed as HER2 positive and eligible for T-DM1 therapy, the majority of breast cancer patients are not, and therapeutic options are limited, especially for those diagnosed with triple-negative disease (ER-, PR-, and HER2-negative). The greater potency of MEDI4276 offered the hope of extending HER2 ADC therapy to patients currently classed as HER2-negative. MEDI4276 induced complete tumor regression in triple-negative PDX models, and extension of study with a further 16 HER2-negative PD models revealed tumor regression in 71% of models as well as tumor stasis in 12% of models. Finally, due to the cleavable nature of the linker in the AZ13601508, MEDI4276 is able take advantage of the bystander effect unlike T-DM1 with its already noted noncleavable linker.

MEDI4276 was the subject of a GLP toxicology study in nonhuman primates, and toxicities were in line with those expected for ADCs incorporating tubulin-binding warheads. Epithelial degeneration was identified as DLT, but significantly, in light of the thrombocytopenia associated with T-DM1, MEDI4276 does not cause dose-limiting adverse effects on monkey platelets. MEDI4276 is currently in Phase I clinical trial for HER2-expressing breast and stomach cancer.

9.8 Conclusion

ADCs such as Kadcyla (T-DM1, trastuzumab, emtansine) and Adcetris (brentuximab vedotin, SGN-30) have opened up a new paradigm for the treatment of cancer. However, ADCs have not yet opened up the therapeutic window as widely as initially hoped. Most ADCs are administered at or close to their MTDs. We have also seen a number of ADCs fail at the Phase II level often through lack of efficacy or overt toxicity [131].

In his 2016 review, Paul Polakis began by pointing out how far the field still has to go to truly generate magic bullets, and set out the mechanisms through which ADC toxicity can arise [132]. Fortunately, many of these issues can be directly addressed through payload and antibody development. A number of new payloads

such as amanitin [133], Auristatin T [134], and indolobenzodiazepines [135] are emerging and beginning to enter clinical trials [136].

Successful treatment of solid tumors remains an important challenge for the field, and the introduction of new warheads and targets, as exemplified by rovalpituzumab tesirine [137] which combines a novel PBD payload with a stem-cell-relevant DLL3 antigen target, is an encouraging development in this area [120]. There is also hope that DNA-targeting warheads will be able to address indications (such as colorectal cancer) that historically have not responded well to tubulin-targeted chemotherapy.

ADCs have also been used preclinically in combination with antibodies that target immune checkpoints. For example, T-DM1-mediated tumor cell death has been shown to render the remaining HER2-positive cancer cells highly susceptible to CTLA4/PD-1 bockade [138].

Finally, ADCs are being applied to therapeutic areas outside oncology. Lehar *et al.* have reported the use of an antibody–antibiotic conjugate against methicillin-resistant *Staphylococcus aureus* (MRSA), which employs a novel quaternary amine linker [139].

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Preface

The number of marketed protein therapeutics [1–3] has increased enormously since the introduction of the first recombinant protein, human insulin, into the clinic several decades ago. Protein therapeutics play a very significant role in many various fields of medicine, and their use continues to steadily broaden. There are several key advantages of protein therapeutics over small-molecule drugs that contribute to this [1]:

- Proteins often exhibit highly specific and complex functions that cannot be mimicked by simple chemical compounds.
- The larger binding interface of a protein therapeutic enables them to be engineered with high affinity for their target.
- Due to their high level of specificity, there is often less potential for protein therapeutics to interfere with normal biological processes and cause off-target effects.
- Recombinant technology allows the production of proteins that provide a novel function or activity.
- Because the body naturally produces many of the proteins that are used as therapeutics, these agents are often well tolerated and are less likely to elicit immune responses.
- For diseases in which the product of a gene is absent or defective, protein therapeutics can provide an effective replacement treatment.
- The clinical development and approval time for protein therapeutics may be faster than for small-molecule drugs [4].
- Recombinant proteins can also be used in combination with both other large molecule, or indeed small-molecule, drugs to provide additive or synergistic benefit.

Many successful uses of protein therapeutics are documented in this volume. However some challenges still remain for the discovery and development of protein therapeutics: (i) The current route of administration is typically parenteral. Development of oral biologics remains largely an aspiration at this time. (ii) Although the production of recombinant proteins is becoming increasingly efficient and cost–effective, it remains relatively expensive compared to that of small molecules. (iii) The body may mount an immune response against the

therapeutic protein. In some cases, this immune response can neutralize the protein and reduce the efficacy of the potential drug.

Taken together, the early success of recombinant insulin production in the 1970s created an atmosphere of enthusiasm and hope, which was followed by an era of disappointment when the vaccine attempts, nonhumanized monoclonal antibodies, and cancer trials in the 1980s were largely unsuccessful. Despite these setbacks, significant progress has been made. With the large number of protein therapeutics both in current clinical use and in clinical trials for a range of disorders, one can confidently predict that protein therapeutics will have a further expanding role in future medicine and may – together with cell and gene therapy – dominate over classical therapeutic approaches based on small-molecule drugs.

Accordingly, this is an appropriate time to review our current knowledge and future perspectives of protein therapeutics as realized in this volume by experts in the field both from industry and academia. It is organized in six sections, first of which introduces the past and present development of protein therapeutics in the chapters on "Early Recombinant Protein Therapeutics" and "Evolution of Antibody Therapeutics." The second section is dedicated to antibodies as the ultimate scaffold for protein therapeutics and is covered in two chapters on "Human Antibody Structure and Function" as well as "Antibodies from Other Species." Discovery and engineering of protein therapeutics are described in the next section comprising detailed chapters on "Human Antibody Discovery Platforms," "Beyond Antibodies: Engineered Protein Scaffolds for Therapeutic Development," "Protein Engineering: Methods and Applications," "Bispecifics," and on "Antibody–Drug conjugates." Physiological and manufacturing considerations are given in the follow-up section including overviews on "Pharmacokinetics," "Safety Considerations," "Immunogenicity," "Expression Systems for Manufacture," and a chapter on "Stability, Formulation, and Delivery." The section on Clinical Applications discusses in detail "Protein Therapeutics in Autoimmune and Inflammatory Diseases, Oncology, Respiratory, and Infectious Diseases." Chapters on "Rescue Therapies" and "Biosimilars" supplement this section. Future horizons and new target class opportunities are the topics of the final section.

The series editors thank Tristan Vaughan, Jane Osbourn, and Bahija Jallal for organizing this volume and for identifying and working with such excellent authors. Last but not least we thank Frank Weinreich and Waltraud Wüst from Wiley-VCH for their valuable contributions to this project and to the entire book series.

May 2017

Raimund Mannhold, Düsseldorf
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A Personal Foreword

To a diabetic patient, it is hard to imagine a world without biosynthetic insulin. For a new parent of a premature baby, Synagis provides potentially life-saving prevention of respiratory syncytial virus. And for someone suffering the devastating effects of rheumatoid arthritis, Humira, the world's first fully human antibody, introduced in 2002, is so effective that it has become the world's best-selling medicine. Today, biologics such as these account for nearly half of all new drug approvals across the globe and nearly 25% of overall sales. In fact, in 2015, 6 biologics were among the top 10 best-selling drugs worldwide. With more than 500 biopharmaceuticals on the market, biologics represent the fastest growing sector of this industry, targeting illnesses such as cancer, asthma, cardiovascular disease, infectious diseases, multiple sclerosis, hepatitis, inflammatory disease, and so many others.

As a student of physiology and biochemistry in Paris, I was struck by the possibility of modifying proteins to increase their potential to treat illness. My postdoctoral work in molecular biology and oncology at the Max-Planck Institute for Biochemistry in Munich allowed me to focus on the analysis of epidermal growth factor receptor (EGF-R and HER2) signaling and to investigate the antitumor properties of the novel secreted tumor-associated antigen 90K.

My later work building the translational sciences function at Sugen further heightened my interest. But the day I met a patient with renal cell carcinoma who was successfully being treated with Sutent, a multitargeted receptor tyrosine kinase (RTK) inhibitor, made me realize that my true calling was to work in the biopharmaceutical industry to discover new drugs to help patients. Today, as the head of MedImmune, the global biologics early research and development unit of AstraZeneca, I am even more excited by the possibilities of using protein therapeutics to not just treat or prevent disease but to provide a durable cure for so many illnesses affecting patients across the globe.

This book dissects the field of protein therapeutics, from its early struggles to its promising future, and provides a thorough look into this dynamic industry. It touches on exciting developments around immuno-therapies for oncology – advancements I never thought were possible in my lifetime. It delves into the considerable energy going into the development of antibody drug conjugates and their potential for new therapies. Other topics include human and nonhuman

antibodies; technological advances in protein therapeutics, including human antibody discovery platforms, nonantibody scaffolds and antibody mimetics; protein engineering and physiological and manufacturing considerations around pharmacokinetics, immunogenicity, safety, and manufacturing; and clinical applications for protein therapeutics.

Protein Therapeutics concludes with a view into innovation of the future, including the potential to target protein therapeutics across the blood–brain barrier for the treatment of diseases ranging from brain tumors to Alzheimer's disease and how protein therapeutics could be delivered intracellularly to gain a better understanding of protein interactions and, for example, modify the RAS/MAPK pathway that could be potentially transformative for the treatment of leukemia and other cancers.

With contributions from recognized academic and industry experts, including Professor Pierre De Meyts, the leading academician in the field of insulin research and one of the fathers of protein therapeutics, and Herren Wu, MedImmune's chief technology officer who offers just a glimpse at potential future innovations, *Protein Therapeutics* will be a valuable addition to a field that is profoundly changing the way we treat disease.

Gaithersburg, MD
December 2016

Bahija Jallal

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Part IV
Physiological and Manufacturing Considerations for Biologics

10
Pharmacokinetics of Therapeutic Proteins

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Therapeutic biologics are biotechnology-derived drugs, including a variety of modalities such as blood and blood components, cytokines, tissue growth factors, vaccines, enzymes, monoclonal antibodies (mAbs), antibody fragments, and antibody-fusion proteins and peptides. mAbs are the mostly commonly employed biological modalities, which are also called immunoglobulins (Igs). Based on the structures of the heavy chain of Igs, five classes of Igs have been identified: IgA, IgD, IgE, IgG, and IgM [1, 2]. Among them, IgG is the dominant form of therapeutic proteins [3]. This is a rapidly growing class of therapeutics for a broad spectrum of indications, ranging from oncology and autoimmunity to orphan and genetic diseases. Most biologics are very large, complex molecules or mixtures of molecules. Because of their high molecular weight (MW) and structural complexity, the pharmacokinetics (PKs) of biologics is different from that of small molecules. They have a unique absorption, distribution, metabolism, and elimination (ADME) pathway. However, PKs of biologics cannot be summarized for the entire class because they comprise wide variety of compounds. In this chapter, the focus will be the ADME and PK modeling of biologics including mAbs, proteins, and peptides.

10.1
Absorption

Unlike small molecules, which are frequently delivered via oral administration, biologics are mainly delivered by parenteral administration, such as intravenous (IV), subcutaneous (SC), and intramuscular (IM) injections. The majority of Food and Drug Administration (FDA)-approved therapeutic proteins are administered intravenously because of their complete and rapid systemic availability in the systemic circulation compared with SC and IM administrations. However, this administration route is not convenient, so extravascular routes such as SC and

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IM administration are alternative convenient delivery modes for biologics. The limitations for these administration routes include the possibility of incomplete bioavailability and potentially higher immunogenicity to the therapeutic proteins.

After SC administration, protein drugs can be transported to systemic circulation directly via blood capillaries or indirectly via the lymphatics depending on the molecular size. Small peptides and proteins with MWs less than or equal to 16 kDa primarily leave the SC injection site into blood capillaries by diffusion [4]. The mechanism of absorption of mAbs with higher MWs after SC or IM administration is thought to be through convective transport across the lymphatic vessels ("lymphatic drainage"). mAbs enter local lymphatic capillaries and pass to the draining lymph nodes, where they bind to target cells. Antibodies not bound there pass to more distant nodes. If still not removed from the lymph flow, antibodies enter the blood stream through the thoracic duct. In addition, receptor-mediated transport such as FcRn (Fc-receptor of the neonate)-mediated transport could facilitate the absorption process.

Absorption of therapeutic proteins from the SC injection site tends to be slow compared to that of small molecules, which can be explained by the slow flow rate of lymph, which is ~100–500 times less than that of the blood. Following SC administration, the time to reach the maximum systemic concentration (t_{max}) in humans for peptides is in the range of hours, while the t_{max} for mAbs is generally several days [5]. For mAbs, SC bioavailability for currently marketed products is in the range of 50–100% in humans [6–8]. The extent of absorption of therapeutic proteins after SC injection could be impacted by metabolism/catabolism in subcutaneous tissues. The reason for low bioavailability of therapeutic proteins in humans may be due to the degradation at the injection site. Catabolism at the injection site may be dependent on the rates of extracellular degradation via proteolysis. For mAbs, rates of endocytosis and interaction with FcRn may also impact the extent of absorption. While the mechanism of FcRn-mediated effects on SC bioavailability is not known, FcRn may protect IgG from lysosomal degradation and is believed to contribute to the relatively high bioavailability of mAbs [9, 10]. Other factors, including the site of SC injection, depth and volume of injection, MW, and the size and charge of proteins, could impact the absorption of biologics after SC administration.

Relatively little work has been done to assess the systemic absorption of therapeutic antibodies following oral administration [11, 12]. The bioavailability of therapeutic proteins after oral administration is negligible. The gastrointestinal tract provides a hostile environment for antibodies and other therapeutic proteins. They will be degraded by proteolytic enzymes after oral administration. The extent of presystemic antibody degradation may impact the extent of absorption. In spite of the fact that FcRn has been detected within the human gastrointestinal epithelium, available data suggest that this FcRn receptor did not enhance the absorption of therapeutic antibodies following oral administration [11]. The utility of oral administration is limited to the treatment of gastrointestinal infection.

Delivery of therapeutic proteins through inhalation and intranasal routes could be an alternative route for rapid absorption. Inhaled insulin combination product

for the treatment of diabetes was approved by the FDA. The bioavailability of inhaled insulin for each of the devices varies, but it is in the range 10–46%, with much of the drug being lost within the device or in the oropharynx or upper airways [13]. Intranasal absorption of a variety of biologics, including calcitonin, oxytocin, growth factors, and interferons, has been extensively investigated [14].

10.2 Distribution

Distribution defines the reversible transfer of molecules from one location to another within the body. Small molecules can easily cross the blood capillary membranes and distribute to organs and tissues, which typically tend to have higher tissue penetration. However, because of their size and hydrophilic nature, therapeutic proteins have limited ability to distribute from the blood compartment to peripheral tissue. The typical central volume of distribution (V_c) for an mAb is approximately 2–3 l, which is similar to the total blood volume. The steady-state volume of distribution (V_{ss}) ranges from 3.5 to 7 l [15]. These reported volumes of distribution after IV administration are close to the plasma volume, suggesting limited distribution into tissues.

In general, a therapeutic protein initially distributes into the plasma volume and then into limited extracellular space. Once inside the tissue vasculature, active and/or passive transport processes govern the movement of molecules across the tissue membranes. Therapeutic proteins are distributed to peripheral tissues by paracellular and/or transcellular movement following IV dosing or by absorption after parenteral injections [10]. Because of the large molecular size and high polarity, paracellular movement of biologics is mainly via convective transport instead of passive diffusion. Transcellular movement of mAbs starts with the antibody's entry into vascular endothelial cells via pinocytosis followed by receptor-mediated endocytosis and phagocytosis. The FcRn recycling pathway can participate in the transcytosis step, in which mAbs can be bidirectionally transported to either the interstitial spaces or the vascular space [16]. However, the mechanistic understanding of the role of FcRn in antibody distribution is not clearly known. Studies with intestinal human cell lines suggest that FcRn transports IgG across cell monolayers, implicating FcRn in the transport of mAbs from circulation to the interstitial fluid of tissues [17].

Since therapeutic proteins are designed to bind their target antigen with high affinity, binding to plasma or tissue targets can influence antibody distribution. mAbs can bind to their targets by specific or nonspecific binding. Nonspecific binding includes FcRn and Fcγ receptor binding, residing in the constant region (Fc) of the mAb. Specific binding mainly refers to the binding between the antigen binding site of the variable region and the targets (e.g., soluble antigen/ligand or receptor). Target-mediated tissue distribution has also been reported for some mAbs [18, 19]. Upon tissue uptake, metabolism/catabolism of protein drugs will

occur in tissues before the remnants of the molecules are excreted from the body as smaller peptides and amino acid degradants, or they are recycled for synthesis into other proteins in the body.

The analysis of therapeutic protein distribution is much more complicated than the analysis of the distribution of most small-molecule drugs. For small molecules, concentrations of drug at the site of drug elimination appear to be in very rapid equilibrium with the concentration of drug in plasma. V_{ss} was estimated from noncompartmental or data-fitting compartmental analysis assuming that all elimination is from the central compartment. However, this assumption may not be valid for some therapeutic proteins, especially for the ones with high-affinity, high-capacity binding in tissue. In addition, the therapeutic proteins with target-mediated elimination could show nonlinear clearance. In such case, the V_{ss} estimated from noncompartmental analysis was underestimated and did not represent the true distribution of therapeutic proteins. The compartmental modeling approach should be used to estimate V_{ss}. Precise analysis of V_{ss} requires concentration data from both plasma and tissues.

IgG antibodies and other low-MW proteins show very little distribution to the brain because of the tight junctions of the blood–brain barrier. In general, antibody penetration into brain (cerebrospinal fluid (CSF)/serum partitioning) has been reported to be > 0.1% in animal models and in human patients [20]. There is some evidence to suggest that specific Fc receptors, perhaps including FcRn, actively efflux IgG from brain tissue [21]. Antibody fragment consisting of only an antigen-binding part can cross the blood–tissue barrier more easily. For example, in the treatment of neurodegenerative diseases, single-chain variable fragments have been shown to successfully penetrate the blood–brain barrier, leading to more effective therapy [22].

Other factors, including MW, blood flow, tissue heterogeneity, structure and porosity, target antigen density, turnover rate, and the target antigen expression profile, can impact the distribution of biologics.

10.3 Metabolism and Elimination

Therapeutic proteins, including mAbs, are unlikely the substrates of metabolizing enzymes of the CYP (cytochrome P) family, even though some of them may exhibit an inhibitory or inducing effect on the metabolic enzymes or transporters [1, 2]. Therefore, generally the metabolism of mAbs is not associated with the metabolic enzymes or transporters; rather, it is mainly via the intracellular catabolism process. This metabolism process may occur in circulation by circulating phagocytic cells or by their target antigen-containing cells such as tumor cells, or may occur in tissues by various cells [23]. Once taken up into cells, the mAbs may be metabolized to peptides or amino acids, or enter endogenous salvage pathways [14].

mAbs are mainly eliminated from circulation or interstitial fluid via several pathways: (i) FcRn-mediated salvage and nonspecific (linear) clearance, (ii) specific or target-mediated clearance (target-mediated drug disposition, TMDD), (iii) anti-drug-antibody-related clearance, and (iv) non-specific endocytosis and pinocytosis, and nonspecific proteolysis that occurs widely in plasma or extracellular fluid and formation of the immunocomplex (ICs) followed by complement- or Fc receptor-mediated clearance mechanisms [24, 25]. This chapter discusses the aforementioned elimination pathways, Fab clearance, as well as clearance variability and renal clearance.

10.3.1 FcRn-Mediated Salvage and the Nonspecific (Linear) Clearance

The FcRn-mediated salvage pathway, as well as the associated nonspecific (linear) clearance, is essentially a recycling process of IgG. Similar to endogenous IgGs, the Fc portion of exogenous mAbs binds tightly to the FcRn, that is, the Brambell receptor, under acidic pH (< 6.5). Then, during the internalization process, the IgG–FcRn complexes do not undergo degradation at lysosome; rather, they are recycled to the apical or the basolateral domain under physiological pH (~ 7.4). At this neutral pH, IgG has virtually no affinity to the FcRn receptors and therefore dissociates and returns to the systematic circulation. The nature of recycling of IgGs via FcRn receptors contributes to the rescue mechanism for IgGs, including medically administered IgGs, thus regulating the clinically observed prolonged half-life of mAbs, which is ~21 days [10, 26, 27]. As FcRn serves the roles of functional protection and as a transcellular vehicle for mAbs, the binding affinity between mAb and FcRn would impact the clearance of the mAbs. Conversely, modification of the Fc domain of IgG may subsequently change its affinity with FcRn and then potentially refine its linear clearance for therapeutic benefits [28]. Examples include differential glycosylation [29] and modification of the isoelectric point via deamination process [30–32], which have limited impact on the FcRn binding and the subsequent clearance rate. In comparison, the methionine oxidation of the Fc fragment appears to reduce the binding affinity to FcRn, thus prolonging the half-lives of the mAbs [33]. Interestingly, the reduction of half-life correlates well with the extent of methionine oxidation and the corresponding FcRn binding affinities [34].

In addition to the therapeutic proteins, FcRn is responsible for the salvage of both endogenous albumin and IgG [34]. Albumin is the most abundant protein in human plasma, averaging 3.5–5.5 g/dl and accounting for 50–60% of the blood plasma proteins [35, 36]. IgGs represent the second most abundant protein in human plasma, averaging 0.7–1.9 g/dl and accounting for about 10–20% of plasma protein [37]. The binding site of albumin and IgG with FcRn is noncompetitive [38]. Despite the competitive binding of endogenous IgG with FcRn, it is not common that FcRn receptors would be saturated by therapeutic proteins, because normally the dose of therapeutic proteins is below 10 mg/kg, which is far below the normal capacity of FcRn recycling of serum IgG [30]. Under normal

plasma IgG concentration, IgG has a half-life of ~ 25 days and a plasma clearance of ~ 10–20 ml/h (~ 3.5 ml/kg/day) [39, 40]. High concentrations of serum IgG are able to saturate the FcRn recycling system, decreasing recycling efficiency and leading to an increase in the fractional catabolic rate of IgG [41]. For example, a disease state may impact on the endogenous secretion of IgG. Multiple myeloma is one such example. In myeloma patients, the IgG concentrations in plasma may approach 10 g/dl, and the half-life of administered exogenous IgG decreases slightly to 18 days, which is partially due to the endogenous high level of IgG [42]. Another example is the malfunctioning of FcRn due to the disease state, such as familial hypercatabolic hypoproteinemia, which results in the deficiency of FcRn function, leading to low concentrations of both IgG and albumin [39, 43]. Conversely, in patients with very low plasma concentrations of IgG but normal FcRn receptor expression, the half-life of the IgG antibody may be prolonged. For example, in primary immunodeficiency patients, their IgG level is generally lower than normal, that is, less than 0.6 g/dl. With IV administration of therapeutic IgG, the patients show a longer half-time of 32–37 days [44–46].

IgGs that are not bound or weakly bound to the FcRn portion are destined to the lysosome for enhanced degradation. Among the four IgG subclasses in humans, the binding affinity to FcRn decreases in the order IgG1, IgG2, IgG3, and IgG4, ranging from 20 nM (IgG1) to 80 nM (IgG4) [47]. As such, even though IgG3 exhibits increased binding affinity with FcRn at neutral pH, its binding affinity decreases under acidic pH, which results in a reduced half-life of ~9 days [48].

10.3.2 Target-Mediated Drug Disposition

It is common to observe the TMDD characteristics of biologics. The Fab binding domains of the mAbs bind to the epitopes of the target on the cell surfaces, and then the IgG–receptor complex goes through endocytosis and gets eliminated from the body. By definition, TMDD is capacity-limited (saturable) because of the finite production and expression of the targets. The elimination rate by the TMDD pathway is impacted by several factors, falling into the following two categories: antigen–target and IgG–target complex-related [38]. The antigen–target-associated factors include the concentration/amount of the target, the elimination of the free mAb (determining the availability of mAb to bind to the antigen), the turnover rate of the specific antigen (determining the availability of antigen), the receptor occupancy of the target, and the location of the target (e.g., soluble and/or cell-surface-expressed). Antigen–mAb complex-associated factors include the reversible (or irreversible) nature of the binding affinity between the mAb and the target and the depletion of the antigen–mAb complex.

TMDD is often obvious at low doses with low antibody concentration. When the mAb doses increase, the contribution of TMDD to the overall clearance is

less because of the progressive saturation of the target receptors by the drug, and subsequently the exposure increases more than dose-proportional compared to the dose [49]. The antigen target generally has two forms, namely the soluble form and as expressed on the surface of the cells. In general, for a soluble target, the mAb is more likely to exhibit a dose-independent linear PK; examples include bevacizumab which targets the vascular endothelial growth factor [50, 51]. The PK of bevacizumab is linear between 1 and 10 mg/kg dose, and the accumulation of bevacizumab to steady-state concentration after long-term treatment can be predicted by a linear two-compartment model [52]. TMDD is more common for the cell-surface targets, where lower doses present faster clearance, such as cetuximab targeting the epidermal growth factor receptor (EGFR), denosumab targeting the receptor activator of nuclear factor kappa-B ligand (RANKL), and obinutuzumab targeting CD20 receptors, to name a few [53–55]. Efalizumab is another example of a nonlinear PK targeting CD11a in human psoriasis patients [56]. At the dose of 0.3 mg/kg, the mean clearance of efalizumab was reported as 322 ml/day. In comparison, the clearance was 11 and 6.6 ml/day at the doses of 3 and 10 mg/kg, respectively [57]. Nonetheless, a linear PK is frequently observed for the cell-surface receptor; examples include pertuzumab targeting the HER-2 receptor at the dose > 2 mg/kg [58, 59]. Additionally, the disease state or the tumor burden may impact on the TMDD component of mAb clearance. For example, daclizumab is employed to target CD25 of activated lymphocyte, and the CD25 is overexpressed in several diseases, including graft-versus-host disease (GvHD), renal transplant, and leukemia [60, 61]. The clearance of daclizumab was reported as 0.042, 0.0314, and 0.015 l/h for the treatment of GvHD, prevention of GvHD, and renal transplant patients, respectively [62, 63]. Another example is obinutuzumab for the treatment of various hematological malignancies [52]. Population PK analysis has shown that the clearance of obinutuzumab, including the initial and the steady state, as well as the rate of clearance decline, was disease-dependent. Comparing patients suffering from chronic lymphocytic leukemia (CLL), B-cell lymphoma (BCL), diffuse large B-cell lymphoma (DLBCL), non-Hodgkin's lymphoma (NHL), and mantle cell lymphoma (MCL), it was found that MCL patients exhibited higher clearance than the CLL patients, whereas BCL or DLBCL patients had the lowest clearance [52]. This observation is attributed to the different disease states, where a significantly higher expression of CD20 receptors on B cells was reported in MCL patients compared with CLL patients [64–66]. Similarly, the clearance is faster in CLL than NHL patients, resulting in C_{trough} and area under the curve (AUC) in CLL patients being much lower than those for NHL patients given the same rituximab dose (375 mg/m^2) [67]. Additionally, for the same disease but with different tumor burdens, the clearance of daclizumab was strongly correlated with the tumor volume, where a higher tumor volume resulted in faster clearance. Also, it was observed that patients showed more than 80% clearance within 48 h after the first dose, and the clearance was significantly reduced after repeated dosing [68]. This observation is consistent with that shown by other mAbs where a higher disease burden resulted in faster clearance and reduced exposure of mAb [69]. In another example, the exposure

of rituximab was shown to inversely correlate with the disease state in NHL [70, 71]. In 166 recurrent low-grade lymphoma patients, the concentration of rituximab increased throughout the treatment cycle. The half-life increased from 76.3 h after the first dose to 205.8 h after the fourth dose, which corresponds to a fourfold decrease of rituximab clearance from 38.2 to 9.2 ml/h. The hypothesis is that the amount of antigen in circulation decreases with treatment, and so is the clearance [72]. In general, TMDD is more frequently observed at lower doses where the receptors are available and not saturated. However, TMDD may occur across all the investigated doses, as is the case with elotuzumab. Elotuzumab has been administered in the dose range of 0.5–20 mg/kg in relapsed or refractory multiple myeloma patients, where the elotuzumab exposure increased more than dose-proportionally [73].

Clearance of immune complexes is another form of TMDD. Certain soluble receptors, such as IgE, with multiple repeated epitopes are likely to bind with two or more antibodies, resulting in various stoichiometric IgE–antibody complexes. Omalizumab is an IgG1K humanized anti-IgE murine antibody, which binds to different forms of IgE with ratios ranging from 1:2, through 3:3, to 2:1 [73, 74]. The clearance of free omalizumab has a mean value of 2.4 ± 1.1 ml/kg/day; however, the clearance of the IgE–antibody complex is much faster, with the mean value of 5.18 ml/kg/day [75, 76]. One hypothesis for the faster clearance of the complex is that the IgE–mAb complex may be rapidly eliminated by phagocytosis, mainly because of its large size [77].

10.3.3 Immunogenicity-Induced Clearance Change

All protein therapeutic agents are potentially immunogenic. It is generally assumed that fully human mAbs are less immunogenic than chimeric or murine antibodies. Immunogenicity, such as induced by an anti-drug antibody (ADA), can alter the clearance of the mAb, mainly due to the binding of ADA to mAbs or the neutralizing effect of Neutralizing antibody (NAb). The bidirectional impact of ADA on PK exposure has been observed, that is, dominantly increasing the clearance and occasionally decreasing the clearance [78]. A recent review of FDA-approved biological products suggested that 29 products reported ADA status and its impact on the PK. Among them, 13 products showed increased clearance and 6 products showed drug-sustaining ADA with reduced clearance [75]. One plausible hypothesis for the decreased clearance is that the ADA–drug complex protects the elimination of biologics from the system with the presence of persistent ADA. Two methods have been commonly reported to assess the impact of ADA on PK, naamely between-subjects and within-subjects comparison [79]. Between-subject comparison is to evaluate the concentration difference between ADA+ versus ADA− subgroups, and the within-subject comparison is for the time-varying concentration of the same ADA+ subject, as shown in Figure 10.1 [78]. Examples of ADA-induced clearance increase are adalimumab and infliximab. For adalimumab, the low trough serum drug concentration is associated

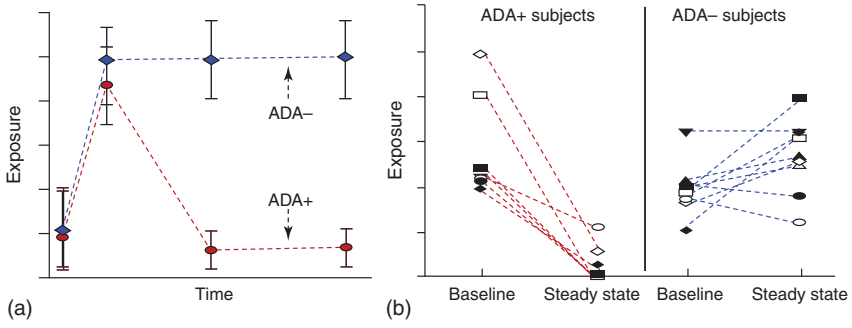

Figure 10.1 Schematics for two separate case examples showing the effect of ADA on exposure by between-subject comparison. (a) Between-subject comparison of exposure time course. (b) Within-subject comparison of exposure at two different time points.

with positive ADA, as established using 168 Crohn's disease patients. This observation was consistent throughout the entire follow-up period ($P < 0.0001$) and was independent of the time points of ADA detection [80]. Similarly, an independent clinical study with 71 patients showed a negative correlation between adalimumab serum concentration and the ADA levels ($P < 0.001$). Specifically, the media adalimumab levels were 6.7, 3.7, and 0.1 g/ml at ADA levels of < 1.5, 1.5–3, and ≥ 3 g/ml-equiv., respectively. It was also reported that the maximum adalimumab level was only 0.5 μg/ml at the ADA level of ≥ 3 g/ml-equiv. [79]. Infliximab was also reported to have an association between low trough level and the ADA-positive NAbs, and the ADA to infliximab was predominantly of the IgG type, with 36% being IgG4 [81]. Examples of ADA decreasing the clearance of biologics are rare, and a thoroughly reported case is the insulin antibody [82]. It has been reported that the anti-insulin antibody may serve as a repository for insulin, thus preventing it from acting on the receptors and from being eliminated [83, 84]. In a study with 23 subjects, Chen and colleagues reported that the $AUC_{0-24\,h}$ for the total insulin was significantly higher for high-ADA patients than for low-ADA patients. Additionally, the calculated steady-state clearance of the total insulin is negatively correlated with the ADA binding, that is, a higher ADA binding level associates with lower total insulin clearance [85, 86]. It is worth noting that the existence of ADA may not have an impact on the clearance of mAbs. This observation may be clinically true, with no impact of ADA on the PK, or the impact of ADA on PK may be interfered by the ADA bioanalytical method [87]. Generally, a low level of titer may have a low impact on the PK, while high titers may be more likely to be evaluated with its impact on exposure. It has been reported that ADA has no impact on pembrolizumab and PK [88, 89]. Pembrolizumab concentrations for patients who were ADA-positive during treatment were in the same range as for ADA-negative patients treated with the same dose of 10 mg/kg q3w [79]. Similarly, nivolumab clearance for the ADA-positive patients during treatment was in the range of clearance for patients tested negative for ADA with same dose of 3 mg/kg q2w [81].

Quite often, the impact of ADA on the clearance or exposure of mAbs cannot be assessed, mainly because of the inadequate data collected in clinical trials or limitation of the analytical method; examples include daratumumab and obinutuzumab [90]. Partially for the same reason, comparison of ADA incidence or magnitude across various mAb products can be misleading, because the ADA results are highly dependent on the sensitivity and specificity of the assay, the sample handling, the timing of sample collection, underling disease state, and co-mediations. Therefore, many FDA-approved mAb product labels have the immunogenicity language to indicate as such.

Drug–drug interactions are not common for therapeutic proteins. However, co-administration of immunomodulators may have an impact on the PK of mAbs via the indirect ADA pathway. Concomitant use of immunomodulators, such as methotrexate or azathioprine, has been reported to reduce the formation of ADA. In a meta-analysis comparing the combination versus the monotherapy of infliximab, the risk ratios of developing ADA and infusion reactions were reduced to 0.6 (95% CI of 0.4–0.9) [91, 92]. The reduction of ADA formation was postulated as the observed reduction of clearance of infliximab and adalimumab, thus resulting in higher systemic exposure [93, 94]. For example, the U.S. product label states that methotrexate decreases the clearance of adalimumab after a single dose and multiple doses by 29% and 44%, respectively, in patients with rheumatoid arthritis (RA) [95]. Subsequently, the adalimumab mean steady-state trough concentrations of were approximately 5 μg/ml and 8–9 μg/ml, without and with methotrexate, respectively [84]. The differential effect of single versus multiple doses may suggest that methotrexate may take time to exert its "perpetrator" effect on the formation of ADA of mAbs. This leads to another hypothesis, namely that immunomodulators like methotrexate may alternate the expression of FcRn expression, and thereby modulate the linear elimination [96, 97].

10.3.4 Fragment of mAbs

Fragments of antigen-specific mAbs have been developed for therapeutic use. Three general modalities, namely antigen-binding fragments (Fab), single change variable fragments (svFv), and the "third-generation" (3G) molecules, illustrate the historical development waves of mAb fragment development [98]. Several Fab products have been approved by FDA, such as abciximab, ranibizumab, and certolizumab [88]. The Fab fragments have faster clearance and thus shorter half-lives, mainly due to their lack of the Fc domain that binds to the FcRn for recycling protection [99]. Additionally, depending on the size of the Fab fragment, it may be eliminated by renal excretion. The suggested cut-off of the molecular size for renal elimination is below ~60 kDa [100]. For example, the free abciximab plasma concentration declines rapidly for ~6 h and then at a slower rate afterward, and abciximab remains in the circulation for ~15 days [101]. The half-lives of ranibizumab and certolizumab were reported as ~9 and 14 days, respectively

[102, 103]. Certolizumab is a PEGylated Fab, with decreased elimination due to decreased renal clearance and proteolysis. Being conjugated with PEG, its half-life is prolonged to ~14 days [103]. Similar to the Fab fragment, svFv has a smaller size compared to the full mAb, so it has improved tumor-penetrating properties but gets cleared rapidly from the system. Therefore, its exposure in the circulation and target tissue may require more frequent administration than the entire antibody. Blinatumomab is a bispecific svFv with anti-CD19/anti-CD3 activities, which targets both T cells and B cells, respectively, in non-Hodgkin lymphoma. Its U.S. product label indicates that the estimated mean (SD) systemic clearance is 2.92 (2.83) l/h, and the mean half-life is very short with the value of 2.11 (1.42) h [104].

10.3.5 Variability

A large variability of biologic exposure has been observed clinically [105, 106]. For mAbs approved for RA treatment, the inter-individual standard deviation ranged from 17% to 44% for clearance [103]. Numerous factors contribute to the PK variability, such as biologic, pharmacologic, and pathophysiologic factors, including but not limited to age, body weight or size, gender and ethnic diversity, serum albumin and LDH levels, disease type and severity, disease burden, and co-medication [14, 28, 39, 50, 51]. In general, the clearance of mAbs increases with body weight and body surface area, and is faster in men than in women [27]. The clearance of rituximab increases by 35% with higher body surface area from 1.4 to 1.9 m^2, and is higher in men than women [107]. The clearance of elotuzumab also increases with increasing body weight [70]. This body-weight effect justifies a weight-based dosing regimen rather than the flat dosing for mAbs. Contradictory to this proposal, another work suggests that body-weight-based dosing may provide limited advantages in reducing the variability over the flat dosing, depending on the magnitude of the body weight effect on the clearance [108]. The authors reported that the effect of body weight is generally little or moderate, and the difference of variability in exposure is less than 20%, which is significantly less than the variability in pharmacodynamics (PDs), efficacy, and safety measures [103]. A comprehensive evaluation of both PK and/or PD for 18 proteins and peptides, between the body-size-based dosing and fixed dosing, suggested that the fixed dosing performed better for 12 out of 18 selected biologics with regard to reducing inter-subject variability in exposure, whereas 6 biologics performed better for the body-size-based dosing [109]. This analysis result is consistent with the evaluation of 12 mAbs in terms of body-size-based dosing versus fixed dosing [110]. In addition, serum albumin has been demonstrated to be associated with clearance of mAbs such as pertuzumab, where the pertuzumab clearance increases with the decreased baseline albumin level [58]. Both IgG and albumin are salvaged by FcRn, with noncompeting nature. However, the low level of albumin may indicate the disease state and a reduced functionality of FcRn for efficient recycling of exogenous mAbs, thus

relating to the faster clearance of mAbs. The decreased state can impact on the nonlinear clearance of mAbs, mainly due to the antigen burden. Normally, the higher the disease burden, the faster the clearance of the mAbs via the TMDD pathway [65, 66]. Further, ADA levels could contribute to the between-subject variability, since the ADA formation is partially dependent on the patients' individual immune response (genetic susceptibility), which is characterized by pronounced heterogeneity, ADA variability over time for the same subject, as well as the pathological mechanism and the activity of the underlying disorder [111]. It should also be noted that co-medication can influence the clearance of mAbs, with cases reported for immune modulators such as methotrexate [84, 88, 90].

10.3.6 Renal Clearance of mAbs

Given the high MW of mAbs, renal elimination virtually plays no role at all [112, 113]. Only low-MW proteins, peptides, and amino acids that are generated by proteolysis in other organs can be potentially excreted renally [114]. Clinical evidence has shown that smaller biologics, such as interleukin-10, growth hormone, and anakinra, exhibit gradual decrease of their clearance with increasing severity of renal impairment [98]. This could also occur to a small extent in the liver (~16%), skin (~33%), and muscle (~24%) [115]. Small-sized mAbs, with MW < 60 kDa, can be eliminated by the renal clearance pathway. In these special cases, renal impairment study in patients is very important for exposure and potential dose adjustment. Reported cases include kineret, a recombinant, non-glycosylated form of the human interleuk-1 receptor antagonist that consists of 153 amino acids and has an MW of 17.3 kDa. In a renal impairment study, the mean plasma clearance of kineret in subjects with mild (creatinine clearance 50–80 ml/min) and moderate (creatinine clearance 30–50 ml/min) renal insufficiency was reduced by 16% and 50%, respectively. In severe renal insufficiency and end stage (< 30 ml/min), the mean plasma clearance declined by 70% and 75%, respectively. It is recommended that dose adjustment should be considered for subjects with renal impairment [116]. Another example is pegintron, which is a covalent conjugate or recombinant α-1b-interferon with monomethoxy polyethylene glycol, with an average MW of ~31 kDa. Because of its small size, renal elimination accounts for 30% of the total clearance. Following multiple doses of pegintron, its clearance is reduced by 17% and 44% for moderate and severe renal impairment subjects, respectively, compared to normal renal function. And the clearance for severe renal impairment is similar to that in patients receiving hemodialysis. Therefore, dose reduction is recommended for patients receiving pegintron with moderate or severe renal impairment [117].

10.4
Pharmacokinetic Modeling of Therapeutic Proteins

In March 2006 [118], six healthy volunteers in a clinical trial were given a synthetic mAb called TGN1412, a drug intended to boost the human T cells in the immune system, but soon all of them became violently ill. TGN1412 is an mAb that was being developed as a medicine to treat leukemia and autoimmune diseases such as RA by triggering T-cell proliferation in diseases. The human T cell is normally activated only when two receptors, namely CD28 and the T-cell antigen receptor, are stimulated. The agonistic mAb TGN1412 bypasses the requirement for T-cell antigen receptor signaling and activates human T cells by stimulating only the co-stimulatory receptor CD28. Preclinical studies showed that TGN1412 was well tolerated in cynomolgus monkeys at doses up to 50 mg/kg/week for four consecutive weeks, and 50 mg/kg was considered as the no-observed-adverse-effect level (NOAEL). The Phase I trial starting dose was calculated primarily based on the NOAEL, which is considered as 50 mg/kg from the repeated dose toxicity studies in cynomolgus monkeys and procedure described in the draft FDA guideline "Estimating the Safe Starting Dose in Clinical Trials for Therapeutics in Adult Healthy Volunteers." A starting dose of 0.1 mg/kg, which is 1/500th that given as a safe dose in animals, was proposed for the first-in-human trial by applying an allometric correction factor of 3.1 and additional safety factor of 160 for the cynomolgus monkey NOAEL. However, the extra precautions were not taken when antibodies were used to stimulate rather than neutralize components of the immune system. The binding of TGN1412 and CD28 receptors is shown in Figure 10.2. TGN1412 with concentration C binds to the free co-stimulatory receptor (R) CD28 to form a TGN1412-CD28 complex (RC). The binding affinity (K_D), total TGN1412 concentration C_{tot}, and total receptor CD28 concentration R_{tot} were represented by Eqs. (10.1) and (10.2), respectively, and Eq. (10.1) for K_D can also be expressed in

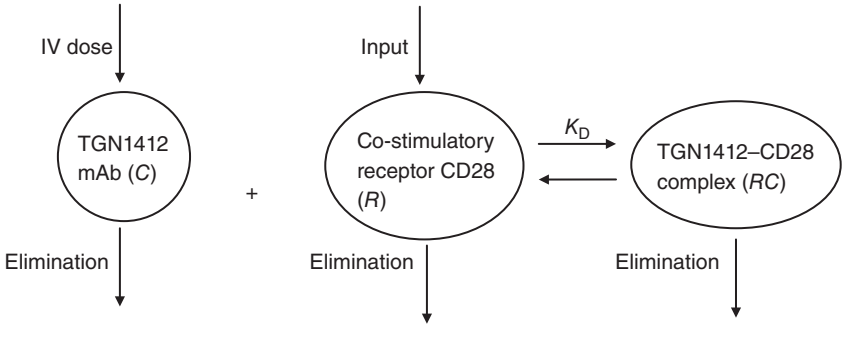

Figure 10.2 Binding of TGN1412 with the co-stimulatory receptor CD28.

Eq. (10.3). Based on Eq. (10.3), the percent of receptor occupancy (%OCC) can be derived as in Eq. (10.4).

$$K_D = [C][R]/[RC] \tag{10.1}$$

$$C_{tot} = C + RC \tag{10.2a}$$

$$R_{tot} = R + RC \tag{10.2b}$$

$$K_D = (R_{tot} - RC)(C_{tot} - RC)/RC \tag{10.3}$$

$$\%OCC = ((C_{tot} + R_{tot} + K_D) - \sqrt{((C_{tot} + R_{tot} + K_D)^2 - 4 \times R_{tot} \times C_{tot}))/(2 \times R_{tot})} \tag{10.4}$$

By substituting the known K_D and the total TGN1412 concentration and total CD28 concentration at that time into Eq. (10.4), more than 90% of the CD28 receptors were bound by TGN1412 with the proposed first-in-human dose of 0.1 mg/kg. Without any knowledge on the behavior of this compound in humans, the receptor occupancy of more than 90% was too high, since this induces massive production of cytokines and uncontrolled inflammatory responses, which were observed in all six healthy volunteers in this trial. The conclusion is that the preclinical development studies that were performed with TGN1412 did not predict a safe dose for use in humans even though current regulatory requirement were met.

An Expert Scientific Group was set up following the very serious adverse reactions that occurred in the first in-human clinical trial of TGN1412 to consider what should be necessary in the transition from preclinical to the first in-human Phase I studies and in the design of these trials, with specific reference to biological molecules with novel mechanisms of action and/or with highly species-specific action, especially toward immune system targets. The group recommended in the final report that a broader approach to dose calculation, beyond reliance on "No Observable Effect Level" or "No Observable Adverse Effect Level" in animal studies, should be taken and that the calculation of the starting dose in first-in-human studies should utilize all relevant information and tools including PK and PD modeling and simulation (M&S).

The development of therapeutic proteins poses additional challenges in comparison with small molecules. The PKs of biologics is often nonlinear, resulting in a disproportionate increase in exposure with increasing dose, which is caused by TMDD in many cases. High-capacity binding to target receptors serves as an important elimination route by target- or receptor-mediated uptake and subsequent intracellular metabolism of the therapeutic proteins. If binding to these receptors is capacity-limited, the cellular uptake may become saturated, resulting in dose-dependent exposure. Because of target-mediated disposition, the identification of an exposure–response relationship becomes more complicated, as the PKs and PDs are now bidirectionally interdependent. As the PKs and PDs of the therapeutic proteins or mAbs are often complex, they should be studied using a

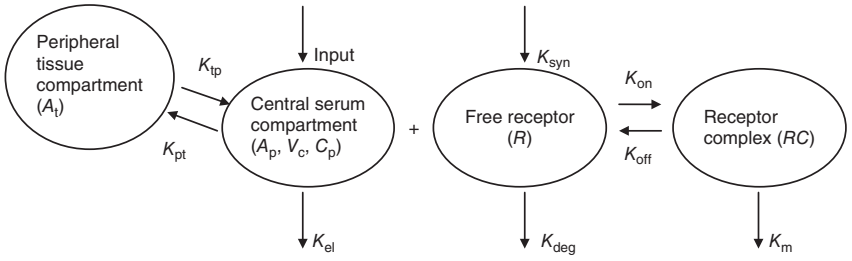

Figure 10.3 Full TMDD model comprising drug distribution and drug–receptor interaction.

rational and powerful tool: PK/PD M&S from the preclinical stage to find an optimal dose that is the safest possible dose with maximum efficacy and to optimize the design of both preclinical and clinical trials.

In order to accommodate the effects of TMDD as well as the concentration–time profiles of therapeutic proteins exhibiting saturable target binding, a generic TMDD model was published by Mager and Jusko in 2001 [119]. A schematic illustration of the full TMDD model is shown in Figure 10.3.

Drug in the central compartment (amount, A_p; apparent volume of distribution, V_c; concentration, $C_p = A_p/V_c$) can distribute to and back from a tissue compartment (amount, A_t; apparent volume of distribution, V_t; concentration, $C_t = A_t/V_t$) with the first-order rate constants K_{pt} and K_{tp}, respectively, be eliminated from the central compartment with the first-order rate constant K_{el}, and bind to the free pharmacologic target (concentration of free receptors, R) with the second-order association rate constant K_{on} to form a drug–target complex (concentration of the drug–target complex, RC). This complex may either dissociate with the first-order rate constant K_{off} or be internally degraded with the first-order rate constant K_m. The free receptor turnover is reflected by the process of synthesis with the zero-order synthesis rate constant K_{syn} and the process of degradation with the first-order rate constant K_{deg}. Input to the central compartment refers to any route of administration of the drug to the body. From the perspective of PK modeling, the above system in the diagram can be described by the following four differential equations with system variables A_p, A_t, R, and RC.

$$\mathrm{d}A_p/\mathrm{d}t = \mathrm{In}(t) - K_{el} \times A_p - K_{pt} \times A_p + K_{tp} \times A_t - K_{on} \times R \times A_p + K_{off} \times RC \times V_c \tag{10.5}$$

$$\mathrm{d}A_t/\mathrm{d}t = K_{pt} \times A_p - K_{tp} \times A_t \tag{10.6}$$

$$\mathrm{d}R/\mathrm{d}t = K_{syn} - K_{deg} \times R + K_{off} \times RC - K_{on} \times R \times C_p \tag{10.7}$$

$$\mathrm{d}RC/\mathrm{d}t = K_{on} \times R \times C_p - K_m \times RC - K_{off} \times RC \tag{10.8}$$

Instead of modeling the change of the concentration of free receptors (R) over time in Eq. (10.7), based on the relationship defined in Eq. (10.2b), the change of

total concentration of receptors (R_{tot}) over time can be modeled, and the above differential equations can be revised as the following, except Eq. (10.6), with the system variables A_p, A_t, $R_{tot,}$ and RC.

$$dA_p/dt = In(t) - K_{el} \times A_p - K_{pt} \times A_p + K_{tp} \times A_t - K_{on} \times (R_{tot} - RC) \times A_p + K_{off} \times RC \times V_c \quad (10.9)$$

$$dR_{tot}/dt = K_{syn} - K_m \times RC - K_{deg} \times (R_{tot} - RC) \quad (10.10)$$

$$dRC/dt = K_{on} \times (R_{tot} - RC) \times C_p - K_m \times RC - K_{off} \times RC \quad (10.11)$$

In the initial condition of the biological system, which is at the biological steady state in the absence of the drug, $R_{tot} = R$ and $dR_{tot}/dt = dR/dt = 0$; derived from either Eq. (10.7) or Eq. (10.10), the initial condition or the baseline concentration of total free receptors is defined as K_{syn}/K_{deg}. Because most computing programs use for PK modeling updates the amounts of drug in all the compartments at time t_j to that at time t_{j+1} using PK formulas, the amount of mAb in the central compartment in either Eq. (10.5) or Eq. (10.9) could be considered primarily as one of the system variables to the model. Although the distribution and elimination of mAb from the central compartment were modeled by first-order rate processes and all first-order rate constants will be estimated based on either Eq. (10.5) or Eq. (10.9), both processes can also be described in terms of clearance (CL) from physiologic point of view. CL is defined as the volume of fluid cleared of drug from the body per unit of time. Based on this different parameterization, Eqs. (10.5) and (10.9) can be revised as the following differential equations, Eqs. (10.12) and (10.13), respectively:

$$dA_p/dt = In(t) - CL \times C_p - Q \times C_p + Q \times C_t - K_{on} \times R \times A_p + K_{off} \times RC \times V_c \quad (10.12)$$

$$dA_p/dt = In(t) - CL \times C_p - Q \times C_p + Q \times C_t - K_{on} \times (R_{tot} - RC) \times A_p + K_{off} \times RC \times V_c \quad (10.13)$$

where Q is the intercompartment (between central and peripheral compartment) clearance. Moreover, Eq. (10.6) is also parameterized as follows:

$$dA_t/dt = Q \times (C_p - C_t) \quad (10.14)$$

Also, the concentration of mAb in the central compartment could be an alternative system variable to consider and revise Eq. (10.9), as in the following example:

$$dC_p/dt = In(t) - K_{el} \times C_p - K_{pt} \times C_p + K_{tp} \times A_t/V_c - K_{on} \times (R_{tot} - RC) \times C_p + K_{off} \times RC \quad (10.15)$$

Equation (10.6) will be

$$dA_t/dt = K_{pt} \times C_p \times V_c - K_{tp} \times A_t \quad (10.16)$$

Choosing different parameterizations and/or different system variables, the full TMDD model can be defined from Eq. (10.5) to Eq. (10.16).

However, depending on the availability of preclinical and/or clinical data – for example, the $K_{on} - K_{off}$ parameters were not uniquely identifiable when the full TMDD model was applied to abciximab PK/PD data – sometimes a simplified TMDD model needs to be used instead of a full TMDD model to fit the preclinical and/or clinical PK and PD data we collected in the trial. A simplified TMDD model called quasi-equilibrium (QE) model was published by Mager and Krzyzanski in 2005 [120]. The key assumption on which the QE model is based is that the binding of the drug to the free target and the dissociation of the drug–target complex are at equilibrium almost instantly, with the characteristic half-life of seconds or minutes, which is faster than the remaining system processes, which include the distribution and/or elimination of drug, target, and drug–target complex, with the characteristic half-life of hours or days, or even weeks. With this large difference on the timescales of the different system processes included in the full TMDD equations, the binding-related parameters K_{on} and K_{off} may become difficult to be estimated precisely based on routine PK sampling schedules, which are often of order of minutes or hours. Under the condition of QE between binding of drug (C_p) to free receptors (R) and dissociation of the drug–receptor complex (RC), the rate of binding $V_b(K_{on} \times C_p \times R)$ equals the rate of dissociation V_d $(K_{off} \times RC)$, and K_D, which is the QE dissociation constant, was defined as $R \times C_p/RC$ or K_{off}/K_{on} based on the equivalence of rate of binding and rate of dissociation at equilibrium. Thus, instead of uniquely estimating K_{on} and K_{off} with the full TMDD model without rich data in the transition phase, only one of the constants may be estimated, such as K_{on}, and the other may be calculated from the relationship $K_{off} = K_{on} \times K_D$, with or without K_D fixed to a known value from *in vitro* experiment. K_{on} and K_{off} are related by $K_{off}/K_{on} = K_D$ and cannot be estimated uniquely with the QE model. The relationship $K_{off}/K_{on} = K_D$ can be applied to Eq. (10.5) through Eq. (10.16), depending on the parameterizations and system variables chosen, to turn the full TMDD model to the QE TMDD model. Alternatively, with the relationships Eq. (10.2a) and (10.2b) and the concentration of drug and receptor measured, the change of another system variable C_{tot}, the total drug concentration over time, can be derived by $dC/dt + dRC/dt$ as in the following:

$$dC_{tot}/dt = In(t) - K_{el} \times C_p - K_m \times C_{tot} + K_m \times C_p - K_{pt} \times C_p + K_{tp} \times A_t/V_c \quad (10.17)$$

Equation (10.10) can also be revised as

$$dR_{tot}/dt = K_{syn} - (K_m - K_{deg}) \times (C_{tot} - C_p) - K_{deg} \times R_{tot} \quad (10.18)$$

C_p in the right-hand side of both Eq. (10.17) and Eq. (10.18) is derived based on the relationship $K_D = C_p \times [R_{tot} - (C_{tot} - C_p)]/(C_{tot} - C_p)$ as in the following

$$C_p = ((C_{tot} - R_{tot} - K_D) + \sqrt{((C_{tot} - R_{tot} - K_D)^2 + 4 \times K_D \times C_{tot}))}/2 \quad (10.19)$$

Then the system can be described by the variables C_{tot}, R_{tot}, and the free drug concentration C_p with the QE model. For the selection between full TMDD model and quasi-equilibrium TMDD model, that the ratio of $\tau_B/t_{char} \ll 1$ is considered one of the conditions under which the QE model is the accurate approximation of the full TMDD model. The characteristic time (t_{char}) was defined as the length of the time interval between PK samplings, probably more accurately, smallest time interval between PK samplings. τ_B, the reciprocal ($\tau_B = 1/(C_{char} \times K_{on}) = \text{Dose} \times K_{on}/V_c$) of $C_{char} \times K_{on}$, was interpreted as the time a receptor resides in the receptor compartment before binding to its ligand; C_{char}, the characteristic ligand concentration, is Dose/V_c for IV bolus drug administration. Although some unknown parameters (V_c, K_{on}) were involved in the definition of τ_B, they may be available from noncompartmental analysis and *in vitro* experiment. Another condition to ensure that the QE model is a reasonable approximation of the full TMDD model is that the drug concentration should exceed the free receptor concentration by an order of magnitude. Based on the QE TMDD model or full TMDD model, some simplifications, but not limited to them, could be further made, sometimes depending on the results of curve fitting of the modeling and the stability of model, based on the following assumptions: (i) the receptor turnover is slow, $K_{syn} \to 0$, $K_{deg} \to 0$, the amount of free receptor is constant; (ii) the rate of internalization of drug–receptor complex is slow, $K_m \to 0$; and (iii) rate of degradation of receptor equals the rate of internalization of drug–receptor complex, $K_{deg} = K_{m,}$ and the amount of total receptor is constant.

In 2008 [121], Gibiansky and colleagues published a quasi-steady-state (QSS) TMDD model based on the situation that the QE condition may not hold when the rate of elimination of the complex is not negligible compared to the dissociation rate. In their model, instead of QE, which was only achieved between the binding of drug to free target and dissociation of the drug–target complex, a QSS was reached between the binding of drug to free target, dissociation of the drug–target complex, and the elimination of the complex, as in the following:

$$K_{on} \times C_p \times R - (K_m + K_{off}) \times RC = 0 \tag{10.20}$$

K_{ss}, the steady-state constant, was derived as $(K_m + K_{off})/K_{on}$, which also equals $K_D + K_m/K_{on}$. With Eqs. (10.17)–(10.19) to describe the system, only Eq. (10.19) need to be revised based on the condition of QSS:

$$C_p = ((C_{tot} - R_{tot} - K_{ss}) + \sqrt{((C_{tot} - R_{tot} - K_{ss})^2 + 4 \times K_{ss} \times C_{tot}}))/2 \tag{10.21}$$

The QE approximation could be considered a particular case of QSS approximation when the elimination rate constant K_m is much smaller than the dissociation rate constant K_{off}; however, an important difference was also pointed out that K_D, the dissociation constant that measures the affinity between the drug and the target, can be obtained from *in vitro* experiments, but K_{ss} can be available only from *in vivo* measurements.

Probably the simplest model to describe the nonlinearity observed in the PKs of biologics is the Michaelis–Menten (M-M) model in which the drug is eliminated from the system by a saturable process described by the M-M equation or combined with the elimination with the first-order rate constant (K_{el}):

$$dC/dt = In(t) - (V_{max}/V_c) \times C_p/(K_m + C_p) - K_{el} \times C_p - K_{pt} \times C_p + K_{tp} \times A_t/V_c \quad (10.22)$$

where V_{max} represents the maximum velocity of drug disposition, such as metabolism or elimination, K_m denotes the concentration at which the velocity is half its maximum value. In the situations where only free or total drug concentration is measured from preclinical and/or clinical studies, the M-M model has been frequently applied to deal with the nonlinearity observed in the PK data. At QE, $RC = R_{tot} \times C_p/(K_D + C_p)$, which can be derived from Eq. (10.3); then Eq. (10.17), which was derived based on the condition of QE assumed in the QE model, can be expressed as follows:

$$dC_{tot}/dt = In(t) - K_{el} \times C_p - K_m \times R_{tot} \times C_p/(K_D + C_p) - K_{pt} \times C_p + K_{tp} \times A_t/V_c \quad (10.23)$$

If $RC \ll C_p$, then C_{tot} can be replaced by C_p and

$$dC_p/dt = In(t) - (K_m \times R_{tot} \times C_p)/(K_D + C_p) - (K_{el} + K_{pt}) \times C_p + K_{tp} \times A_t/V_c \quad (10.24)$$

With $K_m \times R_{tot} \times V_c = V_{max}$ and $K_D = K_m$, Eq. (10.24) is identical to Eq. (10.22). When R_{tot} is time-varying ($K_m \neq K_{deg}$), in addition to Eq. (10.24), Eq. (10.18) is also needed, with $(C_{tot} - C_p)$ replaced by $R_{tot} \times C_p/(K_D + C_p)$. In summary, PKs of mAbs and other therapeutic proteins can be described by the TMDD model. Because of the poor identifiability of full TMDD model parameters, given the typical nonclinical and/or clinical data, appropriate TMDD approximations including QE, QSS, and M-M models can be selected based on the available data and biological considerations. Numerically, the model yielding parameter estimates with acceptable precision, lowest AIC (Akaike's Information Criterion) values, and goodness of fit should be selected through the order of complexity of the models: MM < QE < QSS < full TMDD. Furthermore, the dose-dependent bias observed from the goodness-of-fit plots stratified by dose may indicate that a more complex model is needed to fit the data. Biologically, according to the properties of the drug and the target (soluble vs membrane), the QE or QSS approximation can be expected to hold for soluble low-MW targets with the availability of the concentration of drug, target, or drug–target complex; the MM model will be expected to hold for a membrane target with fast target turnover when only free drug concentration is available.

There are numerous practical examples from the open literature illustrating the use of PK–PD models and simulation in the development of biopharmaceuticals. Luu *et al.* [122] published a typical PK model-based approach to predict

human PKs of a fully human IgG_2 mAb exhibiting TMDD from cynomolgus monkeys. The full TMDD model, which consists of Eq. (10.5) – Eq. (10.8), was used to model the pooled PK data from the single-dose study at 5 or 50 mg/kg and the multiple-dose study at 2, 10, or 50 mg/kg IV bolus dose on cynomolgus monkeys ($n = 14$). In their model fitting, K_{on} and K_{off} were fixed to values from *in vitro* experiment (surface plasmon resonance study); K_m was fixed to the value obtained from another *in vitro* study (fluorescence-activated cell sorting analysis), and K_{deg} was fixed based on half-life, as estimated from the human receptor protein expression data from the literature by assuming that the half-life of the monkey receptor is the same as that of human. The full TMDD model with some fixed parameters reasonably described the single- and multiple-dose PK profiles of the mAb in monkeys. In order to predict human PK profiles based on the model developed from monkey PK data, a body-weight-dependent allometric scaling was applied to the parameters K_{el}, K_{pt}, and K_{tp}; the human values of some mechanistic parameters, K_{on}, K_{off}, and K_m, were also experimentally measured and K_{deg} and amount of receptor were assumed to be same between monkey and human. With the aforementioned model and strategies, the human PK of the mAb was successfully predicted by comparing with actual human PK data from the ongoing Phase I study. As emphasized from the example of TGN1412, predicting the PK of therapeutic proteins in human from preclinical data would be crucial for informing the starting dose and design of first-in-human study. The PK model-based approach, which is based on preclinical *in vivo* PK and *in vitro* cellular data, has been shown to be a reliable and valuable approach for predicting the human PKs of the therapeutic proteins exhibiting TMDD from preclinical data.

Clinically, Ng *et al.* [123] developed a full TMDD PK/PD model to investigate the nature of nonlinear PKs of volociximab, a chimeric IgG4 mAb as an anti-angiogenic $\alpha_5\beta_1$ integrin inhibitor, and to help understand the interactions with $\alpha_5\beta_1$ integrin in cancer patients in the Phase I study. The full TMDD model described in their paper was defined by Eqs. (10.7), (10.8), (10.12), and (10.14) since they parameterized the full TMDD model with clearance instead of first-order rate constant. Two additional modifications they made to the full TMDD model are (i) the addition of a negative feedback loop on the synthesis of receptors, mainly due to the decreased objective function, and (ii) holding the total receptor concentration constant by assuming $K_m = K_{deg}$ because of limited PK/PD data used in the analysis. The model was developed based on the PK data from 21 subjects receiving five 1-h IV infusions at the dose of 0.5, 1, 2.5, 5, 10, and 15 mg/kg. The final model reasonably described the observed serum volociximab concentration and $\alpha_5\beta_1$ integrin binding over time with acceptable precision on the parameter estimates. In order to examine the potential role of the percentage of free monocyte $\alpha_5\beta_1$ integrin levels as a biomarker for subsequent clinical studies, the final model was then used to simulate the percent-free monocyte $\alpha_5\beta_1$ integrin response for various volociximab dosing regimens. The simulations indicated that volociximab doses of >5 mg/kg every 2 weeks were necessary to saturate the monocyte $\alpha_5\beta_1$ integrin sites in a majority of subjects

and that 10 mg/kg or higher dose was necessary to saturate > 95% receptors in most patients (> 93%) from further analysis. The M&S based on the full TMDD model with minor modifications has provided valuable information for examining the nature of nonlinear PKs and the utility of percent-free monocyte $\alpha_5\beta_1$ integrin as a biomarker in the clinical development of volociximab. The PK–PD model-based approach was again successfully incorporated into the development of HAE1 [124], a second-generation, fully humanized mAb that binds to IgE, from preclinical studies to the clinical program. An equivalent QE TMDD model was adopted from the modeling work for the first-generation molecule omalizumab [76]. The model was defined by Eq. (10.17) without tissue compartment ($-K_{pt} \times C_p + K_{tp} \times A_t/V_c$) and Eq. (10.18) with a different set of system variables, namely the measured C_{tot}, measured R_{tot}, C_p, and measured R and RC. With the relationship $(C_{tot} - RC)(R_{tot} - RC)/RC$ defined in the expression of K_D, the equation for RC was defined as

$$RC = ((C_{tot} + R_{tot} + K_D) - \sqrt{((C_{tot} + R_{tot} + K_D)^2 - 4 \times R_{tot} \times C_{tot})})/2 \quad (10.25)$$

Besides Eq. (10.19), K_D was also dependent on the relative concentrations of C_{tot} and R_{tot} because of the complex (RC) in the form of two trimers and a hexamer. The above omalizumab model was used to simulate HAE1 PK/PD profiles to select the Phase I dose and was refined using the Phase I data to provide study design and decision-making support for the Phase II study. In addition to the application of PK–PD M&S in the separated developmental phase, M&S also played a significant role in the above case study through the complete developmental program in supporting acceleration of the development through the cycle of learning and confirmation. A fully human monoclonal IgG1 antibody, which prevents PCSK9 (a soluble protein that binds and regulates low-density lipoprotein receptor (LDLR) activity by binding to hepatic LDLR and thereby reduces low-density lipoprotein cholesterol (LDLc) levels) was under Phase II clinical development [125]. In order to guide Phase II dose and dose regimen selection to provide significant LDLc reduction while minimizing the frequency of dosing, a one-compartment PK model with first-order absorption and combined linear and saturable elimination from the central compartment was defined by Eq. (10.22) without last two terms ($-K_{pt} \times C_p + K_{tp} \times A_t/V_c$). The development of the PK model was based on the data from 80 subjects from both single-dose cohorts who received 10, 40, 150, 300, 600, or 800 mg and multiple-dose cohorts who received either 40 or 150 mg once weekly for 4 weeks. Because only drug concentrations were measured using a validated target-binding enzyme-linked immunosorbent assay (ELISA), the M-M model instead of the full TMDD or QE model was selected to account for the observed nonlinearity in the PK data at low dose levels. After successfully fitting the PK and PD model to the Phase I PK and LDLc data with a sequential modeling approach, a model-based simulation using the final PK/PD model was conducted to support Phase II dose selection to provide the quantitative understanding for the Phase II doses in coronary heart disease patients.

In summary, PK–PD M&S is a powerful tool to enable scientific-rationale-based decisions as well as to maximize the efficiency during drug development.

M&S should preferably start from the preclinical stage, so that the gained knowledge can be carried forward to the next clinical stages in a rational manner. By applying the developed models, the design and power of (pre)clinical trials can be optimized with simulations, minimizing the risk of uninformative trial results and misunderstanding of drug behavior.

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11
Safety Considerations for Biologics

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11.1
Introduction

The goal for a successful drug therapeutic is to modulate a specific biological target implicated in a disease process. However, as is often the case, small-molecular-weight chemicals can interact with other targets in addition to the intended therapeutic target, producing adverse effects referred to as *off-target* or *chemically related* toxicity. This off-target toxicity is often the reason behind the termination of drug candidates, forcing medicinal chemists to strive for that more "selective compound." Technological achievements in recombinant DNA technology together with improved cell culture techniques in the 1970s allowed for not only cloning of the genes responsible for endogenous proteins (and hormones) but also for producing sufficient quantities of these for therapeutic use. In addition, Kohler and Milstein's hybridoma technology produced monoclonal as opposed to polyclonal antibodies [1]. These new technologies provided potential drug candidates with much improved target selectivity; however both their manufacturing process and their complex biological characteristics differ from traditional small-molecular-weight chemicals.

Human recombinant proteins such as insulin, erythropoietin (EPO), cytokines (e.g., interferons and interleukins), and growth hormone formed the first generation of these novel molecules and were developed primarily for hormone replacement therapy or as cancer treatment agents. Insulin (Humulin) in 1982 was the first to be approved and was followed in the next 8–9 years by Interferon α (Roferon A and Intron A), human growth hormone (Humatrope and Protropin), Intertleukin-2 (Proleukin), Erythropoietin (Epogen and Procrit), Interferon γ (Actimmune), and G-CSF and granulocyte/macrophage colony stimulating factor

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(GM-CSF) (Neupogen, Leukine and Prokine) [2–5]. Many of these biologics remain important drug therapeutics today.

Murine monoclonal antibodies (mAbs) comprised the other important group of biologics at this time. The first therapeutic murine mAb licensed in 1986 using this technology, muromonab (OKT3), depleted peripheral T lymphocytes after binding to CD3 and was licensed for acute organ transplant rejection [6–8]. Unlike the recombinant human proteins, these first-generation therapeutic mAbs were not as successful. Immunogenicity following repeated dosing and the weak interaction between murine antibodies with human complement and human Fc receptors significantly limited their therapeutic potential [9, 10]. Technological advances in genetic engineering allowed for the replacement of the murine constant regions with human sequences ("humanized") resulting in mAbs that contained 50–90% human sequences [3]. The immunogenicity was sufficiently decreased compared to murine mAbs and many of these mAbs were licensed for therapeutic use. These "humanized" mAbs were a step closer to the fully human mAbs which were later produced using either phage display technology or from immunization of transgenic mice expressing human antibody genes [9].

This chapter discusses the differences between the "traditional" small-molecular-weight compounds and these "novel" large-molecular-weight compounds and the impact of these differences on nonclinical safety assessment strategy. From a molecular modality perspective, the scope of this chapter includes human recombinant proteins, monoclonal antibodies, fusion proteins, bispecific antibodies, and antibody drug conjugates. Stem cell and gene therapy, vaccines, and synthesized proteins and oligonucleotides (e.g., siRNA, aptamers) are considered out of scope of this chapter and are therefore not discussed.

11.2 Small Molecules versus Large Molecules – A Comparison

Chemically synthesized molecules typically have a MW of about 500 Da, exhibit well-defined physicochemical properties, and can be metabolized, whereas biologics are large, structurally and biologically complex molecules, and are catabolized (Table 11.1). These differences in physicochemical properties and method of elimination are what primarily drive the alternative strategy required to evaluate the nonclinical safety of biologics.

One similarity, however, is that biologics can act as agonists as well antagonists. In general, human recombinant proteins stimulate biological activity following binding to their receptor. Monoclonal antibodies, on the other hand, achieve their pharmacodynamic (PD) effects through a variety of mechanisms [11, 12]. A mAb can act as a targeting agent or as a vehicle for delivery of a payload to a particular cell (e.g., immunotoxin, Ricin A, or a cytotoxic drug). By binding to a cell surface receptor or a soluble factor, a mAb can block an interaction (antagonism). Infliximab (anti-TNFα receptor agent) and Mepolizumab (anti-IL5 soluble factor) are both examples of this mechanism of action [12, 13]. Other mAbs such as

Table 11.1 Comparison between small molecules and biologics.

Characteristic	Small molecule	Biologics
Molecular weight	<500	30 000 – 150 000
Potency (both can be potent)	pM – nM (agonist/antagonist)	pM – nM (agonists/antagonists)
Structure	Well-defined physiochemical properties	Complex physiochemical properties (e.g., tertiary structure, glycosylation, aggregates)
Volume of distribution	Wide range, ≤1000 l/kg	70 ml/kg, 2× plasma
Bioavailability	0 – 100% can be variable	0% via oral, 30 – 90% via sub-cut
PK time frame	Tend to have shorter half-lives (often <24 h)	mAbs tend to have longer half-lives (i.e., > 1 – 2 weeks)
Type of toxicity	Dose-limiting, nonspecific, off-target toxicity	Exaggerated pharmacology (nonspecific protein effects (e.g., anaphylaxis) can occur)
Dose response	Tends to be sigmoid or linear More gets more (i.e. higher dose, greater effect)	Can be nonlinear, bell-shaped More gets longer (i.e. higher dose, longer but not greater effect)
Elimination	Metabolized (metabolites can be more or less toxic than parent and can be DNA reactive)	Catabolized
DNA interaction	Compound and/or its metabolite can be DNA reactive	Do not interact directly with DNA

muromonab can directly stimulate intracellular signal transduction subsequent to binding to a cell receptor. Target cell lysis can also occur following binding to a cell surface receptor through recruitment of an immune response which is mediated by the Fc region. In this situation, the lysis is caused by either an effector cell (e.g., natural killer (NK) cells in the case of antibody-dependent cell-mediated cytotoxicity (ADCC)) or through direct activation of the complement system known as complement-dependent cytotoxicity (CDC)). Alemtuzumab (anti-CD52 mAb) is an example of this mechanism [12]. Therefore, the format of the mAb, in particular, the Fc is an important characteristic to consider during mAb format design and should be decided during early target identification. The implications of the interactions of the Fc region of a mAb with Fcγ and complement receptors and how that can affect the nonclinical safety assessment program is discussed in greater detail below.

Biologics tend to exhibit a low volume of distribution primarily as a consequence of their size, which results in minimal diffusion across vascular endothelium [14, 15]. Binding to plasma or tissue targets can also impact mAb distribution [16]. On the other hand, the chemical properties of small-molecular-weight compounds can allow them to transit through the cell and/or nuclear membrane either by passive diffusion or by active transport in order to bind to their target.

Small-molecular-weight compounds can therefore distribute widely throughout the body. The downside of this attribute is that these molecular compounds can interact with a much larger number of normal cells and/or bind to nontarget cells and produce off-target toxicity [17]. As discussed above, off-target toxicity with small-molecular-weight compounds is often the cause for drug attrition.

Small-molecular-weight compounds can be metabolized, sometimes extensively. Metabolites can be biologically inactive or active or can exhibit greater or reduced toxicity. In addition, metabolism of a small-molecular-weight compound can result in the formation of reactive metabolites, that is, metabolites that can covalently bind to macromolecules resulting in toxicity such as cell death (e.g., hepatocyte necrosis), immunotoxicity, and potential carcinogenicity [17]. Therefore, small-molecular-weight compounds can cause toxicity due to on-target effects (i.e., exaggerated pharmacology), off-target toxicity, and toxicity due to reactive metabolites. Because of these attributes, a nonclinical safety evaluation program is required to assess toxicity in two species, rodent and nonrodent, irrespective of whether the test compound is pharmacologically active or not in that species.

11.3 Toxicity Related to Exaggerated Pharmacology – Importance of Species Selection

In contrast to small-molecular-weight compounds, biologics are exquisitely selective for their intended target and therefore, the majority of toxicity associated with biologics is due to exaggerated pharmacology and examples of off-target toxicity are relatively rare. One of the early misconceptions about human recombinant proteins, however, was that as they were "natural" and "endogenous", they were expected to be safe, that is, if it is pharmacology, it cannot be adverse. However, the dose-limiting toxicity observed in a Phase I clinical trial with Interferon α, characterized by fever, fatigue, loss of appetite, nausea, and chills quickly dispelled this assumption. In addition, this was in sharp contrast to the relatively minor toxicity observed in the rodent toxicity study. This example underpinned the absolute requirement of assessing toxicity in a pharmacologically relevant species and an in-depth understanding of the pharmacological properties of biologics [18, 19]. Studies in non-pharmacologically relevant species can be dangerously misleading as exemplified by the TGN1412 disastrous Phase I clinical trial [20].

Demonstration of target expression and binding and resultant functional activity should all be confirmed in the test species. Additionally, knowledge of target expression and distribution in normal and diseased tissues as well as potential downstream effects should be similar between animal species and humans. In addition, consideration for how the drug candidate is intended to be delivered to patients and how that might differ from the *in vivo* endogenous situation should be known. An example of this is IL-2, a cytokine originally thought to act as a growth factor for T cells, which was later shown to activate other immune cells such as NK cells, monocytes, neutrophils, and macrophages [21–23]. IL-2 was evaluated as

a potential anticancer agent; however, normally IL-2 is released locally in the body, whereas intravenous administration was used in the early clinical trials, resulting in systemic exposure. Dose-limiting toxicities such as vascular leak syndrome and pulmonary edema was observed and believed to result from immune cells (such as NK cells) activated by cytokines rather than by IL-2 itself [3, 24, 25]. In an attempt to abrogate the toxicity of IL-2, alternative dosing schedules and routes of administration were investigated clinically. Decreased toxicity observed following subcutaneous administration was thought to result from a shift in IL-2 distribution from blood to the lymphatics where a greater percentage of IL-2 responsive cells such as T lymphocytes responsible for its efficacy resided and away from the cell types such as the NK cells thought to be responsible for its toxicity [23]. This example illustrates the importance of not only understanding the pharmacological activity of the drug candidate but also understanding the potential downstream effects, how the drug is delivered to the patients, and how that might affect the toxicity. Therefore, species selection is not merely a matter of whether the test compound binds to and activates or blocks the target in the test species.

Although data from *in vitro* and/or *in vivo* studies indicate that similar pharmacology exists between an animal species and humans, the question is what is the quantum of data required. For example, the physiological role of interleukin 7 (IL-7) has evolved over the last 10 years [26]. IL-7 is now known to be involved in both B- and T-lymphocyte development and homeostasis [26, 27]. Although there is a high degree of homology between mouse and human IL-7, studies indicate that IL-7 is critical for differentiation of immature B and T lymphocytes in the mouse, and although it can modulate B-cell maturation in human cells, it does not play the same critical role in humans [27]. Therefore, although the mouse is technically a pharmacologically relevant species, data from toxicity studies will need to be interpreted taking into account the species differences. However, in this case, using the animal species is unlikely to miss any potentially important safety data relevant to the clinic.

A different outcome occurred in 2006 when six volunteers suffered life-threatening adverse effects following administration of TGN1412 (an anti-CD28 monoclonal antibody) in Phase I trial. TGN1412, an agonistic mAb directed at CD28 was postulated to be able to activate T cells resulting in T-cell proliferation without prior interaction of the T-cell receptor complex [28, 29]. *In vitro* assays using peripheral blood mononuclear cells from leukemic patients showed, among other effects, enhanced proliferation of T cells, and TGN1412-activated T cells demonstrated enhanced cytotoxic T lymphocyte (CTL) activity against B-CLL (chronic lymphocytic leukemia) cells [30]. Since TGN1412 did not cross-react with rodent CD28, an anti-rat CD28 antibody (JJ316) was evaluated in rats and showed efficacy in experimental autoimmune encephalomyelitis (EAE) and adjuvant arthritis models. In mechanistic studies, administration of JJ316 resulted in a rapid redistribution of CD4 T cells into the lymph nodes and spleen as evidenced by splenomegaly and lymphadenopathy [28, 31]. It was postulated that tissue cross-reactivity (TCR) engagement was not required for the proliferation of all T-cell subsets because TGN1412 could, by binding to

CD28, directly stimulate T cells [28]. In contrast, in humanized mice [32], an anti-CD28 mAb rapidly depleted peripheral T cells. Efficacy was also shown in a rhesus monkey collagen-induced arthritis (CIA) disease model [33].

The pivotal toxicity study used to support the clinical trial was in cynomolgus monkeys at doses up to and including 50 mg/kg/week. TGN1412 was well tolerated and the high dose of 50 mg/kg was considered to be the no-observed-adverse-effect-event level (NOAEL) and was used to select the clinical starting dose. A 10-fold increase in IL-6 and T cells and CD25/CD69 expression was observed at the lower dose of 5 mg/kg and it was suggested that all CD28 receptors were saturated at the highest dose of 50 mg/kg [33].

Therefore, the nonclinical studies produced very different results by species, in particular there was the lack of obvious effects in the monkeys which were selected as the species that was used to evaluate TGN1412 safety and to set the first dose level for the clinical study. These differences including the lack of *in vitro* potency data using human cells should have been sufficient to question which, if any, species was truly relevant for humans [31, 32].

Shortly following an intravenous dose of 0.1 mg/kg TGN1412, all six volunteers suffered from a massive cytokine storm caused by proinflammatory cytokines which was associated with symptoms such as headache, myalgia, nausea, and hypotension [20]. The volunteers' condition continued to worsen within 12 – 16 h and patients were diagnosed with pulmonary infiltrates and lung injury, renal failure, and disseminated intravascular coagulation. Fortunately, all TGN1412-treated volunteers survived. Although the initial phase of the response was consistent with a cytokine storm seen with other therapeutic mAbs (e.g., OKT3), the latter phases differed and were characterized by early acute lung injury, diffuse erythema with late desquamation, neurologic sequelae, and post-illness myalgias [20]. The first wave (cytokine storm) was likely due to T-cell activation, in particular effector memory T cells, whereas the second wave resulted from by an expansion of $CD4^+$ $CD25^+$ $FoxP3^+$ regulatory T cells (Tregs), that is, as seen in rodents, the beneficial effects of anti-CD28 therapy selective for Tregs [28, 31].

A number of possible explanations for the unexpected toxicity were considered and included a post hoc review of the drug product as well as the nonclinical data. No issues were found with the drug product such as LPS contamination or high levels of aggregation of the mAb and the healthy volunteers were administered the planned dose level. It is now believed that the nonhuman primate (NHP) (cynomolgus monkey) was not a relevant species even though TGN1412 could bind to cynomolgus monkey CD28. Results from polychromatic flow cytometry and intracellular cytokine staining *in vitro* assays showed that activated $CD4^+$ effector memory T cells likely caused the cytokine storm in humans. CD28, however, is not expressed on cynomolgus monkey $CD4^+$ effector memory T cells and is the likely explanation for the lack of responsiveness in this species [29].

Although a number of the potential targets are well conserved, some biologics exhibit human selectivity and no relevant animals models exist. Alternative models such as transgenic animals and homologous systems can be considered and are discussed in Section 5.1.4. While selection of a relevant species and/or model is critical to the scientific quality of the nonclinical data generated, there may be situations in which no *in vivo* models are available. One option could be to use a combination of *in vitro* data and *in vivo* pharmacokinetic (PK) from a nonrelevant species as relevant toxicity data can only be generated in pharmacologically relevant species. However, as stated in ICH S6(R1), there may be situations in which *in vivo* testing in a pharmacologically nonreactive species may have to suffice [34].

11.4
Toxicity Unrelated to Exaggerated Pharmacology

Adverse effects can be caused by biologics which are not due to exaggerated pharmacology but are related to the physiochemical attributes of biologics. Infusion reactions, for example, are a common adverse event in nonclinical studies and clinical trials. Similarly, redness, swelling, and pain can be observed following subcutaneous administration of a biologic and are thought to be a related normal reaction of the skin to a foreign protein [35]. Another potential reaction to a foreign protein is a hypersensitivity reaction which can occur following a single or repeat dosing [36]. Symptoms due to anaphylactoid responses include urticaria, angioedema, bronchospasm, and hypotension [35]. There are a number of factors that influence the probability of these reactions and include presence of preexisting antibodies, aggregation, and/or impurities in the drug formulation [36].

11.4.1
Cytokine Storm

OKT3, a wholly murine mAb, does not cross-react with any CD3 in any traditional laboratory species (only with chimpanzee and gorilla CD3), therefore, not surprisingly, no toxicity was observed in rodent and rhesus monkey toxicity studies. Patients, however, developed flu-like symptoms (pyrexia, chills, chest pain, wheezing, and nausea) within 1–6 h after the first dose. In addition, pulmonary edema including death secondary to the respiratory issues was observed in a small number of patients [37]. These symptoms were attenuated following repeat dosing. Investigation using a hamster mAb directed against the CD3 complex of murine T lymphocytes confirmed that the flu-like symptoms were associated with a release of a number of cytokines including IFN-γ, TNF-α, IL-2, IL-3, IL-4, IL-6, and GM-CSF. The cytokine release syndrome could be prevented by

high doses of methylprednisolone [38]. Subsequent investigations revealed that cross-linking of the mAb by binding to the Fc receptor (FcR) led to the activation of T cells *in vivo* resulting in cytokine release [39]. These adverse effects and the immunogenicity of OKT3 would clearly limit the usefulness of an anti-CD3 therapy for other potential indications; therefore, humanization (to decrease immunogenicity of OKT3) and modification of the Fc, in which the leucines at positions 234 and 235 were replaced with alanines, were carried out [39–41]. The new mAb (hOKT3γ1[Ala-Ala]) unable to bind FcR demonstrated a 3 log-fold decrease in IL-6 and TNFα release *in vitro.* Studies in patients corroborated the *in vitro* findings in that the clinical symptoms of the cytokine release were milder and of shorter duration [39]. There were other differences, however; administration of hOKT3γ1[Ala-Ala] resulted in an increased production of IL-10 and IL-5, whereas other nonbinding mAbs such as HuM291 and OKT3 produced increased IFN-γ [39, 42].

11.4.2 Unexpected Toxicity

At first glance, unexpected toxicity may be regarded as off-target toxicity; however, when the results of the toxicity study are fully assessed what was initially thought to be off target is actually exaggerated pharmacology. Understanding of the full biology of a novel target can be difficult. True off-target toxicity for biologics is relatively rare; however, the number of examples may be underestimated as adverse findings from terminated drug candidates may never be published externally. One published example of potentially off-target toxicity is the apparent testicular, vascular neoplasias and brain lesions observed following 6 months dosing of an anti-IL-13 mAb in rats but not monkeys [43]. A number of lines of investigation were followed, including *ex vivo* TCR, CNS, and/or testes distribution of the biologic or preexisting viral infection; however, no plausible explanation was determined [43].

Hematotoxicity not associated with exaggerated pharmacology typically identified as cytopenias has been noted in both nonclinical toxicity studies and in clinical trials with a number of biologics including both recombinant proteins and mAbs [44]. In general, most of these cases are immune-mediated, related to a specific species and are of low frequency [44]. Although different cell types are affected, thrombocytopenia represents the majority of the cases of hematotoxicity and may be due to a combination of the high number of platelets circulating (200 000–600 000/μL blood) and the large number of FcγRIIa receptors on the plasma membrane [44]. Immune-mediated mechanisms such as ADA-related hypersensitivity which often occur after multiple dosing also account for cases of anemia. Other proposed mechanisms include direct cellular activation, cytotoxicity, and side effects of cytokine and/or complement release [44]. Data from nonclinical toxicity studies have not been found to be particularly predictive for the clinical situation although the number of cases of hematotoxicity in nonclinical studies may be underestimated if the data

resulted in termination of the development of that biologic prior to human exposure [3, 44].

11.5 Regulatory Guidance

ICH S6(R1) is the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) guideline that provides the major source of guidance for biologics. The original guideline was drafted and approved in 1997 [34]. Given their physiochemical and biologically complex characteristics, it was acknowledged that biologics required a different approach than small molecules. ICH S6 has always promoted a case-by-case science-based strategy as the primary toxicity of biologics is exaggerated pharmacology and therefore many of the traditional studies conducted for small molecules are inappropriate for biologics. Similar to other guidelines, ICH S6 is reviewed at specific intervals and because of differences in interpretation from various health authorities, ICH S6 was amended in 2011 [34]. The purpose of the amendment was to clarify a number of areas including species selection, study design, carcinogenicity, immunogenicity, and reproductive toxicity. Throughout the document, the importance of species selection is emphasized as is also an understanding of the limitations of animal studies. For example, for immunomodulators, whose mechanism of action suggests a potential risk but taking into account the limitation of animal models, ICH S6(R1) recommends postmarketing surveillance unless there is a nonclinical study that could mitigate the concern. The guidance included in ICH S6(R1) and how that impacts safety assessment programs is discussed in greater detail in the following [34].

Although ICH S6(R1) provides the bulk of guidance for most of the biologics, ICH S9, the primary guideline for the development of anticancer agents in patients with advanced, late-stage cancer, covers both small molecules as well as biologics [45]. ICH M3(R2) is a multidiscipline guideline and although it mentions biologics, it does recognize that the study types and designs will differ from small-molecular-weight compounds and therefore only recommends the timing for the conduct of nonclinical safety studies [46]. Other guidelines in which biologics are mentioned briefly and could potentially serve as specific functional advice include ICHS 7A (Safety Pharmacology), ICH S5(R1) (Reproductive toxicity information), ICH S1A (need for carcinogenicity studies as stated in ICH S6(R1)), and ICH S3A (toxicokinetics (TK)) [47–50]. Similar to the safety guidelines, there are several quality guidelines that are specific for biologics and these include ICH Q5A-5E [51–55]. The areas that these guidelines include are viral safety, analysis of expression construct of cells, characterization of cell substrates, stability, and comparability. In addition, ICH Q6B covers specific testing and acceptance criteria for manufacturing [56]. A relatively new guideline ICH Q11 covers development and manufacturing of drug substances for both biologics and small molecules [57]. There appears to be a trend with the more recent guidelines

to discuss both biologics and small molecular compounds, that is, ICH S9 and ICH Q11.

11.6 Development Considerations Due to Biological Characteristics

11.6.1 Early Discovery – Information Needed to Set the Stage and Early Studies

Nonclinical development of biologics is greatly enhanced when there is close interaction between the biology, safety assessment, PK, and biopharmaceutical development experts.

11.6.1.1 New Targets/Pathways

As already discussed above, the toxicities observed with biologics, distinct from new chemical entities (NCEs), tend to be a result of exaggerated pharmacology which can usually be predicted from the mechanism of action. Therefore, even at the target identification and selection stage of new biologics, there is great value in considering potential safety liabilities. A brief review of the available target data from a safety perspective should encompass literature data around the biology of the target, reported mRNA and protein expression profiles to understand target distribution; paying particular attention to normal tissue expression, gene and pathway information, and, in particular, to understanding how the target functions in normal and diseased individuals. Data from genetically modified animals (transgenic or knockout) for the target, disease models and human mutations/polymorphisms; data with competitor information on molecules designed to affect similar pathways; sequence analysis; and data on conservation of function between species can often identify key liabilities and enable the provision of advice on and interpretation of safety-related aspects and provide useful contributions to discussions around target selection or deselection (Table 11.2). This early interaction also provides the opportunity to identify where it may be appropriate to include investigation of safety endpoints in biology, pharmacology, and/or PK studies conducted with tool molecules or potential leads.

A more complete target safety review can be performed prior to candidate selection. This very detailed analysis using the same criteria as conducted prior to commit to discovery, while fairly resource intensive and typically taking a safety scientist several weeks to complete, will highlight all potential liabilities from the literature and any internal target validation work performed. This information, updated as a living document throughout the nonclinical phase of development, can provide a record of the evidence for consideration of potential liabilities and how they will be further investigated.

Suggestions for investigation and mitigation of potential issues are useful even at this stage while the development plan is being generated. Sharing the data with

Table 11.2 Key considerations for an early safety prediction evaluation.

Biology of the target/molecule	Target expression profiles	Gene and pathway information	Data from genetically modified animals	Disease models/human mutations, polymorphisms	Competitor information	Immunogenicity	Sequence analysis and conservation of function
Agonism or antagonism of the target	Differences in expression profiles in humans and preclinical species	Differences and similarities between human and preclinical species	Knockout, transgenic pheno-types	Physiological manifestation of alteration of the target	Class-dependent toxicities	*In silico* prediction	Differences and similarities of function between human and preclinical species
Target behavior including internalization and turnover	Differences in expression profiles in normal and diseased tissue	Upstream modulators				Consider impact of ADAs, including immune complexes, cross-reactivity to endogenous target, exacerbation of disease	Protein and mRNA alignment scores between species
Desired receptor occupancy	Distribution of the target within specific cell types	Downstream ligands or receptors affected					
Desired half-life. Consider impact of soluble target on PK	Consider access across barriers, for example, blood–brain barrier (BBB), blood–testis barrier						
Binding affinities to target and FcR in different species Effector function activity, also consider species differences							

all scientists involved ensures key information around potential safety liabilities can help guide the molecule strategy including platform (e.g., mAb, long acting, Fc disabled), screening for appropriate nonclinical species, for example, potential for developmental toxicity issues may require a rodent or rabbit cross-reactive molecule or early identification of the need for a surrogate molecule, and the early medicine vision. This work can facilitate mitigation of potential liabilities or contribute to a decision for early attrition. Various approaches to discharging significant development risks very early in development can be employed, including seeking expert advice from internal and external experts, inclusion of additional endpoints in PK/PD, and/or conduct of early toxicity studies. Depending on the target, these additional investigations could include body weight measurements, clinical pathology analysis, and histopathology investigations. Where additional safety endpoints are included in these studies, it is often beneficial to have control animals with which to compare.

In the lead optimization to the candidate-selection phase of biologic development, the majority of activities are related to selection of the best molecule. A number of key factors are considered, which include potency and affinity for the human target, the desired elimination and PK profile and whether effector function is required or should be reduced/minimized, cross-reactivity with nonclinical species, and stability and production levels of the cell line. Initial PK data will be generated, typically after a single dose at two dose levels running for a period of up to 10 weeks to determine target-mediated clearance and half-life properties of the molecule (Figure 11.1).

11.6.1.2 Alternate Hit/Lead Discovery Approaches for Existing Targets

Lead discovery approaches for existing targets present their own challenges, not least the lack of global harmonization of the regulatory guidance. Biosimilars are for all intents and purposes the same as a biologic (generally approved or in late

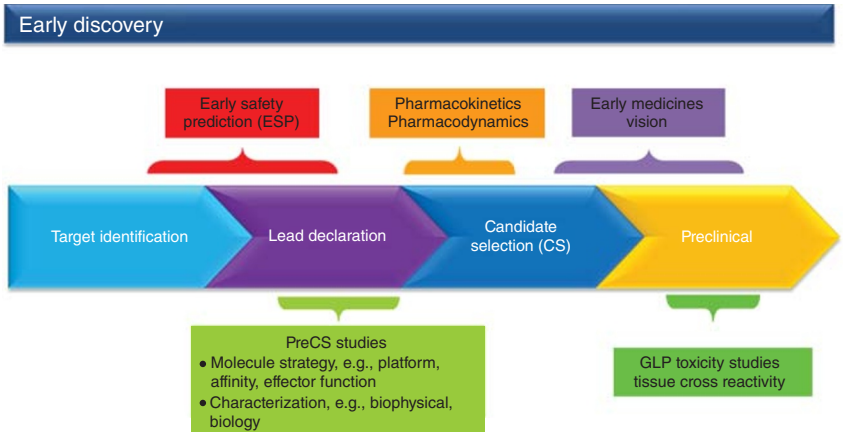

Figure 11.1 Key activities during early discovery of biologics.

development) and demonstrate no clinically relevant differences in safety, purity, and potency, but they are not identical to the original product. This is due to the inherent variability associated with manufacture and therefore they are not considered as "generics" from a regulatory perspective. However, the wealth of information available from the original molecule, known as the reference product, strongly encourages a risk-based, weight of evidence approach [58]. This approach lends itself to focusing strongly on the 3Rs (replacement, reduction, and refinement) when preparing the nonclinical safety development plan and conducting nonclinical studies. With a focus early in development on demonstrating comparability of the biosimilar with the reference product in terms of biophysical and biological characteristics and PK/PD, the nonclinical safety activities will concentrate on *in vitro* and *ex vivo* assays and may include as may be appropriate TCR to demonstrate binding to expected antigenic determinants and *in vitro* cytokine release. The requirement for additional *in vivo* nonclinical safety studies should be considered carefully in light of the available data and, if required, should leverage available data to significantly reduce the numbers of animals used and number of studies conducted [59].

Where a molecule is improved to increase functionality through enhanced affinity binding or the half-life is extended by introducing mutations in the Fc portion of a mAb, for example, the situation is different. These molecules, often referred to as biobetters, cannot be considered the same as the reference product and require much more extensive nonclinical assessment of safety, as is required for a new biological entity. Direct chemical modification of the insulin replacement AspB10 was shown to enhance the efficacy and PK profile. Replacement of its histidine B-10 with an aspartic acid resulted in the fast-acting insulin analog, AspB10 [60]. In addition to its increased binding to the insulin receptor (IR), AspB10 also exhibited a 7- to 10-fold higher binding affinity for the insulin growth factor 1 receptor (IGF-1R). However, when mammary tumors were observed in female rats following chronic administration of AspB10, its development was discontinued [61]. This example provides the cautionary tale that minor changes in molecular structure can lead to significant differences in biological activity and illustrates the importance of appropriate testing in order to understand the activity of a biologic. Rather than changes to the molecular structure, biological activity can also be affected through modification of the glycosylation sites of proteins. Nonetheless, the opportunity often still presents itself to conduct a more bespoke nonclinical safety package utilizing the available nonclinical and clinical data both from the reference product and from other molecules employing the same platform technology. For example, consider reducing the number of animals on the chronic study by reducing the number of dosing groups and the group sizes, and limiting the number of animals on recovery. If time permits, consideration can be given to going straight to the 6 months chronic duration study which is the pivotal nonclinical toxicity study used for registration of biologics for all indications apart from late-stage cancer, taking an interim readout and using data from early PK/PD studies to provide single dose and recovery data to support first in human (FIH) studies [62]. Should all data generated nonclinically and clinically display the

expected profile compared to the reference product and there is no known reproductive risk with the target, there may be justification to consider not conducting reproductive studies.

11.6.1.3 New Lead Optimization Methods

Reagent supply, assay development and validation (Good Laboratory Practice [GLP]) are critical activities that need to occur well in advance of the nonclinical toxicity studies. The work will generally be conducted at a specialized laboratory and may be outsourced. Establishing translational/biology interactions with representation from supporting lines with technical responsibilities, especially if this subteam is set up prior to candidate selection, enables careful consideration and early identification of the most relevant nonclinical Toxicokinetics (TK) (total and/or free or bound), anti-drug antibody and PD assays for use in the toxicity studies.

In addition, understanding the selectivity, binding affinity, and potency of the molecule against the human and nonclinical species target is critical to determine the potential relevance of findings in the toxicity studies to the clinic. A framework is provided in the ICH S6(R1) guidance around the regulatory expectations for species selection [34]. Comparison of sequence homology is a good starting point but on its own is not sufficient. A pharmacologically relevant species should also express the target to which the drug binds in a similar tissue distribution to humans, the target should have a similar role as in human, and the drug must also have a similar functional activity in that species. *In vitro* data providing binding affinity and confirmation of downstream activity, usually in cell-based assays, is therefore required to provide evidence of relevance of the toxicology species selected. The *in vitro* affinity and potency of the pharmacological effects should be similar, for example, within 10-fold in animals and human to enable robust assessment of potential toxicities in the toxicity studies. Lower binding affinity for the target or reduced pharmacological effect should be taken into account in the design of the toxicology studies, for example, by increasing the dosing frequency or highest dose level tested to ensure maximum receptor occupancy for the duration of the study (Table 11.3).

Expression of the target (mRNA and protein/IHC) will have been examined in the literature during compilation of the target safety profile; the data may be further enhanced by immunohistochemistry experiments conducted in-house using lead molecules. Target tissue distribution in the nonclinical species versus human may be identified in a pilot TCR study which includes human and may also include animal (when there is no previous class data) critical organs and tissues as well as those expected to express the target. Not all mAbs are suitable IHC reagents as the correct physiochemical characteristics must be present to result in the appropriate specificity and sensitivity in tissues *ex vivo* [63]. Furthermore, both the execution and interpretation of IHC present challenges that require significant expertise to avoid bias [64]. Although it is not a requirement of ICH S6(R1) to include animal tissues, differences in expression between the nonclinical species and humans may identify potential gaps in the data generated in the nonclinical toxicity

Table 11.3 Examples demonstrating variations in species specificity.

	Biology	Sequence homology	Species specificity	Affinity	Expression	Considerations
Bispecific mAb/ domain antibody (Ab)	No differences in species identified (literature review)	Cynomolgus monkey shared 96% and 93% identity at the amino acid level with human targets	No cross-reactivity demonstrated with rat, mouse, or rabbit for either target. Cross-reactivity demonstrated with cynomolgus monkey for both targets. Therapeutic mAb/dAb was less potent for both cynomolgus targets when compared with that for human targets	Binding affinity of human and cynomolgus monkey targets differed; human 0.151 and 0.167 compared with 0.0019 and 0.3156 in cyno	Soluble targets; limited expression to immune cells	Demonstrated drop-off in potency against cynomolgus targets was taken into consideration when selecting doses in the toxicology study; doses increased eightfold
mAb	Known differences in pharmacology of target in rodents; in the mouse, it stimulates only the target receptor, whereas in man it stimulates both the target and another receptor in the pathway	Cynomolgus monkey shared 96% identity at the amino acid level with human target	No cross-reactivity demonstrated with rat, mouse, or rabbit target. Cross-reactivity demonstrated with cynomolgus monkey target	Therapeutic mAb binding affinity for glycosylated human target and cynomolgus monkey target were similar; IC_{50} value for human glycosylated target was 0.036 µg/ml; for cynomolgus monkey glycosylated target, 0.03 µg/ml, respectively	Soluble target; limited expression to immune cells	Negates the utility of the rodent as it would not explore the human toxicity potential of neutralizing target with therapeutic mAb
Defucosylated Fc enhanced mAb	Conservation of function across species (literature review). Shed form of the target extracellular domain present in human and cyno serum	Human target has extracellular region of 54 a.a. that retains 91% homology with cynomolgus target compared to 62–64% homology in rodents	No cross-reactivity demonstrated with mouse target. Cross-reactivity demonstrated with cynomolgus monkey target	Target affinity in appropriate range compared to human (4-fold) – as ADCC activity binding to cyno and human Fc also compared and confirmed to be within appropriate range	Limited expression in normal tissues and cell types outside expected immune cell lineage	Cynomolgus monkey only relevant preclinical species. Doses and dosing regimen mimicked clinical study

studies and aid in the interpretation of the toxicity findings in the nonclinical toxicity studies. The data supporting choice of nonclinical species is generated during lead discovery and is generally part of the biological package of work conducted.

Although any species appropriate for use in nonclinical toxicity studies can be considered, the most frequently used species for nonclinical safety testing of biologics are rodents (rats and mice) and NHPs. The most typical NHP used is the cynomolgus monkey, largely because of the high degree of cross-reactivity noted with biologics in this species. They also have the advantage of a smaller size thus requirement for less compound, high degree of background data, and ease of manual handling; although the rhesus monkey may also be used.

Another area of consideration for monoclonal antibodies, when determining a relevant nonclinical species, is the effector activity ADCC, antibody-dependent cellular phagocytosis (ADCP) and/or complement fixation and IgG half-life, via FcRn binding, of the Fc domain. This is of particular importance when there is enhanced affinity for the Fc receptors to increase cytotoxicity activity, following modifications including amino acid mutations in the Fc portion of the mAb or glyco-modifications such as fucose depletion of the Fc-linked oligosaccharides. These modifications can enhance effector function in humans by increasing the binding activity to FcγRIIIa or C1q, or increase agonistic activity through optimal FcγRIIb binding. However it should be noted that Fc receptors and complement factors have different sequences and vary in their affinity for specific IgG subclasses between different species. For example, human IgG1 binds cynomolgus monkey FcRn with 2-fold higher affinity than human FcRn and binds both mouse and rat FcRn with 10-fold higher affinity than human FcRn. There are also differences between species in the repertoire of Fc receptors expressed on the various effector cells. A high level of intraspecies heterogeneity of immunoglobulins and their Fc receptors has also been reported [65–70]. Despite this, there is some evidence in the literature that NHPs represent the most relevant species for evaluating immune cell effector functions arising from Fc engagement of IgG1 antibodies with its receptors [71, 72]. It is important that for each type of Fc modification and antibody isotype, the choice of test system should be carefully considered. *In vitro* experiments using human and NHP cytotoxicity assays can provide useful comparative information regarding potency (e.g., increased ADCC or CDC activity) arising from the Fc modification. This approach can also be used for those antibodies with Fc regions modified to prevent complement activation or binding by Fc receptors.

Human FcRn, FcγRIA, FcγRIIA, FcγRIIB, FcγRIIIA and FcγRIIIB transgenic mouse strains that replicate the unique pattern of human gene expression have been generated [73]. Introduction of these transgenes into mouse strains deleted for their mouse counterparts has resulted in humanized FcγR animals which can potentially be useful as a nonclinical, *in vivo* platform for the evaluation of Fc-engineered human IgG or Fc-based therapeutics.

Other nonrodent species which may be considered include the rabbit, mini-pig, and dog, among others. It is generally believed that the dog is not suitable for nonclinical safety testing of biologics because of the tendency toward type III hypersensitivity reactions, for example, developing anaphylactic-like reactions following exposure to human serum albumin leading to death [74, 75]. In recent years the mini-pig has become a more popular choice of nonrodent species, replacing the dog for toxicity testing of NCEs and can also be considered when testing biologics [76]. However it should be noted that unlike humans and NHPs transfer of immunoglobulins and other large molecules do not cross the porcine placenta therefore there is an assumed lack of embryofetal exposure making the mini-pig unsuitable for developmental toxicity testing of biologics [77].

Some biologics, particularly those for use in oncology and infection, may be directed against targets that are either not expressed at all or expressed at very low levels in healthy animals. Demonstrating relevance of a species where the target is expressed at very low levels can be challenging and may include complex *in vitro* and *ex vivo* assays to demonstrate target engagement and/or pharmacology. Efforts to induce the level of target *ex vivo*, for example, stimulation of the cell type expressing the target, such as activated T cells, or the use of primary cells may be required to demonstrate functional activity of the drug in that species. Where the target is not present in any healthy nonclinical toxicity species, consideration can be given to the use of disease models in addition to or instead of standard toxicity studies. For example, in antibody products directed against foreign targets such as bacterial or viral targets, inclusion of safety endpoints on biology models of disease should be considered [34]. Although a more relevant model for the human situation, it requires some consideration as there a number of challenges associated with this approach. Limitations of using disease models to assess safety risk include lack of sufficient background data and/or heterogeneity in the disease model, preventing adequate interpretation of potential toxicity, lack of historical controls, and generally a short duration of exposure as most disease models have a limited lifespan [78]. One approach where there is no previous human safety data with a particular therapeutic against a foreign target is to generate some safety data in a nonrelevant species, typically a rodent prior to dosing in humans. ICH S6(R1) indicates a short-term toxicity study in one species to be justified by the sponsor can also be considered with no additional toxicity studies, including reproductive toxicity studies, required [34]. TCR studies should be conducted to determine any potential off-target binding of the therapeutic to non-disease-infected tissue both in the nonclinical species and human. This approach can aid data interpretation if toxicity is observed and support the justification of a minimal toxicology program.

Where rodent and nonrodent species are shown to be cross-reactive, there is an expectation that toxicity studies should be conducted in both species [34]. There is also a regulatory expectation to provide justification of why species is not relevant,

for example, when a single-species (typically NHP) approach is proposed. Lack of binding by the drug or greater than 10-fold lower affinity and/or potency is likely to be considered sufficient justification as increasing the pharmacology in that species cannot be altered through increasing the dosing frequency or dose levels to provide reasonable comparison with what is expected in humans. A species may also be excluded as irrelevant when immunogenicity results in neutralization of the drug in a high proportion of the animals of a particular species, which cannot be overcome to ensure sufficient numbers maintain adequate exposure throughout the study.

Although great apes such as the chimpanzee have been used in the past, their use is banned in the United Kingdom since 1997, and has been restricted or banned in many other countries since, including New Zealand, Netherlands, Spain, and Australia; so also the use of wild, caught primates (unless exceptionally and specifically justified, and approved by the Secretary of State) as only animals bred in captivity can be used for research purposes [79, 80].

Assays such as ligand binding and cell-based assays are often used to determine PK and PD activity of biologics such as direct effects on cell phenotype, for example, cytotoxicity or proliferation. For monoclonal antibodies with a fragment antigen binding (Fab) region which binds to the target or ligand and an Fc region which binds to Fc receptors such as FcRn, FcγR1, FcγRII, and FcγRIII, a combination of ligand and Fc-binding assessments will provide greater understanding of target and effector activity. Cell-based assays demonstrating mechanism of action are generally used for drug substance or drug product testing in biologic development. These assays, although much more complex, tend to be more sensitive to minor differences. Identification of key PD endpoint(s) required for inclusion on the toxicity studies will then require development and validation.

All the bioanalytical assays developed for use in the nonclinical studies should be validated to demonstrate reliability and reproducibility for the intended use [81]. This can be a challenge especially early on in drug development as many of the cell-based assays used to address immune function and safety can be very complex.

Drug safety, PK, and efficacy can be altered in the presence of ADAs and the impact in animal studies, as in the clinic, can be varied. There can be little or no effect observed, for example, when the ADAs are non-neutralizing and any immune complexes are cleared effectively, to very severe effects which eradicate the pharmacology of the drug or compromise the health of the animal, even causing death. The most severe clinical responses to ADAs have been observed with non-mAb products, for example, ADAs which cross-react with endogenous proteins such as EPO and GM-CSF [82–84]. Biologics, in particular those of human origin or humanized products, often cause immunogenicity in animals as they are recognized as foreign but this is generally not predictive of immunogenicity in humans. Understanding immunogenicity in the nonclinical studies, however, is important for interpretation of the findings and occasionally may provide information on potential toxicities that can be monitored in the clinic; for example, administration of homologous recombinant thrombopoietin to mice and

monkeys resulted in severe thrombocytopenia following the occurrence of neutralizing ADAs, which was predictive of the response in the clinic [85].

For an immune response to occur, the drug must interact with different types of immune cells including T and B cells and antigen-presenting cells. When the target for a mAb, for example, is located on an immune cell, this increases the potential for immunogenicity. There are a number of methods available which can be used to identify T-cell epitopes or major histocompatibility complex (MHC)-binding ligands to help predict the immunogenic potential of protein-based therapeutics including, *in silico* predictions, *in vitro* peptide/MHC-binding assays, and *in vivo* HLA transgenic mouse models. These tools which can be used for nonclinical immunogenicity screening are increasingly being incorporated into the product developing strategy; using protein technologies to remove these sites while retaining structure and function is most effective to reduce the potential for immunogenicity [86].

The presence of ADAs can sometimes be detected nonclinically after a single low dose in the initial animal studies to determine PK profile. These molecules are likely to be highly immunogenic following repeat dosing at low doses over a prolonged period and a further animal study to investigate doses for the nonclinical toxicity studies may be required. The most common strategies to minimize the impact of immunogenicity in nonclinical studies includes use of high doses to induce tolerization, more frequent dosing to induce tolerization, and administering an initial loading dose to increase exposure levels early in the study. It is thought that these approaches overwhelm the immune system, reducing the ability to clear the drug by inducing a state of tolerance or perhaps saturating the FcRn receptor thus enabling higher drug exposure to be maintained in more animals throughout the study [87, 88]. Adding animals to dose levels where some level of immunogenicity is predicted can increase the likelihood of having a valid dose group at the end of the study. As it is not possible to completely predict biological behavior such as immunogenicity when determining comparability of biosimilars with the reference product, there is often a need for postmarketing observational studies to detect differences in ADA incidence and magnitude [89, 90]. Changes in PD activity, TK, and/or evidence of immune-mediated reactions, for example, glomerulonephritis and vasculitis, will be determined in the toxicity study to understand the antidrug antibody response.

Prior to conduct of the nonclinical toxicity studies, nonclinical dose formulation stability studies at the appropriate dilutions for the dose levels selected are conducted to verify the concentration of the antibody solution which will be used for dose administration. Owing to the complexity of the assays conducted, which should be able to detect antibodies at low concentration and which should demonstrate stability under the conditions of use; they are generally conducted under the auspices of the testing laboratory releasing the drug product rather than the safety assessment dispensary. Drug concentrations of doses administered on the study are determined using spectroscopy by quantifying protein concentration following measurement of absorption at 280 nM. More recent techniques, for example, the Solo VPE system, determine analyte concentration based on the

Beer–Lambert law and slope derived from absorbance measurements made at multiple pathlengths, thus reducing the need for time-consuming dilutions and replicate sample preparations.

Not all toxicities observed following administration of biologics can be attributed to exaggerated pharmacology. Depending on current biological knowledge of the target, pharmacology not predicted from the desired mechanism of action may result in unwanted effects or the release of cytokines and/or acute phase proteins may lead to undesirable sequelae. These findings may or may not be identified in the nonclinical species and will remain a risk for the clinic (e.g., anti-CD28). Inclusion of *in vitro* assays to predict adverse reactions due to cytokine release in humans is therefore recommended [91–93]. The current assays available can only identify if cytokine release may be produced by a molecule, they are not able to determine a level at which cytokine release may occur in humans. When in development, the molecules with which such an assay should be conducted and, indeed, the assay format itself is still a subject of much discussion across the industry.

11.6.1.4 **Feasibility/Tractability Assessment**

Occasionally, no pharmacologically relevant species is identified that can be used to conduct nonclinical pharmacology or toxicity studies, for example, when only human and chimpanzee are pharmacologically relevant or formation of neutralizing ADAs limits dosing in a cross-reactive toxicity species. A number of alternative approaches may be considered, including use of homologous molecules, "humanized" transgenic or gene knockout animals, and disease models. There are a number of factors which must be considered before employing any of these approaches [94] (Table 11.4).

Homolog Homologs are species-specific molecules and to have any relevance to the clinical candidate, they must display similar target affinity and selectivity and show comparable potency in appropriate functional assays. As development of a homolog incurs the same resource, costs, reagents, assays, etc. as the candidate molecule, it is equivalent to developing two molecules in parallel and is not a decision to be taken lightly. However, this approach may provide the only opportunity to assess certain endpoints, for example successful mating and pregnancy may be examined in rodents or rabbits to evaluate fertility, which is not practicable to measure in NHPs because of their high spontaneous abortion rate and low fertility. During process development of a homolog, care must be taken not to introduce differences that may have an impact on the biological, PK, or immunogenicity characteristics when compared to the clinical candidate. These changes comprise, but are not limited to, process impurities and/or contaminants, formulation and biophysical properties including which epitope it binds, and glycosylation profile. It cannot be assumed because the homolog is specific for a particular species, it will have the same pharmacological activity as the candidate molecule will in human. The homolog may need to be a different Ig isotype, particularly if there is ADCC or CDC activity, than the clinical candidate to show effector function, for example the closest mouse equivalent to human IgG1 is IgG2a. Although

Table 11.4 Benefits and considerations of alternate approaches to nonclinical safety assessment.

	Homolog	Disease model	Transgenic/Knockout (KO) animals
Benefits	Where the target is only pharmacologically active in human or chimpanzee it can provide hazard identification	Where the target is only pharmacologically active in human or chimpanzee it can provide hazard identification	Where the target is only pharmacologically active in human or chimpanzee it can provide hazard identification
	When ADAs limit subchronic or chronic dosing with clinical candidate, it can provide hazard identification	Provides opportunity to investigate safety where target is present at low levels or not at all in healthy animals and is upregulated in disease	Conditional knockouts provide the opportunity to investigate antagonism of a particular target following development
	Reduces conduct of studies in an irrelevant species and typically homologs are developed for lower order species, that is, rodents thus supporting NC3Rs philosophy	May be only relevant model for human situation, for example, biologics against a foreign protein	Transgenics, where a human version of the gene of interest has been inserted into the genome of another species (usually mouse), provide the opportunity to evaluate toxicity and efficacy but limited/no exploration of dose
	If pharmacology has been generated in a disease model using the same species, it can provide a margin of safety in that species Use of standard toxicological species provides confidence in interpretation of findings and often enables inclusion of appropriate endpoints which have previously been validated in this species, for example, immunotoxicity endpoints Can provide the opportunity to evaluate fertility, that is, mating and successful pregnancies, which is not practical in NHPs	If pharmacology is also generated, it can provide a margin of safety in that species	Provides opportunity to investigate safety where target is not present at all or present at low levels in healthy animals

(*continued overleaf*)

Table 11.4 (*continued*)

	Homolog	Disease model	Transgenic/Knockout (KO) animals
Limitations	Codevelopment of homolog with clinical candidate	Difficult to interpret findings against background of disease pathology	Genetically modified is not the same as administering a biologic, that is, conditional KO or agonist because of the potential for redundancy
	Reagents and assays are required to develop PK, PD, and ADA assays, some of which will be specific for the homolog	May have limited life-span-limiting duration of exposure possible	Pharmacological challenge or physiological stressors may unmask subtle phenotypes previously unknown
	Confirmation of affinity, specificity, and PD of homolog required	Translation of disease model to human disease may be poor, for example, significant differences in the immune system, which may limit translation of any safety endpoints	Genomic change or deletion may be embryolethal or reduce lifespan of the organism thus reducing or limiting opportunity to evaluate toxicity
	Significant resources and cost required to develop and characterize a homolog	Risk of immunogenicity with humanized molecule in nonclinical species	Significant resource, cost, and time associated with generation, development, and validation of genomically modified strains
	Risk of immunogenicity with homolog in nonclinical species	Model should be relevant for human situation	Species differences in structure and function of the gene of interest must be shown to reflect that in human for it to be relevant
		May require cross-reactive molecule for disease model species	Risk of immunogenicity with humanized molecule in genetically modified species
		Lack of historical control data makes interpretation difficult	Lack of historical control data makes interpretation difficult
			Limited blood volume, particularly in mice, may result in large number of animals required to conduct an appropriate safety package
			May not reflect the full physiological effect if the target pathway involves other ligands as only one or two human genes will be inserted in transgene

it might be expected that a species-specific homolog will not produce antidrug antibodies in that species, it is not a given. Thus, evidence to understand human relevance of the homolog in the nonclinical species should be provided as for the candidate molecule and any differences in potency, PK, and immunogenicity must be accounted for in the study design and interpretation. However, how similar the homolog is to the clinical candidate can only be fully evaluated after the clinical candidate has been fully assessed in the clinic. Use of a homolog in a standard toxicological species can provide greater confidence in the interpretation of findings and may enable inclusion of appropriate parameters which have been validated previously in this species, for example, immunotoxicity endpoints. Furthermore, for assessing safety using a homolog in the same species in which pharmacology has been generated, a margin of safety may be determined for that species.

Animal Models of Disease In certain situations it may be appropriate to consider generating safety information in animal models of disease, for example, when the biologic is directed against an antigen on an infectious agent, that is, a foreign target such as bacteria or virus, or when the target is induced or has a different disposition as in the disease or oncology setting but is not expressed or expressed at very low levels in healthy animals, or when the molecule is not cross-reactive in nonclinical species.

For molecules against foreign targets, ICH S6(R1) indicates a short-term toxicity study may be conducted in one species, to be justified by the sponsor; typically this will be in a rodent [34]. Alternatively, the biologic can be tested in an animal model of disease which is also used to evaluate efficacy. Often these models have a limited life span which can limit the duration of exposure in which to assess safety endpoints. For biologics against a novel foreign target, it may be relevant to take both approaches prior to conducting clinical studies in healthy volunteers where there is no benefit. These approaches are appropriate if there is no off-target binding to noninfected tissue. TCR studies may be conducted to determine the potential for off-target binding in human and nonclinical tissues. These studies may have challenges around preparation and use of infected cells or tissue or pseudoinfectious reporter virus particles, for example, as positive control material, due to either the hazard classification and handling requirements and/or the destruction of the bacterial or viral epitope during various fixation methods. It is generally not expected to conduct additional studies such as reproductive or developmental toxicity studies.

When disease models are used to assess safety endpoints, it is important the model is well characterized to be able to interpret potential toxicity against a background of disease pathology. Extensive work prior to conduct of the definitive toxicity study is likely to be required to generate sufficient historical control and disease data to enable interpretation and may use significant numbers of animals. Similarity of the model to the human disease situation should be considered in the translation of any safety endpoints. As in most toxicity studies, there is a risk of immunogenicity following administration of a humanized molecule

to a nonclinical species. Despite the many challenges, use of disease models to provide hazard identification may provide the only opportunity to investigate safety prior to administrating the molecule to humans.

Transgenic and Knockout Animals Use of recombinant DNA technology to introduce humanlike changes into mice can provide a powerful tool with which to evaluate the efficacy and safety of biologics. These changes can include the transfer of human genetic material into mice but also the deletion of genes or the manipulation of genes already present. As with disease models, transgenic or knockout (KO) animals also need extensive characterization not only to understand the pharmacological activity of the model compared to humans but also to generate phenotypic background data on the model to allow proper interpretation of safety endpoints. Often, genetically modified animals are developed on background strains which are not typically used for toxicity studies.

There are a number of considerations when genetically modified animals are used. Altering the genetic makeup of an animal prior to birth is not the same as administering a biologic which modulates a protein in the adult. In some cases, the genomic changes introduced are associated with embryonic lethality or significantly reduce the lifespan of the animal, which may not reflect the physiological change observed in humans and will result in limited opportunity to evaluate toxicity. In addition, there may be compensation of the absent target through redundancy in the pathway. Complex diseases such as systemic lupus are extremely difficult to replicate, so the full physiological effect of the disease may not be manifested in the transgenic mouse [95]. Alternatively, lack of adverse effects in mice that specifically lack an endogenous gene and therefore do not express the related protein may suggest that inhibition of the same target in humans is unlikely to be deleterious [96].

Significant resource is associated with the generation, development, and validation of genetically modified mouse strains and both development timelines and costs can be significantly increased when this approach is used. Owing to the breeding of genetically modified mice over recent years, the number of animal experiments has increased, with breeding animals now accounting for more than half of all the animal experiments in Britain [97]. To date, the technical challenges associated with genetic modification has resulted in chimeric animals which do not possess the full complement of genetic changes. These animals must then be genotyped and crossbred repeatedly to create homozygotes, with each stage using many animals. The advantage of having a more human relevant model however is the reduction in the use of NHPs. For example, genetically modified mice have replaced the need for monkeys when testing the polio vaccine [98]. Recent technological advances, such as development of the CRISPR Cas9 system of modifying DNA, are greatly improving the specificity of gene alteration and allowing modification of several genes at once [99]. The precision of the CRISPR system, which can alter both copies of the gene in single-celled embryos while being more reliable, requires considerably fewer animals. In the future, it is probable animal

usage will be reduced overall, while more accurate models of human disease will be reproduced.

Even when there is a high degree of protein conservation through evolution, there may be major differences between the homologous proteins of different species, such as that observed with the pentraxin family. Their evolution along with the innate and adaptive immune systems and their interactions with complement and Fc receptors has resulted in species differences in ligand specificity, their activity as acute phase reactants, and their behavior following ligand binding [100]. The discovery that serum amyloid protein (SAP), a pentraxin family member, is a component of amyloid fibril deposits that cause systemic amyloidosis has resulted in the development of monoclonal antibody therapies targeting SAP [101, 102]. A major challenge in studying systemic amyloidosis has been developing an animal model that is in accordance with the phenotype of the human disease, given the differences between species. Although murine models have been developed, they generally do not reflect the pattern of amyloid deposition or reflect the toxicity observed in the human condition [103]. Consequently, assessing the safety of an anti-SAP mAb that does not cross-react with the standard toxicological species and that targets a protein which demonstrates significant differences in biology between species poses a major challenge. The development and characterization of a refined transgenic model of systemic amyloidosis which has been shown to better represent the human disease has finally provided the opportunity to assess the safety of these molecules nonclinically [104]. The characterization of this transgenic mouse confirmed that the mAb did not cross-react with mouse SAP and that human SAP was limited to systemic circulation only. Indeed, data generated using the C57BL/6 hSAP/+ mouse has been used successfully to provide hazard identification information to support an FIH coadministration clinical study [102]. It is important to confirm that the human ligand/receptor in the transgenic mouse model is expressed as in human and is retained over time.

In conclusion, the acceptability of alternative approaches of assessing safety endpoints in disease models or genetically modified animals, or using a homolog molecule must be considered in light of the risk versus the benefit for the human population, whether that will be human volunteers or patients, which will be tested in clinical studies and following registration. These approaches are not without significant challenges and incur significant cost, resource, and time to develop; however, they may provide the only opportunity to assess safety endpoints when there is no cross-reactive nonclinical species or the target is not expressed in healthy animals and the opportunity to address potential safety liabilities unique to the disease setting and thus should be contemplated accordingly. The use of an alternative approach to address safety assessment must be justified to regulatory authorities to demonstrate validity of the model employed.

In the case of bispecific T-cell engager antibodies (BiTEs) which engage T cells via CD3 interaction *in vivo* toxicity studies cannot be conducted because of the lack of cross-reactivity of the CD3 portion of the BiTE biologic with the CD3

receptor on T cells of nonclinical species. Instead cell-based system using cocultures of human PBMC and target cells can be used to establish a dose response of the activity of the BiTEs [105]. The most sensitive *in vitro* assay endpoint(s) can be used to determine the clinical dose range and identify minimal anticipated biological effect level (MABEL).

11.7 First in Human (FIH) to Registration

The nonclinical data package to support clinical dosing can be considered to be a continuum from information obtained in early discovery and the nonclinical toxicity package. In particular, the following aspects enable the justification of the nonclinical species selected: (i) the known biology of the target; (ii) the downstream consequence of target engagement; (iii) target turnover in humans and the pharmacologically relevant nonclinical species. In addition, potential PK markers may have been identified that could be utilized to confirm the expected pharmacology following binding and/or effector function activation (e.g., CDC or ADCC).

11.7.1 Cross-Reactivity Study

Cross-reactivity studies in tissues are generally conducted on biopharmaceutical constructs that contain complementary domain regions, for example, mAbs, dAbs and Fabs. Prior to 2011, *ex vivo* TCR studies were used to justify and/or deselect nonclinical species on the basis of the presence or absence of target binding (positive staining) that was observed. However, ICH S6 (R1) guidance emphasizes that this assay is not appropriate for the selection of pharmacologically relevant nonclinical species as only target antigen binding is demonstrated without any confirmation of pharmacological activity [34]. In accordance with ICH S6(R1), a TCR study should be conducted using a panel of human tissues only. Suitable positive control tissue or artificially prepared tissue (e.g., antigen-absorbed beads embedded in resin) should be evaluated. In addition, a matched negative control (isotype mAb or bispecific framework matched molecule) should be evaluated at the same concentrations as the therapeutic molecule to enable interpretation of the staining pattern observed and, in particular, differentiate between background (nonspecific staining) and positive (target antigen) staining. Because cross sections of tissues are used in the TCR studies, both cytoplasmic and membrane positive staining may be observed. Since the cytoplasm is not typically accessible to biologics *in vivo,* the positive cytoplasmic staining observed is considered not to represent any significance *in vivo.* If any unexpected binding is noted with any human tissue, or toxicity is observed in the *in vivo* toxicity studies, then a selected panel of nonclinical species tissues can be evaluated to assist in the interpretation

of the unexpected positive staining or toxicity observed. In the case of bispecific molecules, the whole bispecific rather than each component should be evaluated.

11.7.2 Safety Pharmacology

Safety pharmacology assessments should be conducted prior to FIH clinical studies. ICH S6 supplemented by ICH S7A provide guidance on the assessments [47]. Safety pharmacology endpoints (neurobehavioral, respiratory, and cardiovascular function) can be evaluated in stand-alone studies following a single administration or incorporated into the design of repeat-dose studies in which assessments are conducted following multiple dosing. The selection of the most appropriate study design (stand-alone or inclusion into a repeat-dose study) should take into consideration the biology of the target and/or mechanism of action with respect to potential effects on neurobehavior, respiratory, or cardiovascular function. For example, if potential effects on neurobehavior and/or respiratory function are not anticipated, then limited assessment (cage-side observation) of these endpoints could be considered. When safety pharmacology endpoints are included into repeat-dose study designs, consideration should be given to the time points selected for these assessments as interpretation of safety pharmacology data can be impacted by other study procedures that may be scheduled at the same time, for example, blood sampling, or dosing.

In vitro electrophysiology studies to assess the potential for delayed ventricular repolarization (e.g., human Ether-à-go-go-related gene (hERG) assay) are not conducted with biologics, as they have very low potential to interact with the extracellular or intracellular (pore) domains on hERG channel. This low potential for delayed cardiac repolarization is based on their physical size and high target specificity which make them unable to cross the plasma membrane to interact with and inhibit the central pore or nonspecifically block the external "toxin-binding site" of the hERG channel [106–108]. The absence of hERG channel interaction of biotherapeutics has been demonstrated using two specific anti-hERG polyclonal antibodies that bound to epitopes on the hERG channel but did not interact with the external pore region, further supporting the low QTc prolongation risk [109].

11.7.3 In Vivo Studies

In general, the proposed clinical dosing strategy with respect to route of administration, dosing duration, frequency of dosing, and dose range anticipated in the clinical studies should be taken into consideration when designing repeat-dose toxicology studies. In addition, the drug substance and formulation used in the toxicology studies should be representative of the clinical batch and the clinical formulation that will be used in the clinical studies that the toxicology studies support. Demonstration of the comparability of biopharmaceutical drug substance

used in the toxicology studies and that in the clinical Phase 3 studies and post-marketing with respect to the manufacturing process (including residuals) is a key component of the marketing application.

The toxicity studies should be performed in pharmacologically relevant species that will result a similar pharmacological response following biologic administration as that expected in humans (previously described in section 11.6.1.3). If the biologic has a comparable safety profile in 4- or 13-week toxicity studies in NHPs and rodents, or the findings are understood in terms of mechanism of action, then longer-term general toxicity studies in one species are usually considered sufficient [34]. The rodent species should be considered for longer-term studies unless there is a scientific rationale for using nonrodents (e.g., unacceptable immunogenicity).

Guidance on the timing of repeat-dose toxicity studies with respect to the stage of clinical development is provided in the ICH M3(R2) [46]. In general, the duration of the toxicity studies should match or exceed (up to 6 months for chronic indications and for anticancer agents not in the scope of ICHS9) the duration of the planned clinical study in the target patient population. Repeat-dose toxicology studies of 6 months in duration are considered sufficient to support registration for chronic dosing indications and studies of 3 months in duration are considered to be sufficient to support advanced cancer indications (ICHS9) [45].

A recovery/off-drug period should be included in the study design of at least one repeat-dose study. The dose level(s) selected for the off-drug period assessment should be justified on the basis of pharmacological, PD, clinical dose level, or exposure levels. The purpose of the off-drug period is to examine reversibility, which, in most instances, will be exaggerated pharmacology. The demonstration of complete recovery is not necessarily considered essential. Importantly, the off-drug period is not to assess any potential delayed toxicity or just to assess potential for immunogenicity. For example, a single dose or five weekly doses of PF-05280586 (proposed rituximab biosimilar) or rituximab in cynomolgus monkeys at doses $\leq$20 mg/kg, resulted in marked depletion of peripheral blood B cells by 4 days. Following an off-drug period, near to complete repletion of B cells was observed by day 92 in the single-dose study or partial (22–60%) repletion was observed after 121 days in the repeat-dose study [110]. An alternative option is to incorporate frequent (real-time if possible) monitoring of PD in the off-drug period, which might be useful in determining whether to terminate the off-drug/recovery groups earlier than planned or to extend the recovery period [111].

The dosing frequency employed in the toxicity studies should either match or be more frequent than the dosing interval anticipated in the clinical studies. In general, the dosing interval in repeat-dose toxicity studies should be at least every one half-life. However, if immunogenicity was observed in previously conducted short-term repeat-dose studies that resulted in the faster clearance of the biologic, then more frequent dosing and/or higher dose levels may need to be considered in the design of chronic (3 and/or 6 months) toxicity studies.

11.7.4 Selection of Dose Levels

In order to provide the rationale for dose selection for repeat-dose toxicity studies, an understanding of the PK/PD relationship for the biologic and markers for the functional activity, such as binding and any effector function activity, is desirable. If the PK/PD relationship is understood, ICH S6(R1) suggests that the high dose should be the higher of the following:

- a dose at which maximum intended pharmacological effect is achieved in the nonclinical species or
- a dose that provides 10-fold exposure multiple over the maximum exposure to be achieved in the clinical studies.

Generally, three therapeutic treatment groups are planned to be used in the toxicology study design; the low dose generally approximates a low multiple of the anticipated clinically efficacious dose and the mid-dose is an even multiple between the low- and high-dose groups. However, the requirement to evaluate the biologic at three dose levels has been questioned, especially in the case of antagonists [112]; the maximum PD effect in healthy nonclinical species is often achieved at the low dose selected. Therefore, the evaluation of two dose levels (a low multiple of the anticipated clinically efficacious dose and a dose that provides an approximately 10-fold exposure multiple over the maximum exposure to be achieved in the clinical studies) can also be considered.

11.7.5 Pharmacokinetics/Pharmacodynamics

In general, the distribution and clearance of mAbs are predictable and depend on whether the mAb target is soluble or membrane bound. Because of their large molecular weight (compared to a small molecule), the distribution of mAbs is initially restricted to the vascular space with slow distribution to tissues. The clearance of mAbs can be target mediated and/or mediated by the effector function (if present). The long half-life of mAbs is attributed to the interaction of the Fc portion of IgG molecule with the neonatal Fc receptor (FcRn) expressed on various cell types, the internalization of the mAb into a cell endosome without being degraded, and then being released back into the circulation [113]. Modified and improved binding of the Fc can result in even longer half-lives [114]. In contrast the half-life of proteins such as cytokines is typically short.

The disposition of bispecific biologics (dual dAbs, Fabs, diabodies) that are based on IgG molecules is similar to that of intact mAbs and their distribution is determined by molecular weight, physicochemical properties including charge, and other structural features such as binding to Fc receptors. The PK profile of bispecific biologics demonstrate that their volume of distribution is equal or slightly larger than the plasma volume (3–8 l), representing the vascular space and interstitial space of well-perfused organs [115]. The movement of bispecifics

from the systemic circulation to the interstitial space is similar to other large proteins, predominantly facilitated by convective transport. The subsequent removal from the interstitial space back into the vascular space is regulated by lymphatic drainage [116]. For example, the PK of dual-variable-domain immunoglobulin (DVD-Ig), anti-IL-12/IL-18, was shown to have a PK profile and tissue penetration comparable to normal IgG therapeutics [117]. Compared to full-length mAbs, smaller bispecific formats (55–60 kDa) demonstrate a more uniform distribution. Bispecific biologics lack the FcRn binding site and therefore cannot interact with the FcRn salvage pathway. Therefore, the half-lives of these molecules are significantly short when compared with mAbs. For example, the F(ab′)2 molecule H22 × Ki-4, with MW 104 kDa, exhibited a half-life of 11.1 h in patients with Hodgkin lymphoma, 21 compared to the 2–3 weeks for most mAbs [118]. BiTEs have a moiety which specifically binds to T cells (via CD3). After binding to T cells in circulation, their tissue penetration may be restricted owing to the size and charge of the resulting complex [119]. To enable greater tissue penetration, a CD3- signaling motif with a low binding affinity to the immune cell is selected, which maintains the bispecific plasma concentration below the dissociation constant for the immune antigen-binding domain. These properties reduce distribution via circulating immune cells considerably. For example, blinatumomab has a volume of distribution of $1.61 \pm 0.74\,l/m^2$, similar to that of mAbs. It has a short serum half-life of 1.25 ± 0.63 h and a relatively high clearance of approximately 2 l/h, which is the consequence of a lack of FcRn-mediated recycling and its low MW (55 kDa) that allows for glomerular filtration and subsequent renal metabolism [119]. Regardless of the type of biologic (mAbs, dual dAbs, BiTEs), correlating PK with PD provides a model to guide clinical dose-level selection, enable modeling to predict at which dose levels efficacy may be noted, and inform on safety in the clinical studies [120].

11.7.6 Immunogenicity

Immunogenicity, that is, the generation of ADAs is not an unexpected response in healthy animals to a foreign protein (such as a mAb) and incidence of ADAs in, for example, monkeys, could range from a few to all animals [121]. ADAs are evaluated in toxicology studies to aid in the interpretation of the nonclinical exposure and toxicity data. The generation of ADAs can result in a decrease in exposure in the toxicity study. If an insufficient number of animals were exposed to the mAb for the entire duration of the study, the toxicity of the biologic was most likely not fully characterized.

Antidrug antibody testing in the toxicology studies is not mandatory, however, because the study results cannot be predicted, ICH S6(R1) recommends that samples be collected and archived for potential future analysis. If PD-exposure data from the toxicology studies indicate that immunogenicity occurred, then

the ADA samples can then be analyzed. However, if the PK and PD data show full correlation (reduction in pharmacological effect with reduction in systemic exposure), then ADA formation could be inferred without potentially confirming or charactering the ADA response. In the absence of a PD marker, further characterization of the ADA noted (e.g., neutralizing ADA) should be carried out in at least one study. For example, in monkey 4-, 13- and 26-week repeat-dose studies, mavrilimumab was found to be immunogenic and the immunogenicity response observed had a profound impact on clearance of mavrilimumab in these studies [122]. In cases where immunogenicity is anticipated to interfere with the interpretation of the toxicology studies; it may be necessary to adapt the design of nonclinical studies to minimize or overcome the immunogenicity observed. For example, dosing through may be an option when ADA are detectable but are not neutralizing/clearing the exposure to the biologic; where ADA are neutralizing, selecting higher dose levels may be an option to overwhelm the ADA response and/or induce tolerance [123]. Importantly, the nonclinical immunogenicity data are not used to predict immunogenicity rates in patients. It should be noted that FDA Guidance for Industry Immunogenicity Assessment for Therapeutic Protein Products suggests that the nonclinical data may be helpful in "describing the consequences" of immunogenicity [124].

11.7.7 Immunotoxicity

On the basis of their pharmacology (e.g., agonists), an assessment of cytokine release can be included both as a toxicology endpoint for potential immunomodulation and inflammation and as a potential PD marker. If immune modulation and potential immunotoxicity are anticipated with the biologic peripheral blood lymphocyte subset analyses (using flow cytometry) and an assessment of immune function (T-cell-dependent antibody response (TDAR)) should be evaluated in one of the repeat-dose toxicology study designs. Also the assessment of potential effects of the biologic (as a consequence of placental transfer) in the offspring can be determined in the design of the pre- and postnatal development study. For example, a reduction in anti-KLH IgM and IgG levels was observed in the T-dependent antigen response assay following repeat-dose administration of Siltuximab (human-mouse chimeric IgG1 mAb targeting soluble human IL-6) in cynomolgus monkeys [125]. The reduction in IgM and IgG response to antigen challenge was not unexpected based on the known pharmacology and confirms a potential of blunting an immune response to vaccination in humans.

11.7.8 Reproductive Toxicity

ICH M3 (R2) and ICH S6 (R1) provide guidance on the timing of the reproductive toxicity studies for biologics.

11.7.8.1 Fertility

An assessment of potential effect of a biologic on fertility is required before the initiation of Phase III clinical trials [48] for all non-advanced cancer indications. In the case of advanced cancer, fertility studies are not required [45]. As with all aspects of nonclinical safety evaluation, an assessment of developmental toxicity is conducted using only pharmacologically relevant nonclinical species. If the rat and/or rabbit are pharmacologically relevant nonclinical species, then fertility studies can be conducted in these species. For example, rabbit male and female fertility studies were conducted with Tabalumab (IgG4 mAb targeting B-cell activating factor). In both studies, weekly IV dosing was employed and in addition to the standard fertility endpoints, a PD endpoint (B-lymphocyte count) was included. The assessment of potential effects on fertility was limited to animals administered the mid and high dose levels; data from the low dose was excluded as the systemic exposure achieved was below a meaningful exposure multiple relative to the anticipated clinical exposure. No effects on male or female fertility were observed [126]. When the NHP is the only relevant species, it is recognized that mating studies to directly assess fertility are not practical because the low fertility and high spontaneous abortion rates indicate that very high animal numbers are required to show a meaningful effect. In addition, pregnancy cannot be confirmed until gestation days 18–20, therefore the assessment of the number of successful pregnancies per mating as a measure of fertility is not considered to be practical. When the NHP is the only relevant species, the potential for effects on male and female fertility can be assessed by evaluation of the reproductive tract (organ weights and histopathological evaluation) in repeat-dose toxicity studies of at least 3 months duration using sexually mature NHPs. If the known biology of the target or previous findings (repeat-dose studies) is indicative of a fertility risk then specialized assessments such as menstrual cyclicity, sperm count, sperm morphology/motility, and male or female reproductive hormone levels can be evaluated in a repeat-dose toxicity study.

11.7.8.2 Embryofetal Development and Pre- and Postnatal Development

To enroll females of childbearing potential (FCP) into clinical trials prior to the conduct of reproductive toxicology studies, highly effective methods of contraception should be included in clinical trials to manage the potential risk.

If the rat and/or rabbit are relevant nonclinical species, then both species should be used for embryofetal development (EFD) studies. However, if both species are relevant, and embryofetal lethality or teratogenicity has been identified in one species, then evaluation in the second species is not required [34]. It is important to note that placental transfer of IgG across occurs at different time points across species. For example, although in rat, IgG1 has been detected in the yolk sack, the majority of IgG1 transfer in rodents occurs primarily postnatally via milk, whereas in humans and NHP, placental transfer is later in embryofetal development (late second trimester onwards) in humans and NHPs. Therefore, rodent fetal exposure to the therapeutic is likely to be lower when compared to that in humans. A rabbit (pharmacologically relevant species) EFD study was conducted

with tabalumab [126]. As the placental transfer of IgG4 has been shown to be similar in rabbits, monkeys, and humans, this species was considered appropriate for the EFD assessment [127]. No adverse parental, reproductive, or developmental effects were observed at any of the dose levels. However, one mid-dose female died before scheduled euthanasia and three low-dose rabbits aborted. As no unscheduled deaths or fetal losses were noted at the highest dose tested, these sporadic events were not considered to be tabalumab related. Of note, 44% and 36% of low- and mid-dose dams had significantly reduced systemic exposure, below the anticipated clinical exposure [126].

For biologics with pharmacological activity only in NHPs (e.g., cynomolgus monkeys) and with sufficient pregnancy prevention precautions in place in clinical trials, the pre- and postnatal study can be conducted during Phase 3 and the report submitted with the marketing application. For the evaluation of EFD when only the NHP is a pharmacologically relevant species, in most instances an enhanced PPND (ePPND) study design is utilized as this study design combines both the EFD and PPND into one study. The ePPND study allows for the evaluation of pregnancy outcome, viability, and external malformations at birth following a natural delivery. The monkeys are monitored by ultrasound for the progression of pregnancy. Skeletal effects in the developing fetus are evaluated by X-ray and visceral abnormalities are evaluated at necropsy. Exposure to the biologic in systemic circulation and milk (dams only) together with PD can be monitored in the dams and offspring. In addition, dependent on the biology of the target, endpoints to monitor pharmacology endpoints can also be included; the duration of follow-up and endpoint assessments will depend on the anticipated pharmacological activity and the time required for partial or full recovery of the PD effect. For example, immunomodulatory drugs may affect lymph node cellularity, and offspring may need to be followed for a long duration to evaluate the impact on lymph node development. In the case of rituximab, in the ePPND study, neonates NHPs were followed up to postnatal day 180 after weaning to demonstrate the full recovery of B cells [128]. In the case of golimumab (anti-TNFα), an assessment of neonate immune competence showed golimumab had no effects on lymphoid organs, lymphocyte subset populations, or immune function [129].

Of note, studies in NHPs are only useful for hazard identification because the number of animals per group is generally lower than for a rodent or rabbit study. Another consideration is that reproductive failure, through abortions and still births in early and late pregnancy and neonatal losses within the first few weeks is significant and common among NHPs, including cynomolgus monkeys. Therefore, assessment of historical data with respect to such losses should be taken into consideration when determining the number of pregnant females per dose group included into monkey ePPND study design [130]. Monkey ePPND study essentially provide hazard identification, therefore the ePPND study could potentially be conducted at a single-dose level that provides a 10-fold exposure multiple over the clinical therapeutic drug level. However, a scientific justification based on data from completed toxicology studies that demonstrate saturation of

target binding would need to be provided to justify the dose level selected. For products pharmacologically active only in NHPs and where the pharmacology raises a concern for embryofetal development, product labeling should reflect the potential risk or concern without conducting a developmental toxicity study in NHPs, and administration to females of child-bearing potential should be avoided/contraindicated.

11.7.9 Genotoxicity and Carcinogenicity

Biologics, such as monoclonal antibodies, do not directly interact with DNA or other chromosomal material [34]. Therefore, genetic toxicology studies are not performed.

If chronic dosing (>6 months) or repeated intermittent use in the clinic is anticipated, an assessment of the carcinogenicity potential should be conducted. In most cases a weight of evidence approach can be used for the biologics that utilizes data from multiple sources such as the published literature (e.g., human disease genetic data, transgenic animals, knockouts, animal disease models), class effect information, target biology and mechanism of action, *in vitro* data, chronic toxicity data, and clinical data. The information from these various sources may be sufficient to inform clinical risk so that additional nonclinical studies are not needed. If the weight of evidence indicates a concern for carcinogenic potential, rodent bioassays would not be warranted, and the risk can be addressed in product labeling and clinical risk management. When the weight of evidence is unclear, additional nonclinical studies to provide a better understanding of the target and the downstream consequence of biologic engagement should be considered to address or mitigate the concern.

11.8 Selection of a Safe Starting Dose for First Time in Human Clinical Study

The selection of the starting dose level for an FIH study considers all the data on the biologic (pharmacology, mode of action (agonist or antagonist) and downstream signaling consequence, potency/affinity and receptor occupancy), PK profile and half-life in humans, and the relevant nonclinical species. Following the adverse outcome in the TGN1412 healthy volunteer study, the guideline on strategies to identify and mitigate risks for FIH clinical trials with investigational medicinal products provides a framework that should be used as component of a risk mitigation strategy when conducting FIH studies [131]. The guidance emphasizes the importance of determining the pharmacologically active dose (PAD) as well as the full pharmacological dose/concentration – response curve. In addition, the guidance introduces the concept of identifying MABEL and how MABEL is utilized in the selection of a safe maximum recommended starting dose (MRSD) in humans. MABEL represents the lowest animal dose/exposure or concentration

required to produce pharmacological activity *in vivo* and/or *in vitro* in animal/human systems. MABEL may also be defined as the minimum acceptable biological effect level, depending on the level of concern for the pharmacological response. In addition, the selection of MRSD should also demonstrate an adequate safety margin (based on dose and exposure) to the NOAEL identified in the repeat-dose toxicity studies. An integrated PK/TK-pharmacology approach was utilized to inform the mechanism of action and dose selection for the FIH clinical trial with CFZ533, a human IgG1 targeting CD40. A mechanistic PK/PD model was created on the basis of a robust understanding of the biology of the target and the proposed biological/physiological consequences of target activation/inhibition and the target-related findings (effects on germinal center in lymph nodes) observed in the monkey repeat-dose toxicity study. This model enabled the selection of the clinical starting dose and dose range [111]. In another example, PK/PD modeling of nonclinical rat, mouse, and monkey from pharmacology and toxicology studies was successfully used to determine MABEL dose level of PF-04840082 (humanized prototype anti-Dickkopf-1 IgG2 antibody). The PK/PD modeling enabled estimation of antibody non-target-mediated elimination, Dkk-1 turnover, complex formation, and complex elimination. The target-mediated drug disposition model was translated to human to predict MABEL dose level and the anticipated efficacious dose by incorporating information on typical IgG2 human PK, antibody-target association/dissociation rates, Dkk-1 expression, and turnover rates [132]. In the case of truly human specific biologics, such as bispecific T-cell engagers (BiTE) which engage one or more human receptors, *in vitro* data has been used to determine the MABEL dose. For example, MEDI-565 BiTE is a bispecific single-chain antibody of the BiTE class that transiently links carcinoembryonic antigen (CEA) on cancer cells with CD3 on T cells [105]. Although cross-reactivity of CEA was demonstrated in cynomolgus monkeys, no cross-reactivity with cynomolgus monkey CD3 was observed. Furthermore, differences in functional characteristics were observed with surrogate BiTEs. Instead, a cell-based system using cocultures of human PBMC and CEA-positive target cells were used to establish a dose response of the activity of the molecule. *In vitro* assays measuring cytokine release, T-cell proliferation, and tumor cell lysis used to identify dose levels achieved 20% maximal effect (EC20) levels. The most sensitive measure of MABEL with MEDI-565 was considered to be MEDI-565-induced lysis of tumor cells.

11.9 Summary

Successful nonclinical safety evaluation of biologics begins with an understanding of the fundamental differences in the type of toxicity exhibited by biologics, that is, exaggerated pharmacology and nonspecific immunological reactions, for example, immunogenicity. Therefore, species selection for which the test biologic is pharmacologically active becomes the single, most important decision for the design of the nonclinical safety program. In order to select a relevant species,

a considerable understanding of the target biology, including target expression and distribution, how the target functions in normal and diseased human tissue, understanding of any polymorphisms in animal and human, and downstream pathways is necessary. Data demonstrating the comparability of the selectivity, binding affinity, and potency of the test molecule between human and nonclinical species is critical and assists in determining the relevance of findings in the toxicity studies to the clinic. An additional area of consideration for mAbs when selecting the nonclinical species is the Fc domain and how selecting the isotype or changes to the Fc can affect half-life, effector function, or agonistic activity of the test mAb. In situations where no pharmacologically relevant species can be identified, alternative strategies such as use of homologous proteins or transgenic animals may need to be considered.

The design of the nonclinical safety package to support clinical dosing should be based on a number of factors including species relevance, expected pharmacology, data from biology studies, and PD/PK profile. The ICH S6(R1) guideline provides guidance on the type of studies needed and the design of those studies. Potential toxicity following repeat dosing, functional changes (i.e., safety pharmacology parameters), and depending on the proposed clinical plan, toxicity following chronic dosing and/or reproductive/developmental toxicity should be collected. Certain assessments are not necessary for biologics, for example, genotoxicity or *in vitro* electrophysiology studies. The potential immunogenicity of the test molecule, although not an unexpected response in animals to a foreign protein, needs to be evaluated in toxicity studies to aid in the interpretation of the PK and toxicity as ADAs can result in decreased exposure and/or toxicity. Finally, the results from the toxicity studies together with the pharmacology data (mode of action, downstream signaling, etc.) and PK profile will enable the selection of the starting dose in the first clinical trial; the starting dose may often be driven more by the pharmacology than the toxicity data. Although the study designs for biologics will differ from small-molecular weight compounds, they will also differ for each biologic; the goal of nonclinical safety evaluation remains the same, that is, to provide sufficient safety data to proceed safely into clinical trials and identify safety endpoints or biomarkers to evaluate during the trials.

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12
Immunogenicity of Biologics

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12.1
Introduction

This chapter is intended to provide a broad mechanistic overview of how biologics can be immunogenic, how the risk of immunogenicity can be minimized during drug development, and, from a regulatory perspective, how the risks of immunogenicity can be managed. In order to condense an incredibly complex subject, we have focused the discussion on monoclonal antibodies wherever possible, although examples of immunogenicity from other classes of drugs have been included where important lessons can be learned. Defining drug immunogenicity is not particularly straightforward as various definitions of immunogenicity have been proposed. One of the earliest definitions proposed by Burnet stated that immunogenicity was determined by the presence (or absence) of self and nonself components of an antigen [1]. We now know through increased understanding of the immune system that immunogenicity needs to be defined in broader terms, such as any substance that elicits an immune response. For the purposes of this chapter, this broad definition is not particularly useful, so we have restricted the definition of immunogenicity to "any drug that elicits an undesirable immune response as a consequence of the secondary pharmacodynamic properties of the drug." From a simplistic perspective, it is the nature (structure and physicochemical properties) of the antigen (protein therapeutic) that typically dictates the type and magnitude of the immune response in a host. This holds true for patients receiving treatment with modern protein therapeutics where the administered proteins can be highly immunogenic. The actual strength of the immune response will also depend on the genotypic and phenotypic background of individual subjects, including level of immune tolerance, as well as the dose regimen. For example, patients receiving replacement therapies for hemophilia and enzyme replacement disorders will not be immunologically tolerant to repeat dosing of

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the replacement protein therapies owing to reduced or absent expression of these proteins in the target tissues. For protein therapeutics that are immunogenic, the immunogenicity frequency and/or impact in the patient population is typically highly varied, although immunogenicity is rarely associated with significant morbidity or mortality. One of the most well-studied historical cases in which immunogenicity had a major impact on the safety and toxicity of a protein therapeutic was in patients treated with recombinant erythropoietin (EPO, Eprex™) [2–4] in which 175 patients between 1998 and 2004 developed pure red cell aplasia (PRCA) due to the induction of anti-EPO antibodies. The reason for the apparent sudden increase in Eprex™ immunogenicity has been extensively debated [5–7]; however, the most plausible explanation is that a combination of factors including suboptimal formulation, altered primary container, and failure to observe the recommended storage conditions contributed to bypassing of immune tolerance in isolated cases [8]. In spite of this transient increase in the incidence of anti-EPO antibodies, the relative frequency of EPO-induced immunogenicity in patients remains extremely low (3 cases of PRCA in 5948 patients studied during a 3-year period since October 2006 [3]). It is clear from the EPO example that immunogenicity-related risks can be minimized by careful control of the product formulation-primary container combination and reinforcement of supply chain storage conditions, but the question as to which elements have the greatest influence on immunogenicity remain unclear. It is now well established that immunogenicity has to be considered on a case-by-case basis as the importance of factors that drive immunologic responses in patients varies depending on the (often unique) properties of the protein therapeutic.

12.2 Mechanistic View of Immunogenicity: Innate and Adaptive Immunity

12.2.1 Innate Immunity

12.2.1.1 Dendritic Cells

As with any antigen encountered by the immune system, protein therapeutics (including monoclonal antibodies) can interact with the innate immune system almost immediately after administration. The translation of innate to adaptive immunity requires that such proteins, which are typically deposited in peripheral "target" tissues, gain access to lymphoid organs and cells. One of the main functions of specialist professional antigen presenting cells (APC), namely, dendritic cells (DC), is to transport protein antigens to secondary lymphoid organs, whereupon DC can present peptides derived from proteins to rare antigen-reactive T cell clones in the recirculating lymphoid pool. DC essentially function as sentinels of the immune system and this is facilitated through their ability to form networks throughout tissues in the body. DC are identifiable through their characteristic "dendritic" morphology (Figure 12.1) and they have a unique capacity

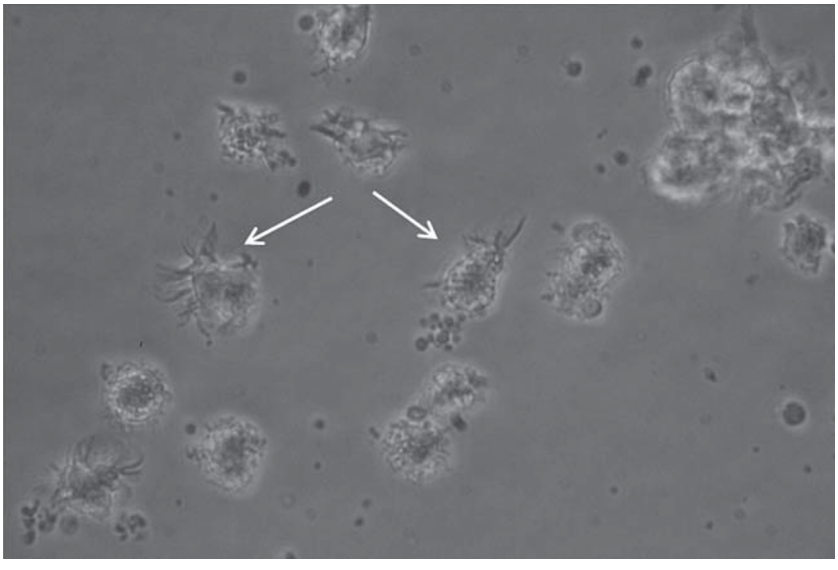

Figure 12.1 Human monocyte-derived dendritic cells (white arrows) produced by *in vitro* culture of monocytes with IL-4, GM-CSF, and TNFα.

to endocytose proteins, and process and present peptide epitopes to T cells. DC are also present in the blood and can be separated on the basis of expression of a variety of phenotypic markers including CD11c. Monocyte DC ($CD11c^{hi}$, MDC) and plasmacytoid DC ($CD11c^{lo}$, pDC) are both presumed to derive from bone marrow precursors, although they differ in function in response to different immunological challenges. Plasmacytoid DC that secrete Type 1 interferons (IFN) represent about 0.4% of peripheral blood mononuclear cells (PBMC) and play an important role in mediating the antiviral response, autoimmunity, and tolerance. Monocyte DC can be subdivided into MDC1 and MDC2 – MDC1 are most common (0.2–0.5% of PBMC) and often considered "conventional" DC with a high capacity to stimulate T cells, whereas MDC2 are very rare (<0.1%) and have been attributed with a specialist function during wound repair. Under steady-state (e.g., in the absence of inflammation) conditions, DC are involved in "tolerizing" the immune system against self and nonharmful environmental antigens. However, as part of their sentinel function DC also have a high capacity to home to sites of inflammation through chemokine receptors such as CCR6 that binds to MIP-1a secreted by inflamed epithelium. This is of particular relevance when considering that the preferred route of administration of many protein therapeutics, such as antibodies, is subcutaneous and delivery via this route nearly always results in some level of local tissue inflammation, swelling, and erythema. Indeed, even intravenous administration of protein therapeutics will enable efficient access to the DC network through lymphatics and tissues to which the molecule is targeted, particularly for long-acting protein therapeutics.

After activation, for example, in inflamed tissue, DC migrate to lymphoid organs where they locate to the T cell areas. It is here that protein-loaded DC form stable contacts with cognate antigen-specific T cells. These contacts persist for at least a day and this occurs under both steady-state conditions when DC can be tolerogenic, as well as upon DC maturation when immunity develops [9]. The fact that DC constitute just a small percentage of the cells in a lymph node means that it is essential to maximize interactions with T cells. This can be achieved through their size and pervasive cell shape (long, extended "dendrites") which enable them to scan T cells circulating through lymphoid tissues.

12.2.1.2 **Endocytosis of Proteins by DC**

In order to efficiently process and present protein antigen to T cells, DC that have not undergone full maturation retain a high capacity to endocytose proteins. Endocytosis by immature DC is facilitated by a continuous process of nonspecific phagocytosis in which the extracellular milieu is effectively sampled for presentation to circulating T cells. Other mechanisms of endocytosis include micropinocytosis and specific receptor-mediated processes such as uptake through clathrin-coated pits, Fc receptors, lectin receptors, and scavenger receptors. This process of antigen uptake normally occurs in the network of DC established in the body's peripheral tissues. Receptor-mediated endocytosis can occur through an array of endocytic receptors and facilitates DC differentiation, rapid antigen uptake and processing, as well as the presentation of peptide T cell epitopes. Indeed, it is this distinct and unique capacity for endocytosis and antigen processing that enables these cells to efficiently translate innate to adaptive immunity. Owing to the very high rate of uptake, even protein therapeutics present at low concentrations (particularly in particulate form) can be efficiently endocytosed, processed, and presented by DC to T cells [10–12].

Much work regarding receptor-mediated endocytosis in DC has focused on the C-type lectin receptors (CLRs) which can either be Type II transmembrane proteins with a single, carboxy terminal lectin domain, or Type I proteins with multiple lectin domains. Studies have revealed the sugar recognition properties of some of these lectins, for example, CD209 recognition of mannose and fucosyl residues on fungi and viruses [13, 14] but in many instances little is known about the natural ligands, which could include sugars on protein therapeutics (particularly those expressed in non-mammalian systems). Internalization through endocytic receptors can enhance the immunogenicity of protein therapeutics in three ways: (i) rapid uptake, (ii) efficient antigen processing and presentation to T cells, and (iii) induction of DC differentiation to provide effective costimulation to T cells.

The fact that the endocytic receptors can bind a wide variety of ligands (from both natural and synthetic sources) may be an important consideration for immunogenicity, particularly when developing products expressed in bacteria or fungal systems that may contain no or nonhuman glycosylation profiles, respectively. Indeed, in the context of therapeutic antibodies, DC also express high levels of FcRs (including FcγR and FcεR), which bind antibody through the Fc region. Internalization via this class of receptors is rapid and traffics

therapeutic antibodies to the endosomal pathway enabling the effective processing and presentation of the antibody therapeutic, such that peptide epitopes derived from a non-germline antibody sequence can be presented to T cells in the context of major histocompatibility (MHC) class II. DC differentiation can also be induced by signaling through endocytic receptors and result in expression of T cell-specific costimulatory molecules. In this context, DC are able to orchestrate antigen-specific T cell function into effector (immunity) or regulatory (tolerance) pathways. The most well studied of these costimulatory molecules is the B7 family which comprise both positive and negative regulators of T cell function. CD80 and CD86 are well characterized as co-stimulatory molecules, and compared to other APC, DC can express the highest levels of CD86 which competes for binding to CTLA-4 or CD28 expressed on activated T cells to either dampen or enhance T cell activation, respectively. It is worth noting that this type of costimulation alone, however, is not sufficient to induce immunity and it has been shown that DC express a wide range of other accessory molecules, especially TNF super family members (e.g., CD40) and cytokines (e.g., IL-12), which influence T cell differentiation and memory.

12.2.1.3 **Innate Immune Receptors**

Germline-encoded pattern recognition receptors (PRRs) expressed on professional APC such as macrophages and DC as well as various nonprofessional immune cells are responsible for sensing the presence of microorganisms. They do this by recognizing a wide range of ligands comprising structures conserved among microbial species, which are called pathogen-associated molecular patterns (PAMPs) (Table 12.1). Recent evidence indicates that PRRs are also responsible for recognizing endogenous molecules released from damaged cells, termed *damage-associated molecular pattern*s (DAMPs). These families include transmembrane proteins such as the toll-like receptors (TLRs) and CLRs, as well as cytoplasmic proteins such as the Retinoic acid-inducible gene (RIG)-I-like receptors (RLRs) and NOD-like receptors (NLRs). With the exception of some NLRs, the sensing of PAMPs or DAMPs by PRRs upregulates the transcription of genes involved in inflammatory responses. These genes encode proinflammatory cytokines, Type I IFN, chemokines and antimicrobial proteins, proteins involved in the modulation of PRR signaling, and many other as yet uncharacterized proteins. Activation of DC through ligands binding to PRR results in the production of proinflammatory cytokines that positively regulate immunity. There are experimental data that suggest protein therapeutics may interact with PRR although the mechanism of interaction has not been elucidated [16]. Recent studies suggest that there may be two possible routes for activating DC through PRR: firstly, the presence of minute amounts of excipients in the products (e.g., host-cell proteins derived from the expression system and/or known PRR ligands such as LPS); secondly, the presence of "stress-induced" aggregated particles derived from the product [12, 16]. Early experimental data indicate that for artificially stress-induced aggregates generated using different antibody therapeutics binding to TLR2 and TLR4 may be important in activation of the innate

Table 12.1 TLR ligands identified from natural, synthetic, and protein therapeutic sources.

TLR	Natural ligand	Synthetic ligand	Protein therapeutic[a)]
TLR1 and TLR2	TriAc lipopeptides	Pam3CSK4	
TLR2	Peptidoglycan, phospholipomannan, tGPI, mucins, hemagglutinin, porins, lipoarabinomannan, glucuronoxylomannan, HMGB1		Stir stress IgG aggregates (2–10 mM)
TLR2 and TLR6	DiAc lipopeptides, LTA, zymosan	FSL1, MALP2, pam2CSK4	
TLR3	dsRNA	Poly I:C	
TLR4	LPS, VSV glycoprotein G, RSV fusion protein, MMTV envelope protein, mannan, glucuronoxylomannan, glycosyl inositol phospholipids, HSP60 and 70, fibrinogen, nickel, HMGB1		Stir stress IgG aggregates (2–10 mM)
TLR4 and TLR6	OxLDL, amyloid-β fibrils		
TLR5	Flagellin		
TLR7	ssRNA	Imidazoquinoline compounds, imiquimod, resiquimod, loxoribine	
TLR8	ssRNA	CpG's	
TLR9	DNA, hemozoin		

Adapted from Lee *et al. Nature Reviews Immunology* 2012 [15].
a) Reference [16].

response pathway [16]. Furthermore, the same antibody aggregates were able to bind to FcγRI on the surface of DC providing a synergistic activation signal. Molecules that interact with PRR are also internalized rapidly and enter the antigen-processing pathway resulting in efficient presentation of T cell epitopes. Given the diversity of endocytic receptors and PRR expressed by DC, it would not be surprising to find that protein therapeutics may bind to one or more of these receptors and induce DC differentiation as well as upregulate antigen presentation.

From the description above, it is clear that the milieu in which T cells encounter epitopes presented in the context of MHC class II on DC determines the type of immune response (namely inflammatory, noninflammatory, and regulatory). The mechanism by which DC orchestrate the T cell response is dependent on the integration of signals received from innate receptors at the time of antigen encounter. For example, when DC encounter two distinct stimuli such as thymic stromal lymphopoietin (TSLP) and/or CD40L, the cells differentiate similarly with increases in

MHC class II and CD86 expression as well as adopting a highly "dendritic" appearance. However, stimulation of DC with either TSLP or CD40L alone results in either naïve T cell differentiation into inflammatory Th2 cells that secrete TNFα (along with IL-4, 5, and 13), or into Th1 cells, respectively [17]. DC are also subject to different pathways of regulation. Negative molecules that can act on both TLR and cytokine receptor signaling are the suppressor of cytokine signaling (SOCS) proteins [18]. As a consequence of negative regulation, DC are able to expand and/or differentiate different types of suppressive pathways. These include IL-10-producing Forkhead box P3 (FoxP3) − Tr1 cells [19] and $CD25^+$ $CD4^+$ $FoxP3^+$ suppressors [20]. Thus, for protein therapeutics, uptake by DC alone may not in itself be sufficient to stimulate immunity; however, if this is combined with engagement of innate receptors on the surface of DC that results in DC differentiation and upregulation of costimulatory molecules, then T cell activation and differentiation into effector or regulatory T cells is likely to follow.

12.2.2 Adaptive Immunity

12.2.2.1 Antigen Processing

APC such as DC and macrophages have a high capacity for macropinocytosis [21] and phagocytosis [22], reflecting their central role in the triggering of naïve immune responses. Within these cells, antigen uptake is followed by a stepwise process of degradation as the protein becomes exposed to various enzymes and proteases during trafficking through the endosomal pathway. This sequential process ultimately delivers protein to the lysosome where proteases are activated via a drop in pH leading to a highly efficient degradation of the protein [23]. One of the key steps in antigen processing is the reduction of disulfide bonds and this is facilitated by IFN gamma-inducible lysosomal thiol reductase (GILT), which is abundantly expressed by professional APC, particularly DC, and catalyzes disulfide bond reduction under acidic conditions [24, 25]. The action of GILT serves to open up folded proteins, enabling digestion by cathepsins (including: B, S, and L) and asparaginyl endopeptidase (AEP) [26]. AEP is highly specific for exposed asparagine residues and is a key enzyme for the ability of APC to degrade antigen [27] such that mutations to asparagine residues in a protein therapeutic can potentially modify the repertoire of T cell epitopes presented [28]. Clearly, there must be a balance between protein antigen destruction and peptide survival as a highly active proteolytic environment could degrade proteins to such an extent that MHC class II loading is no longer possible, that is, peptide length is reduced to below the minimal core 9mer binding peptide. It has been shown that while macrophages, which target protein antigens for destruction, contain high levels of proteases in their lysosomes, DC lysosomes contain much lower concentrations of proteases in a higher-pH environment, resulting in a limited capacity for degradation that favors antigen presentation [29]. Furthermore, the sequence and structure of protein antigens may also affect rate and outcome of degradation; the presence/absence of disulfide bonds

and their location influences antigen processing and epitope selection [30] as does the presence or absence of protease sites [28] with T cell epitopes more likely found in ordered highly structured regions of proteins that are more resistant to protease cleavage than highly accessible flexible/disordered loops [31–34].

As discussed in detail in the sections above, the majority of innate receptors expressed by DC facilitate endocytosis, and result in more efficient presentation of peptides to T cells by delivering protein therapeutics to the endosomal pathway. For protein therapeutics that comprise human germline sequence against which patients are normally tolerant (such proteins can be considered "immunotolerant"), for example, endogenous proteins such as the Fc domain of IgG, enhanced uptake will not lead to immunity due to tolerance in the T cell compartment. However, if the immunotolerant protein therapeutic is processed differently, for example, through the inclusion of an asparagine mutation (as discussed above) or other mechanism that leads to cleavage of normally inaccessible protease cleavage sites, presentation of cryptic T cell epitopes may result in T cell activation and subsequent immunity. Presentation of cryptic T cell epitopes is of concern when dealing with protein therapeutics that may have similar (or identical) amino acid sequences but differ through minor amino acid mutations and/or posttranslational modifications, for example, glycosylation, deimidation, and deamidation. Such modifications may influence the immunogenic potential of protein therapeutics having highly similar or even identical primary amino acid sequence. This effect is difficult to predict owing to the fact that different APC can process the same protein slightly differently [35]. The challenge of predicting the impact of antigen processing on immunogenicity is exemplified from studies where two IgG1 allotypes G1m3 (CH1 domain), non-G1m1 (CH3 domain) termed "G1m3" versus "G1m17" (CH1 domain), G1m1 (CH3 domain) termed "G1m1,17" were tested for immunogenicity against mismatch IgG1 allotypes in healthy human donors [36]. The two allotypes differed by three amino acids where the G1m3 positions 12 and 4 are E and M (CH3 domain) and position 120 is R (CH1 domain); for G1m1,17 the CH3 domain (positions 12 and 14) are D and L, and the CH1 domain (position 120) is K. Data from an early experimental study suggested that G1m1,17 donors responded to cryptic T cell epitopes in IgG Fc comprising the G1m3 allotype and that mismatch between donor and therapeutic IgG1 may be a factor in enhancing the immunogenicity of therapeutic antibodies [36]. However, this was not supported by the majority of clinical data, which shows that patients receiving rituximab and adalimumab did not show an enhanced capacity to develop antidrug antibodies (ADA) if their IgG1 allotype did not match that of the therapeutic antibody [37, 38]. Furthermore, subsequent experimental evidence using methods to directly measure antigen processing using *M*HC-*A*ssociated *P*eptide *P*roteomic*s* (MAPPs), which allows for identifying the sequences of human leukocyte antigen (HLA)-DR associated peptides directly from human DC, showed that mismatch homozygous donors with trastuzumab variants comprising either G1m3 or G1m1,17 allotypes did not result in differential antigen processing [39].

Antigen processing by professional APC, such as DC, is a complex process that can be affected by a large number of variables, for example, protein uptake, tertiary structure, disulfide bonds, and maturation status of DC. For protein therapeutics, it is apparent that not only may changes in amino acid sequence potentially affect immunogenicity but also the presence of different conformational species present in the product formulation, such as subvisible particles. Indeed, under experimental conditions, stress-induced particles of antibodies have been shown to affect the presentation of MHC class II binding peptides in mDC using MAPPs [40]. Stress-induced particles can result in antigen processing and presentation of alternative MHC class II binding peptide clusters, and these alternative peptides (against which immune tolerance may be lacking) have the potential to become cryptic T cell epitopes. The combined effects of particulates on antigen processing and presentation (including enhanced uptake) coupled with activation of an innate immune response could result in an increase in immunogenicity. Indeed, the effects of subvisible particles on immunogenicity have been highlighted in a variety of preclinical models, most notably immune-tolerant transgenic mice expressing human interferon beta (huIFNβ). In such studies, the presence of aggregated/particulate IFNβ (for which the huIFNβ mice are normally tolerant) results in ADA responses [41]. Although it is clear from these studies that particles with different properties, for example, size, hydrophobicity, loss of tertiary structure, and covalent bonding, vary significantly in the ability to enhance immunogenicity. Furthermore, the precise mechanisms that contribute to the enhancement in protein immunogenicity through the presence of aggregates/particles is more complex than stimulation through innate receptors and an effect on antigen processing.

Once peptides have been processed from the protein, they encounter the classical MHC class II molecules (comprised of three highly polymorphic loci HLA-DR, -DQ, and -DP) in the late endosomal compartment. HLA class II molecules are heterodimers consisting of two chains, α and β, that form a peptide-binding groove open at both ends and allowing for peptides of variable lengths to bind in an extended conformation facilitated by a network of hydrogen bonds [42]. Expression levels among the classical HLA class II molecules are highest for HLA-DR [43, 44] which may be the reason why it is the best-studied HLA class II isotype; the β-chain of HLA-DR molecules is highly polymorphic and there are more than 1700 alleles identified so far in the worldwide population [45], whereas there are only three α-chain alleles. In contrast, both the α- and β-chains of HLA-DQ and HLA-DP are polymorphic, giving rise to potentially thousands of combinations; however, HLA-DQ diversity is limited by incompatibility of certain α/β chain pairings [46] and particular HLA-DP α/β combinations have been found to dominate the potential repertoire [47]. Despite the apparent polymorphic diversity of the classical HLA molecules, the peptide-binding repertoire is not unlimited and there is considerable overlap in peptide-binding specificities both within and between HLA types [48].

The HLA class II α- and β-chains are synthesized in the endoplasmic reticulum of APC where they form multimeric complexes with trimers of the invariant chain

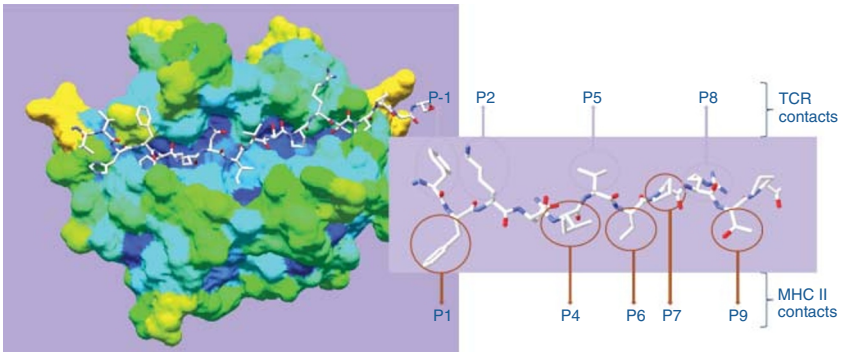

Figure 12.2 Peptide binding to HLA class II molecules. (a) Top view of peptide (stick model) sitting in the binding groove of MHC class II (surface model). The surface is colored to indicate the depth of cavities, with dark blue indicating the deepest pockets. (b) Lateral view of peptide orientation in the binding groove. Residues with side chains oriented downward make contact with MHC class II, whereas side chains oriented upward potentially contact the TCR [63]. Figure prepared using Swiss-PdbViewer (Guex, N. and Peitsch, M.C. (1997) SWISS-MODEL and the Swiss-PdbViewer: An environment for comparative protein modeling. Electrophoresis 18, 2714–2723.) using PDB structure file 1FV1.

(Ii-chain) chaperone [49] that stabilizes the "empty" MHC class II molecules, blocks the peptide-binding groove, and prevents premature peptide loading [50]. The Ii-chain also contains a dileucine motif that targets both the Ii-chain and the associated MHC II to the late endosomal compartments [51, 52] where they encounter peptides derived from degraded antigens in an acidic environment together with a cocktail of proteases, including cathepsins S, L, F, and AEP [26]. These proteases mediate the sequential degradation of the Ii-chain to a peptide of approximately 20 amino acids (*Cl*ass II *A*ssociated *I*nvariant chain *P*eptide, CLIP) [53] that contains a core MHC II binding motif and thus remains associated with the MHC II peptide-binding groove [54–56]. The nonclassical HLA molecule DM is a molecular chaperone which facilitates the exchange of the CLIP peptide with high-affinity antigenic peptides within the late endosome to form the stable peptide/MHC II complex [57–59]. HLA-DM may also remove low-affinity peptides and replace them with high-affinity ones in an iterative process that leads to accumulation of high-affinity peptide/MHC II complexes [60]. The activity of HLA-DM is modulated by a second nonclassical molecule, HLA-DO [61], which may serve to inhibit HLA-DM peptide loading, particularly in certain subsets of APC, but the significance of this interaction is poorly understood. The binding of antigenic peptides within the HLA class II binding groove is dependent on the properties of the amino acid side chains of the peptide at specific positions (termed *anchor residues*) within the core HLA class II binding register [62] (comprising nine amino acids, p1–p9, Figure 12.2). As a consequence, 9mer binding motifs can be determined from the sequences of proteins that may bind to specific HLA class II molecules. However, the issue of peptide binding is more complicated than analysis of the core 9mer as the HLA class II binding groove

is open ended, and can accommodate peptides of varying sizes. Therefore. the influence of residues (p-1, p10, and p11) that are positioned outside of the core 9mer are also considered to be of importance [64, 65]. Peptide elongation, in general, has been reported to result in increased MHC class II molecule affinity with an optimal peptide length of approximately 18–20 amino acids [65] and in peptide-elution experiments from DC, lengths between 18 and 25 amino acids were commonly observed for naturally presented peptides [66, 67]. Ultimately, the peptide/MHC II complexes are trafficked to the cell surface where they are available to be engaged by T cells via the T cell receptor (TCR).

12.2.2.2 T Cell Recognition of MHC–Peptide Complexes

TCRs bear strong structural similarity to antibodies in that they are created from a repertoire of Vα and Vβ gene segments that possess three variable CDRs interspersed with less-variable framework regions. In contrast to antibodies, only the CDR3 loops are highly variable and diversity is created solely by CDR3 V(D)J recombination without the influence of somatic hypermutation [68]. As a result, the interactions between TCRs and peptide/MHC II complexes are relatively weak, compared to antibody/antigen interactions, but are highly specific and sensitive thus allowing both distinction between rare foreign complexes from abundant self-complexes and recognition of very low frequency complexes [69, 70]. During development, thymocytes undergo thymic selection [71] resulting in the generation of a repertoire of TCRs with a diverse range of antigen-binding specificities that are augmented by the fact that, owing to inherent plasticity in the engagement of peptide/MHC II complexes, more than one complex may be recognized by the same TCR [72–74]. The solved crystal structures of several TCR/peptide/MHC complexes have shown a general and relatively predictable pattern for the engagement of the peptide MHC complex by the TCR where the TCR is diagonally oriented along the long axis of the bound peptide with Vα oriented toward the N-terminus of the peptide and Vβ oriented toward the C-terminus [74]. CDR1 and 2 of the Vβ domain generally make contact with the MHC α-chain and CDR1 and 2 of the Vα domain contact the MHC β-chain. The hypervariable loops of the TCR (CDR3 of both chains) lie over the peptide [74]. This arrangement results in a relatively consistent pattern of interaction between the TCR and specific peptide side chains where those orientated away from the MHC II binding groove, that is, p-1, p2, p5 and to a lesser extent p3 and p8 (Figure 12.2 and Table 12.2), are the principal contact residues [74].

Engagement of a peptide/MHC II complex by the TCR is essential, but not sufficient, for the stimulation of an immune response. On $CD4^+$ T cells, CD4 serves to stabilize the TCR/peptide/MHC II interaction and a TCR complex is assembled that includes the accessory molecules CD3 and ζ-chain which are responsible for signal transduction and intracellular signaling. This interaction generates "Signal 1"; however, on its own, this will lead to T cell anergy, whereas a second signal, "Signal 2," is required for T cell activation. This is provided by non-antigen-specific interactions, principally involving CD28 on the T cell and B7 proteins (CD80 and CD86) on the APC that leads to a synergistic interaction between CD40L and CD40 [75]. While this represents early steps in the T cell activation

Table 12.2 Values provided are the total number of peptide contacts (either side chain or main chain) from six crystal structures including both human and murine peptide/MHC II/TCR complexes (1u3h, 1j8h, 1fyt, 1ymn, 1zgl, 1d9k) [74].

	MHC bound peptide contact with the TCR												
Pocket number	−2	−1	1	2	3	4	5	6	7	8	9	10	11
Side-chain contacts	2	25	0	32	21	1	38	0	18	26	0	1	0
Main-chain contacts	5	4	0	8	5	0	1	5	5	9	0	0	0

pathway, further complex ligand/receptor interactions, cytokine interactions, and interplay with a large number of regulatory molecules such as CTLA-4, PD-1–PD-L1, ICOS–ICOSL, GITR–GITRL, OX40, and OX40L serve to manage the nature, extent and longevity of the immune response, reviewed in Refs [75, 76]. Depending upon the intensity, quality, and quantity of the signal generated and the maturation status of DCs, activated T cells may polarize into a variety of T cell subsets that mediate different arms of the immune response (including Th1, Th2, Th17, Th21, and Treg) [77–80].

12.2.2.3 Immunogenicity Risk Mitigation by Protein Engineering

Detailed knowledge of the mechanisms involved in the generation of an immune response and the key properties of protein antigens that stimulate these responses allows for the possibility that protein therapeutics can be modified to escape immune recognition. This can be done at the level of antibody/antigen interaction by using "veneering," that is, the identification of B-cell epitopes which are removed by mutation in an attempt to make a protein "invisible" to antibody and thereby reduce/eliminate cross-reaction with antibodies already present in patient sera [81, 82]. However, the modified protein will still contain T cell epitopes that can drive the immune response; therefore, this type of engineering may result in the modified or other surface regions of the protein therapeutic being recognized, instead, via B-cell epitope spreading, a process that has been well described for autoimmune disorders [83, 84].

A more robust approach is to target T cell epitopes within the protein therapeutic because, as discussed above, these form the essential link in the activation of T cells by APCs and subsequent stimulation of a high-affinity, isotype-switched antibody response. T cell epitopes can be identified either using *ex vivo* human T cell assays [85, 86] or by using computational methods [87, 88]. T cell assays can be used for the accurate and high-resolution mapping of T cell epitopes using synthetic peptides 15 amino acids in length that overlap by 12 amino acids and spanning the entire sequence of the protein of interest. These assays provide information on the frequency of individual donor responses plus the magnitude of response and hence can be used to rank the strength of T cell epitopes and prioritize them for removal by protein engineering. Computational methods tend to be highly overpredictive as they do not "consider" all the processes that are

involved in the development of an ADA response, for example, protein endocytosis, antigen processing, and recognition of the peptide/MHC II complex by the TCR. As a result, there is a risk of overengineering proteins beyond what is required to successfully remove T cell epitopes. Clearly, overengineering a protein therapeutic reduces the probability that the deimmunized product will retain full activity. An alternative computational approach is to use an epitope avoidance strategy that incorporates databases of both T cell epitopes and peptides that have been demonstrated to be immunologically silent using *ex vivo* human T cell assays in combination with HLA class II binding algorithms [89] to filter sequence segments prior to assembly into complete protein therapeutic sequences. This approach is most useful for proteins that belong to extended families of closely related sequences such as therapeutic antibodies.

Whilst *ex vivo* human T cell assays provide the most accurate method for identifying T cell epitopes, *in silico* methods may still be required to identify the core nine amino acids that interact with HLA class II (Figure 12.2), and are useful for identifying changes that can be made to this core 9mer that effectively reduce the affinity of the peptide for HLA class II and therefore disrupt its ability to generate a T cell response. For each T cell epitope, a series of single-point mutations can be designed for the elimination of the epitope, followed by the iterative testing of individual mutations for protein activity. Once active protein variants for each T cell epitope have been identified, combined variants can be generated with combinations of mutations in two or more epitopes in order to identify the final active deimmunized protein variant [90]. Depending upon the location of the T cell epitope within the protein structure, it may not be possible to remove the epitope by targeting the HLA class II contact residues; however, as illustrated by Figure 12.2b and Table 12.2, TCR contact residues provide alternative mutation targets, as do residues such as p-1 that lie outside the core 9mer. Occasionally, choices may have to be made as to which T cell epitope should remain unmodified in order to retain sufficient activity and this underlines the value of being able to rank T cell epitopes in order of potency. Engineered proteins can then be tested in *ex vivo* human T cell assays to confirm that their ability to stimulate T cell responses has been effectively removed [85, 90].

12.2.2.4 Immunogenicity and the Properties of Antigens

As discussed above, the presence of protein aggregates/particles, particularly in the form of subvisible particles, on the immunogenicity of protein therapeutics (particularly antibodies) has been an area of significant research. The potential effects of aggregates on immunogenicity range from providing adjuvant-like properties by interacting with innate receptors on DC through to enhanced protein uptake and presentation of cryptic T cell epitopes as a result of differential antigen processing. Antigenic properties of aggregated particles have also been proposed particularly in the context of eliciting T-independent (TI) immune responses [91]. While this may be possible, it seems somewhat unlikely given that marginal zone B-cell activation (only B1 cells respond to TI-Type 2 antigens) results in low-affinity IgM (occasionally class switching to low-affinity IgG3)

antibody responses. Furthermore, TI responses normally arise in response to polymeric antigens and high-order structures with regular repeating epitopes, for example, DNP-ficoll, viral coat proteins, and capsular polysaccharides of encapsulated bacteria [92]. These antigens present conformational epitopes spaced 5–10 nm apart in a repeat array-like format that are typically observed in higher-order protein structures rather than aggregated protein particles formed in solution [93]. T cell help is therefore a requirement for the development of ADA, which, in patients, have undergone class-switch recombination (IgG1-4 and IgE responses), somatic hypermutation (high-affinity IgG and IgE), and show a memory-like humoral immune response.

Posttranslational modifications have been observed to directly affect the antigenicity (i.e., the ability of an antigen to bind to the B-cell receptor, BCR) of the protein therapeutic. The amino acid sequence of antibodies can, for example, facilitate the presence of unique glycosylation sites in the constant regions of both heavy and light chains as well as the variable domain. The anti-EGFR antibody cetuximab contains an α-1,3-linked galactose sugar in the Fab fragment which has been shown to bind to preexisting IgE antibodies specific for galactose-α-1,3-galactose in pre-sensitized patients [94]. The result was that cetuximab induced severe anaphylactic reactions in some patients treated with the antibody [94]. Cetuximab antigenicity was reduced by expressing the antibody in Chinese hamster ovary (CHO) cells (rather than the mouse myeloma Sp2/0 cell line) which lack α-1,3-galactosyltransferase that forms the terminal galactose-α-1,3-galactose epitope by placing a terminal galactose residue in an alpha-linkage to another galactose. IgE antibodies from patients treated with Sp2/0 expressed cetuximab did not bind to CHO-expressed antibody [95]. Other posttranslational modifications to the antibody sequence may increase immunogenicity including glycation, deamidation, deimidation, and oxidation of amino acid side chains [96, 97]. These posttranslational modifications have the potential to increase immunogenicity through the formation of B-cell epitopes (e.g., development of anti-CCP antibodies in rheumatoid arthritis patients [98]) as well as T cell epitopes by facilitating peptide recognition through the TCR as well as the presentation of cryptic T cell epitopes during antigen processing [96, 97].

12.2.2.5 **Immunological Tolerance**

Competence in the adaptive immune system is achieved through diversity of antigen receptors that have the capacity to bind to the universe of antigens. For both T and B cells the respective TCR and BCR repertoires are generated by random recombination events that take place during lymphopoiesis in primary lymphoid organs. The consequence of random recombination is that a proportion of lymphocytes are produced that bear self-reactive antigen receptors. In order to avoid uncontrolled autoimmunity, antigen receptors are "screened" during early lymphocyte development against self-antigens presented by stromal cells in the lymphoid organs. This process of central tolerance is more rigorous for T cells than B cells as, without T cell help, humoral responses in the periphery are typically short lived and of low affinity. For T cells, central tolerance is achieved by the

deletion of autoreactive thymocytes through negative selection in which $CD4^+$ $CD8^+$ thymocytes with low- or intermediate-affinity TCRs are positively selected and thymocytes with higher-affinity TCRs are negatively selected and undergo clonal deletion. Intriguingly, high-affinity interactions can also signal thymocytes to differentiate into the (FoxP3)-expressing Treg lineage, resulting in a Treg repertoire skewed toward self-recognition [99, 100]. Treg regulate the immune system through mechanisms of peripheral tolerance to prevent auto- and uncontrolled immunity. While the origins and nonoverlapping function of different Treg subsets is beyond the scope of this chapter, Tregs *in vivo* can broadly be divided into thymic-derived (tTreg) and peripherally-induced Treg (pTreg). Owing to the plasticity and stability of Treg subsets *in vivo*, the markers used to determine the origin of different Tregs is far from clear, and such markers can include FoxP3, CD25, Helios, GARP, CTLA-4, and neuropilin. The role of Treg, in general, is to respond to inflammation and integration of environmental cues to limit collateral damage to host tissues [101]. Subdivision of this role between the different subsets of Treg is observed such that tTreg typically act as sentinels of systemic and tissue-specific autoimmunity, while pTreg serve a distinct and essential function in controlling adaptive immunity to restrain allergic-type inflammation at mucosal surfaces.

It is clear that the context under which a T cell is stimulated determines its function and that APCs are the key regulators of Treg development both in the thymus [100] and periphery [102]. Treg regulates the immune response against protein therapeutics, and, for antibodies, this could involve both tTreg as well as pTreg. Indeed, under steady-state conditions, that is, in the absence of innate response signals, DC are tolerogenic and suppress immunity through induction of Treg. Presentation of T cell epitopes derived from protein therapeutics to naïve T cells under these circumstances will not result in the development of an adaptive immune response. Several strategies have sought to reduce immunogenicity by inducing tolerance to the protein therapeutic. For antibody therapeutics specific for cell surface targets, induction of "high-zone" tolerance has been demonstrated by pretreating patients with a nonbinding version of the antibody. This approach was based on the observation that high doses of soluble monomeric xenogeneic human γ-globulin could induce tolerance in mice to immunogenic challenge with the aggregated form [103]. It was therefore reasoned that pretolerization to a highly immunogenic antibody therapeutic could be achieved by pretreatment with a soluble, monomeric, nonbinding variant. Alemtuzumab proved to be the ideal model to test this hypothesis as it is highly immunogenic with ADA responses observed in >60% of patients after single dosing for the treatment of rheumatoid arthritis [104]. A nonbinding variant (SM3) of alemtuzumab was generated by a single-point mutation in CDRH2 in order to retain existing B- and T cell epitopes present in the parental alemtuzumab sequence [105]. Subsequent pretolerization of patients with multiple sclerosis using SM3 resulted in a >70% reduction in ADA, clearly demonstrating that the immune response can be modulated through the induction of tolerance to a protein therapeutic [105]. Indeed, it may be possible to avoid the need to generate a therapeutic "tolerogen" by dosing to induce tolerance. It has been proposed that three-dose induction

therapy with another highly immunogenic antibody, infliximab, can facilitate tolerance induction in Crohn's patients [106], although immunogenicity with this antibody is more conventionally controlled using immunosuppressant drugs such as methotrexate and antihistamines (diphenhydramine) to control ADA, anaphylaxis, and infusion reactions.

New methods of delivery of protein therapeutics are being developed to exploit natural tolerogenic processes in order to reduce immunogenicity. One such method targets protein therapeutics to erythrocytes and takes advantage of the fact that a daily rate of approximately 1% of erythrocytes undergo apoptosis and are cleared by the liver and spleen. It has been observed that apoptotic cells such as erythrocytes are a source of tolerogenic antigens although the exact mechanisms are not fully understood [107, 108]. One possible explanation is that under steady-state conditions, presentation of antigen to T cells in the absence of inflammation, in particular, signals from the innate immune response such as TLRs [109], may facilitate antigen-specific T cell deletion or anergy [110]. Thus, by targeting normally immunogenic proteins to erythrocytes via constructs (scFv fusions or erythrocyte-binding peptides) specific for glycophorin A, immunogenicity can be reduced by T cell deletion [111]. Indeed, the effective deletion of antigen-specific T cells was demonstrated using the clinical therapeutic enzyme *Escherichia coli*-derived L-asparaginase-II (ASNase), which was chemically conjugated to glycophorin A binding peptide (ERY1) [112]. Administration of the ASNase-ERY1 conjugate to mice not only abrogated the development of antibody titers by >1000-fold but also increased the pharmacodynamic effect of the drug 10-fold in mice and tolerized mice to multiple subsequent doses of the wild-type enzyme.

Tolerance can also be achieved through the administration of immunosuppressant drugs for example, coadministration of methotrexate with adalimumab may reduce the incidence of ADA [113] in rheumatoid arthritis patients. Clearly, systemic delivery of immunosuppressant drugs is not always desirable and new methods have been developed using tolerogenic synthetic nanoparticles (tNP) which are loaded with rapamycin [114]. These tNP have been show in experimental models to induce antigen-specific tolerance when the tNP were conjugated to peptides or proteins. Administration of tNP-conjugated therapeutics induced durable tolerance through the induction of Treg and Breg cells even in the presence of potent TLR agonists [114]. Hemophila A mice that were primed against FVIII could also be tolerized with tNP-FVIII molecules. These novel delivery systems offer the possibility of delivering unmodified and potentially immunogenic protein therapeutics in a tolerogenic environment, thus avoiding undesirable, and sometimes detrimental, humoral immune responses in patients.

12.3 Immunogenicity of Protein Therapeutics in Autologous Cell Therapies

The advent of autologous cell therapies that utilize the transduction of recombinant receptors into patient-derived T cells has proved extraordinarily successful

as a treatment modality. One such therapy is chimeric antigen receptor T cell (CAR-T) therapy in which primary autologous T cells are transduced *ex vivo* with CAR specific for tumor targets [115]. However, this type of treatment has led to some significant safety- and toxicity-related issues particularly around cytokine release syndrome (CRS) as well as the immunogenicity of the transduced recombinant receptor (typically a scFv). While it could be argued that CRS, which occurs within minutes to hours of infusion, might be related to primary pharmacodynamics and therefore falls outside our definition of immunogenicity, it is characterized by elevations in cytokines particularly IL-10, IL-6, and IFN-γ which are measurable in most patients with CRS. It is therefore possible that cytokine responses of this nature contribute to increasing the immunogenicity of the therapy. The most advanced CAR-T therapy is used for B-cell acute lymphoblastic leukemia (B-ALL) and comprises a membrane-bound mouse-derived scFv specific for human CD19 coupled to an intracellular signaling domain from CD28 and CD3 ζ-chain [116]. The use of murine IgG variable domain sequences in CAR-T therapy in itself can result in human anti-murine antibody (HAMA) responses in patients. Such HAMA responses can comprise both IgG and IgE antibodies [117, 118] and result in anaphylaxis that at present is only managed by limiting exposure to murine sequences by restricting the expression of CAR to a few days and terminating infusions upon detection of either IgE HAMA or development of anaphylactic responses in patients [119].

12.4 Regulatory Context

The evaluation of undesirable immunogenicity is an essential feature of the development of therapeutic peptides, proteins, and advanced-therapy medicinal products [120, 121]. Sponsors need to present a sufficient weight of evidence in the Marketing Authorization Application (MAA) dossier to enable a reliable assessment of the scale of negative impact of unintended immunogenicity on the overall clinical benefit-to-risk balance. Regulators will assess the incidence and magnitude of treatment-emergent immune responses using a combination of bioanalytical and clinical indices, including the sensitivity of the methodology applied to detect immunologically relevant signals (e.g., ADA), and potential for cross-reactivity of humoral and cellular immune responses with endogenous antigens [122]. The impact of clinical immunogenicity could preclude marketing authorization, particularly if clinical efficacy is marginal.

Regulatory agencies have adopted a risk-based approach to assess potential immunogenicity of therapeutic proteins and peptides during the whole life cycle of the product. This risk-based approach acknowledges that the level of risk should take both the severity of consequences and the probability of occurrence into account, as well as uncertainty associated with detection of pertinent risks for the particular product and target population [120, 121]. Table 12.3 illustrates the diversity of immunogenicity-related risks that have been identified for authorized

Table 12.3 Identified immunogenicity risks for approved therapeutic proteins and antibodies.

Product	Clinical impact of immunogenicity	References
Epoetin alfa	Cross-reactive neutralizing ADA causing antibody-mediated PRCA	[123]
Cetuximab	Severe allergic reactions in pre-sensitized subjects due to cross-reactive antibodies to nonhuman glycan posttranslational modification	[95]
Pegloticase	Enhanced clearance, loss of efficacy and infusion reactions associated with PEG-reactive antibodies	[124]
Infliximab	Immune complex-related hypersensitivity and loss of efficacy	[125, 126]
Clostridial collagenase	One report of systemic hypersensitivity on re-treatment of a Dupytren's syndrome patient	[127]
Rituximab	Loss of efficacy in patients with severe pemphigus and rare cases of hypersensitivity reactions	[128, 129]
Adalimumab	Loss of efficacy and increased incidence of injection site reactions	[130, 131]
Somatropin	Possible reduction in PK, PD *and/or* efficacy in very rare cases	[132]
Insulin	Possible reduction in PK, PD *and/or* efficacy in very rare cases	[133]
Follitropin-alfa	Negative impact not identified	[134]
Bevacizumab	Negative impact not identified	[135]
Trastuzumab	Negative impact not identified	[136]

Key to abbreviations: ADA, anti-drug antibody; PRCA, pure red cell aplasia; PEG, polyethylene glycol.

therapeutic proteins. The consequences cover the full range from highest severity outcomes to no apparent impact. It is noteworthy that many of these risks were identified only during wider post-authorization exposure, underlining the need to incorporate ongoing evaluation of immunogenicity in the long-term risk management plan.

There is considerable uncertainty about how product-intrinsic (e.g., protein-related) and extrinsic (e.g., manufacturing process-related) factors may interact to influence the clinical immunogenicity. Given that any one, or a combination of factors, could contribute to immunogenicity, it is generally advisable that a holistic multidisciplinary approach be taken [137]. This approach links an understanding of the structural attributes of the product to systems biology, control of the manufacturing process and product quality, genotypic, and phenotypic characteristics of the target population as well as conditions for therapeutic use. Use of a carefully structured list of questions addressing intrinsic and extrinsic factors can help to guide the identification of pertinent risks, and then to align appropriate evaluation and mitigation activities [138].

As explained earlier in this chapter, there is increasing recognition that product-related variants and process-related impurities may influence innate and adaptive immune responses. Accordingly, analytical characterization and product quality control play a primary role in the management of immunogenicity-related risks of therapeutic proteins. Equally, it is understood that *in silico, in vitro*, and *ex vivo* methodologies can provide instructive information about the nature of product-related risk factors and the mechanistic basis of undesirable immunogenicity – thereby enabling risk mitigation strategies (such as deimmunization) to be employed during the earlier phases of product development.

12.5 Application of the "Risk-Based Approach" for Undesirable Immunogenicity

12.5.1 Linkage to Product Life Cycle

Immunogenicity-related risks for novel investigational products should, where possible, be assessed from the earliest stages of the development process. The focus can be refined during successive developmental stage-gates to reflect the accumulation of knowledge at each stage and thereby, to enable preclinical, clinical, and post-authorization activities to focus on evaluation of the most pertinent product- and target-specific factors. Because the nature of the risks depend on the interaction of different intrinsic and extrinsic factors, the weight of evidence required for any particular product will need to be aligned with both the specific structural features of the molecule and the proposed conditions of therapeutic use. Detection of unexpected signals during early stages of development should then be taken into account in the iterative immunogenicity risk assessment process.

12.5.2 Initial Risk Assessment for Lead Candidate Selection

Ideally, an initial immunogenicity risk assessment would be performed at the lead candidate selection stage. The initial risk assessment would seek to identify factors that could influence the undesirable immune response to administration of the investigational therapeutic protein to defined clinical populations. For therapeutic antibodies, this should include assessment of the biodistribution and function of the cognate antigen because the immune response could be enhanced if on-target binding resulted in activation of immune effector cells and/or enhanced uptake into APC [139].

Relative ranking of intrinsic immunogenic potential could be performed using a combination of the methods discussed earlier in this chapter, benchmarking results of *in silico, in vitro*, and *ex vivo* analyses against related products for which the clinical immunogenicity profile has been established.

For example, for an antibody-related therapeutic, the following potential intrinsic risk factors might be relevant:

- Presence of high-affinity, promiscuous MHC class II binding motifs associated with unique variable domain including the antigen-binding regions (CDRs);
- Site-directed mutations in IgG Fc regions, introduced to modify effector functionality or stability;
- Immunoglobulin scaffolds that may expose normally cryptic B- and T cell epitopes.

Regulatory and commercial risks associated with these factors can be mitigated, often to a large extent, by applying the "risk minimization" methodology discussed earlier in the chapter. Identification of incremental risk would provide an opportunity to apply molecular engineering options, for example, to deimmunize an otherwise promising investigational candidate prior to further development. Although limited in quantity, there are clinical data verifying the utility of *in silico* tools to estimate intrinsic immunogenic potential that did manifest as detectable immune responses in patients [140].

Information on relative immunogenic potential of a novel candidate might also influence choice of the host cell for manufacture if structural features (e.g., addition of N-terminal methionine or signal peptide sequences) associated with expression in microbial organisms were identified as increasing the risk of binding to MHC class II. Incorporation of non-native amino acids into therapeutic peptides might also introduce novel Tcell epitopes – a risk that could be identified by *in silico* or *ex vivo*/*in vitro* analysis of lead candidates.

Understanding relative intrinsic immunogenicity from the earliest stage may also inform critical decisions associated with Chemistry, Manufacturing and Control (CMC) strategy, and guide the interpretation of causality of unexpected clinical immunogenicity. For example, efforts to reformulate an apparently immunogenic candidate might be less productive if the cause of immunogenicity were related primarily to intrinsic molecular features, rather than solubility. Conversely, more resource might be applied to reformulating a product that was known to have a relatively low intrinsic immunogenicity. Most importantly, observations of clinical immunogenicity could be rationalized as being the expected outcome of intrinsic immunogenic potential, as opposed to manufacturing, product quality, or formulation variables; and that the ultimate approvability of the candidate would depend on demonstration of an unequivocally positive overall clinical benefit versus risk in the target population(s).

12.5.3 Early Screening to Identify "Cryptic" B-Cell Epitopes

Unexpected immunogenicity of an investigational single-domain antibody was found to be associated with a cryptic B-cell epitope formed at the C-terminus of a 12 kDa single-domain antibody comprising a humanized Ig V_H region [141, 142]. This resulted in hypersensitivity reactions following first administration to

healthy volunteers in a Phase 1 study. The mechanism of these hypersensitivity reactions appeared to involve binding of preexisting antibodies to the C-terminus of the single-domain antibody. Modification of one amino acid residue at the C-terminus substantially reduced binding to preexisting antibodies, which translated into decreased incidence and severity of clinical hypersensitivity reactions.

This case illustrated both the difficulty of predicting immunogenicity of a modified version of a therapeutic protein, as well as the value of screening novel candidates *in vitro* for binding capacity to preexisting antibodies to identify, and thereby avoid, unexpected immunogenicity. Ultimately, only clinical evaluation in relevant populations will enable an accurate assessment of immunogenicity but, potential risks for human subjects can be identified early to enable a more cautious approach to administration of an investigational product and monitoring the consequences.

12.5.4 Control of Product Quality

A number of extrinsic, product quality-related factors could influence immunogenic potential of a therapeutic protein. Figure 12.3 describes recognized variables that could interact to affect immunogenicity in an unpredictable manner. While not feasible to predict how a particular combination of these variables might interact with the intrinsic immunogenicity of a therapeutic protein, there is a regulatory expectation for the individual variables to be controlled within prespecified limits [137]; suitability of the product quality control strategy is then qualified by the results of the immunogenicity evaluation in pivotal clinical studies using the drug product-primary container combination to be commercialized.

The role of innate immune effector cells in enhancing adaptive immune responses merits particular attention in respect of therapeutic monoclonal antibodies because of the potential for the IgG Fc region to interact with Fcγ receptors expressed on monocytes [143]. Binding affinity for all three activating

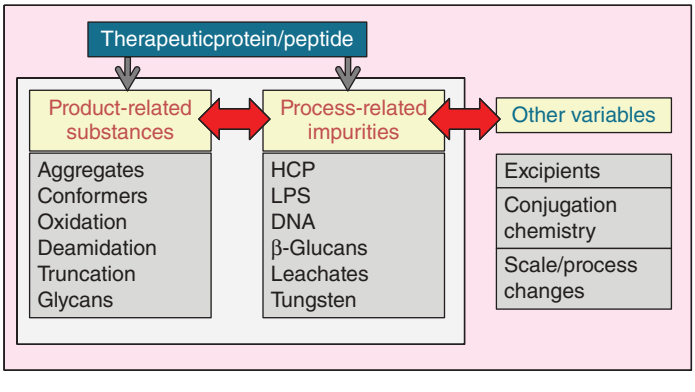

Figure 12.3 Potential interacting CMC variables that may influence immunogenicity.

Fcγ receptors is substantially enhanced by the formation of dimers or aggregates of human IgG_1 [144], which may explain the observation that such aggregates can activate innate immune effector cells [16] in addition to enhancing antigen uptake and presentation by DC [12, 40].

Given that therapeutic antibodies for subcutaneous administration are often formulated at a relatively high concentration (e.g., 100 mg/ml), demonstration of effective minimization of dimerization or aggregate formation in the drug product-primary container combination represents a critical feature of the immunogenicity risk minimization strategy. Since residual tungsten-related impurities in prefilled syringes have been identified as a risk factor for enhancement of protein aggregation [145, 146], appropriate selection of the primary product container also forms part of this strategy. In addition, it is prudent to evaluate stability of the monomeric protein at physiological pH and temperature to understand the propensity for oligomer formation following administration, and whether this could be related to the level of unpaired cysteine residues in the hinge region of the IgG molecule.

Although representing an unusual case, the example of cetuximab (described in Section 12.2.2.4) is a salutary warning concerning the influence of the choice of host cell for manufacture of a therapeutic monoclonal antibody [95]. Both the qualitative (α-1,3-linked galactose and *N*-glycolyl neuraminic acid) and quantitative-spatial (high levels within Fab regions) nature of the nonhuman posttranslational glycosylation associated with the Sp2/0 host cell resulted in a protein that has the potential to induce severe systemic hypersensitivity reactions in pre-sensitized subjects; subjects are routinely pretreated with diphenhydramine to diminish this risk. Accordingly, the decision on choice of host cell for any glycosylated therapeutic protein should be based on a careful assessment of potential immunogenicity. In the context of a biosimilar cetuximab development, the severity of consequences associated with the posttranslational glycosylation profile of the originator cetuximab would favor choice of an alternative cell line, for example, CHO, which produces a more humanlike glycosylation profile. This would be acceptable in the EU regulatory framework if there were no significant difference in clinical efficacy of the biosimilar cetuximab compared to the reference product.

12.5.5
IND-Enabling Safety Studies

Although studies in nonhuman species are not predictive of immunogenicity in humans, it may be necessary to monitor ADA formation of novel therapeutic proteins in preclinical safety studies to understand their potential impact on drug exposure [147]. Measurement of ADA in nonclinical studies should be evaluated when there is (i) evidence of altered PD activity; (ii) unexpected changes in exposure in the absence of a PD marker; or (iii) evidence of immune-mediated reactions (immune complex disease, vasculitis, anaphylaxis, etc.). A potentially important limitation is the sensitivity of the ADA assay to interference by the

relatively high circulating drug concentrations achieved with the drug levels administered in nonclinical toxicology studies, which may result in underdetection of ADA. Accordingly, it may be more valid to apply a generic immunoassay to measure immune complexes [148].

In the case of human or humanized monoclonal antibodies, a substantial host immune response to the xenogeneic protein is expected; this may include a strong humoral response to the human IgG Fc region, which is unlikely to occur in humans by virtue of immune tolerance to syngeneic amino acid sequences. Nevertheless, antibody-mediated toxic effects may well be observed and, if so, it would be prudent to demonstrate a causal link to immune complex formation to exclude these signs as being relevant for the no-observed-adverse-effect-level [149, 150]. Attempting to "dose-through" the host immune response by increasing the dose of the investigational therapeutic protein, in order to exceed the ADA level to achieve exposure to the free drug, may be counterproductive if it simply serves to increase immune-complex-related toxicity.

In the case of novel therapeutic products that have a high degree of structural and functional homology with endogenous factors in different nonhuman species, for example, GLP-1 peptides, the specificity and functional impact of host immune responses in animal toxicology studies may reflect those likely to occur in humans. This possibility should be taken into account when planning the preclinical safety program. Simply regarding nonclinical studies as being "not relevant" would be unwise, as there are numerous examples where regulatory agencies have requested supportive data to understand the consequence of immune responses to products that share common structural features, particularly for cytokines in the TGF-β family for which ADA responses could cross-react with related endogenous proteins.

Directly comparative nonclinical studies for different versions of the same protein therapeutic may also be useful for evaluation of incremental risk for modified versions of a first-generation product; this is illustrated by recently approved versions of coagulation factors [151, 152].

12.5.6 First-Time-In-Human Studies

Typically, immunogenicity evaluation represents a secondary endpoint in the first-in-human clinical study of an investigational therapeutic protein or peptide. Descriptive analysis of the scale of immunogenicity – reflected as the incidence and magnitude of the ADA response allied to potentially immune-mediated adverse events – are expected, in addition to correlation of pharmacokinetic (PK) parameters to the ADA response. In the case of therapeutic monoclonal antibodies, the AUC_{0-t} and AUC_{0-inf} parameters may represent the most sensitive clinical correlates of ADA formation, particularly if the drug concentration assay detects only the unbound, active drug [153].

ADA detected in a suitably qualified ligand-binding assay should be evaluated for specificity, titer, and capacity to neutralize a relevant biological function of the

therapeutic protein. Clear distinction between neutralizing and non-neutralizing ADA may be confounded by the different sensitivities of the assay methods used – often, these may represent the same population of ADA in terms of binding specificity. Potentially, ADA could enhance or reduce the rate of clearance of a therapeutic protein in addition to direct neutralization of biological function, leading to altered clinical activity. Comparison of ADA levels relative to drug concentration (unbound and total) and target ligand concentration can be helpful to distinguish between these different effects.

In some cases, the immunogenicity analysis performed in the Phase 1 study has provided important information on the scale of the risk. For example, systematic investigation of potential causes of a high incidence of low-titer ADA observed in a Phase 1 study of ATR-107, a fully human anti-IL-21 receptor antibody candidate for treatment of lupus and other autoimmune diseases, revealed how the enhanced presentation of a T cell epitope identified by *in silico* analysis could be related to expression of the target antigen on DC [154]. Moreover, by applying novel methods for evaluating binding to, uptake by, and activation of APC, the clinical results effectively validated the application of nonclinical tools to identify a candidate having a relatively high risk of clinical immunogenicity.

12.6 Clinical Proof of Concept and Beyond

Monitoring of undesirable immunogenicity should be performed in all subjects treated with therapeutic proteins and peptides in Phase 2 and Phase 3 clinical studies [120, 121]. Correlation of measures of ADA incidence, titer and neutralizing capacity with active drug concentration, pharmacodynamics (PD) markers, efficacy, and incidence and severity of potentially immune-mediated adverse events enables assessment of the dynamic relationship of the treatment-emergent ADA response to clinical manifestations. Since many factors may interfere with signals detected in the bioanalytical assays applied to measure drug concentration and ADA, these assays require careful validation to understand sources of bias in relevant test sample matrices [155]. Measures of drug concentration and ADA may not be independent variables because ADA may influence accuracy of the PK assays [156], and residual drug might reduce sensitivity of the ADA assay [157]. Since the apparent relationship between the ADA response and clinical parameters can be substantially influenced by the type of assay format used [158], it is essential always to interpret bioanalytical results in relation to relevant clinical endpoints to assess actual immunogenicity of a product candidate in a particular therapeutic setting. Monitoring circulating immune complexes and complement activation should be considered if there were clinical signs consistent with serum sickness. Potential allergic-type hypersensitivity might trigger follow-up investigation of antigen-specific IgG and *ex vivo* basophil activation testing.

Sponsors are encouraged to investigate causality of unexpected immunogenicity of novel candidates prior to marketing authorization. The impact of immune-mediated adverse events in approvability depends on a number of factors, including unmet medical need for the product and magnitude of efficacy relative to risk mitigation opportunities for routine clinical use. Uncertainty about the scale of immunogenicity-related risks at the time of marketing authorization may require post-marketing commitments that involve monitoring of immunogenicity in randomized controlled interventional clinical studies, as exemplified by the recent approvals of two different therapeutic antibodies for treatment of hypercholesterolemia [159, 160]. Understanding the impact of patient-related variables such as genotype, levels of immune tolerance and immune competence, comedications and comorbidities may also help to minimize the extent of ongoing risk management provisions.

12.7 Future Perspectives

On the basis of the primary role of MHC class II binding affinity in influencing the relative intrinsic immunogenicity of peptides derived from therapeutic proteins, it would be expected that the HLA genotype of treated subjects would influence immunogenicity. Although there are data to demonstrate such a relationship for infliximab [161], HLA genotyping is not yet a routine feature of randomized clinical trials. More frequent HLA genotyping could contribute to the identification of subjects at heightened risk of undesirable immunogenicity. Novel methodologies for inducing immune tolerance to therapeutic proteins [112, 114] show considerable promise for overcoming the negative clinical impact associated with immunogenicity of therapeutic proteins, particularly in therapeutic settings of severe disease or absence of alternative treatment options.

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13
Expression Systems for Recombinant Biopharmaceutical Production by Mammalian Cells in Culture

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13.1
Introduction

Since the approval of the first recombinant biopharmaceutical produced by engineered Chinese hamster ovary (CHO) cells in 1987, namely tissue plasminogen activator (tPA) [1, 2], bioindustrial production of recombinant therapeutic proteins by this method has increased dramatically. Currently, 6 of the top 10 best selling medicines are recombinant proteins (predominantly monoclonal antibodies (mAbs)) produced by CHO cells, with many new mammalian-cell-derived recombinant therapeutic proteins in development [3].

Over this time, progressive improvements in manufacturing technologies – from genetic vector engineering to process engineering – have substantially intensified the production processes, enabled control of product molecular heterogeneity, and, critically, reduced development time [4]. Disruptive innovations such as new technologies for protein product engineering [5–7], clone isolation [8], disposable processing [9], product/process monitoring [10, 11], purification [12], continuous manufacturing [13, 14], and a host of other incremental developments (e.g., synthetic media design; [15]) continue to speed new therapeutic products into the clinic. However, the basic "input" components of the mammalian-cell-based manufacturing process remain the same: a genetic vector is used to engineer a transformed mammalian cell that proliferates *in vitro* to produce a complex glycoprotein product – vector, cell, medium, and process. Therefore, manufacturing systems, which are still largely based on fed-batch upstream cell culture (and driven also by the dominance of monoclonal antibody products), have tended to converge on optimized platform technologies (e.g., [16]) that interface these components efficiently to reduce the time spent on the

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critical path to the clinic. Moreover, as product pipelines diversify away from mAbs to include a greater proportion of non-natural engineered protein formats, such as bispecific antibodies and fusion proteins, early diagnosis of product "manufacturability" has become an essential part of the development process [17, 18]. The advent of "biosimilar" and "biobetter" products in development has highlighted the link between the critical quality attributes of the product, production process variables, and product performance characteristics (e.g., bioactivity, immunogenicity, equivalence). In this context, quality by design (QbD) is a key regulatory concept leading to improved product and process design [19, 20].

The science and engineering base underpinning mammalian-cell-based biopharmaceutical manufacturing has, in recent years, undergone similar disruptive change in that fundamentally new tools, resources, and concepts have emerged. Arguably, the most significant scientific development is the advent of "CHOmics," that is, genome-scale quantitative cataloging, description, and bioinformatic analysis of the genome and cellular machinery of CHO cells at different layers of cellular organization. Next-generation DNA sequencing technologies have enabled large CHO cell genome [21–23], epigenome [24], and transcriptome [25, 26] datasets to be obtained rapidly at relatively low costs. New high-throughput mass spectrometry-based proteomic [27, 28] and metabolomic [29] analyses provide complementary datasets, although these require specialist laboratory infrastructure, where commercial service providers are not generally available.

Importantly, facile techniques to specifically edit the mammalian cell genome are now available [30–32], and these methods are beginning to be utilized to engineer CHO cell function *in vitro*. For example, genome editing to remove α-1,6-fucosyltransferase (Fut8) activity from CHO cells has already enabled the production of recombinant mAbs lacking core fucose with increased bioactivity [33]. Noncoding RNAs (e.g., miRNAs) are also beginning to be used as tools for CHO cell engineering [34, 35]. These short (~22 nt) RNAs are potentially attractive tools for CHO cell engineering, as a single miRNA, which will not contribute to the translational and synthetic burden associated with the expression of functional effector genes, can repress the translation of hundreds of target transcripts to regulate complex cellular processes that may improve CHO cell factory performance *in vitro* such as resistance to apoptosis [36], improved recombinant protein production [37–39], and cell proliferation [40, 41].

Despite the availability of these powerful new tools and resources, the real challenge now facing CHO cell engineers in this new era of systems biology, computational biology, and bioinformatics is how to rationally employ or mine genomic resources and analyses to drive the development of useful, new functional phenotypes, that is, to predictably link the genotype to the phenotype [42–44]. In the same way the QbD paradigm is driving manufacturing process development, a design-led *modus operandi* must drive cell factory engineering, where it is carried out *in silico,* using computational models of core CHO cell functions that are relevant to functional performance such as central carbon metabolism [45–47] and N-glycosylation [48, 49]. In this context, the current global interest in mammalian

synthetic biology [50] may be usefully applied to engineered mammalian cells, which already utilize synthetic multigene constructs. This will require the development of new genetic parts and systems (e.g., [51, 52]) that will make precision, and even bespoke, product-specific genetic engineering of CHO cells a reality.

There is no doubt that challenges lie ahead. For example, a confounding feature of the CHO cell "quasi-species" [53] is clonal variation in functional performance deriving from significant genetic variation between CHO cell families and clones [54, 55]. Thus the same genetic engineering solution may not be applicable to all CHO cell types, or clonal isolates, or it may vary with respect to the core "input" variables: vector, cell, medium, and process. In this respect, computational prediction of the functional consequences of engineering interventions will ultimately be as necessary as it is for other traditional engineering disciplines. To enable both significant reductions in development times and improvements in production yields for the new non-mAb products, there is a need to shift from screening-led to design-led technologies; embedding prediction and design of product manufacturability at an earlier stage in the bioprocess development to speed products into the clinic.

In this chapter, we cover the current core technologies used to engineer mammalian cells for biopharmaceutical production. We anticipate that further radical changes to biopharmaceutical manufacturing processes toward flexible and disposable manufacturing facilities [56] will harness advances in cell engineering technology.

13.2
Host Cell Systems

13.2.1
Chinese Hamster Ovary (CHO) Cells

Although other murine myeloma lines (NS0, SP2/0) and human-sourced cell lines such as human embryonic kidney (HEK293) and the immortalized human fetal retinoblast cell line PER.C6 [57] have been utilized [58–60], CHO cells are currently the predominant production vehicle of choice. From 1982 to 2014, 35.5% of approved protein therapeutics utilized CHO cell hosts [58]. In 2014, 5 of the top 10 best selling recombinant proteins were produced by CHO cells [3, 61], with combined global revenues of ~US$140 billion in 2013 [58].

Transformed CHO cells were first isolated in 1957, deriving from a Chinese hamster (*Cricetulus griseus*) primary cell culture [62]. Since their initial isolation, CHO cells have been subjected to multiple genetic engineering strategies and adaptation processes. This has resulted in the establishment of several lineages, including CHO-K1 ($DHFR^{+/+}$), DUKX-B11 ($DHFR^{-}$), CHO-DG44 ($DHFR^{-/-}$), CHO-S (suspension adapted), CHO-K1SV (GS-KO), and hundreds of other industrial clonal-derivative CHO cells used for biopharmaceutical production [53, 63, 64]. While all CHO cells are auxotrophic for one or more amino acids

(e.g., proline, arginine, cysteine) as per the ancestral proline-deficient (pro-)CHO cells [43, 65], all derived cell lines have significant metabolic and genomic differences.

The first biopharmaceutical produced in CHO cells was manufactured in the DUKX-B11 cell line (originally isolated from CHO-K1) [53, 66]. This cell line was created to carry a deleted dihydrofolate reductase (DHFR) locus and a mutation on the other DHFR locus, making them DHFR-deficient and unable to reduce folate to tetrahydrofolate [53, 66], which is an essential precursor for purine, thymidylate, and glycine/serine metabolism [67]. Co-transfection of a DHFR gene with the gene of interest (GOI) enabled the easy selection of recombinant cells in a medium lacking hypoxanthine and thymidine. The CHO-DG44 cell line (from a different mutant CHO-Mtx-RIII) was established using the same approach, but it carried a full deletion of the two DHFR loci [53, 68]. Another descendent of CHO-K1 has been utilized with an alternate metabolic selection/amplification system. These cells are co-transfected with a glutamine synthetase (GS) gene and GOI, and then cultured in the absence of glutamine, with an added GS inhibitor (methionine sulfoximine (MSX)), thus selecting recombinant cells (CHO – GS system) [69]. Recently, endogenous GS activity has been removed from CHO-K1 cells via zinc finger nuclease (ZFN) technology-mediated knockout of GS to improve selection stringency [70]. Although other easily applicable antibiotic selection techniques are available, such as neomycin, hygromycin, and puromycin selection [71], metabolic selection/amplification techniques (DHFR and GS) are the most used selection systems for biopharmaceutical production. This is mainly due to the ability of DHFR and GS systems to offer a higher degree of selectivity and gene amplification than antibiotics systems. Another derivative of the original CHO isolate was quickly adapted to suspension growth in Thompson's lab [72], giving rise to the CHO-S cell line. At the time, the suspension CHO-S cell line was inherently different to the DUKXB-11, CHO-DG44, and CHO-K1 populations since these cell lines were maintained as adherent cultures but have subsequently been adapted to suspension cultures [53].

The plethora of different metabolic selection and culturing techniques has resulted in functional heterogeneity between the various CHO host cell lines [53]. To characterize this, much effort to use "CHOmics" technologies has been made in order to characterize the genetic [21], proteomic [27, 73, 74], and transcriptomic [75, 76] differences between CHO cell lines. Compared to the original hamster karyotype (22 chromosomes), 13 of the CHO-K1 chromosomes differed as a result of deletions and rearrangements. CHO-K1 and DG44 populations each contained a modal chromosome number of 20 [21]. Genomic studies on seven different cell lines from three different lineages and two culture modes (i.e., suspension and adherent) identified (i) important changes in the expression levels of key anti-apoptotic (e.g., Akt and Bcl-xL, NF-κB, protein kinase A) and pro-apoptotic (e.g., endonuclease G, IκBα, BAX) genes, and (ii) the abundance of single-nucleotide polymorphisms (SNPs) (>3.7 million, 67% shared among all CHO cell lines) and indels (>500 000) between the hamster genome and the different CHO cell lines [54].

This propensity of CHO cells (as per other transformed mammalian cells) for genetic instability also underpins their utility in cell line development. For example, genetic variants with unusual phenotypic traits that enable large-scale recombinant protein production (e.g., suspension growth in a synthetic environment, elevated IVCD performance, and high recombinant protein productivity) can be isolated. Unfortunately, such genetic instability also predisposes CHO cells to genetic drift, which may manifest itself as production instability or altered fed-batch culture performance over repeated subculture [55]. Another major attribute of CHO cells is their inherent incompatibility to human pathogens, making CHO cells safe expression hosts due to their inability to propagate human viruses that are of potential concern (i.e., polio and the human immunodeficiency virus) [43, 77, 78]. This alone sets them apart from equally able human production hosts.

CHO cells can easily be adapted to suspension culture, making it easier to maintain multiple liters of bioreactor cultures producing kilograms to tons of the product [79]. They can be grown at high densities in culture, and they adapt readily to synthetic environments. Because of regulatory concerns, import regulations based on its geographical origin, purification issues, and costs associated with serum, chemically defined serum-free media formulations have been developed, offering cost-effective advantages compared to bovine serum supplementation. Among these advantages are greater reproducibility in media compositions and culture conditions, reduced downstream purification challenges, cost-effective formulation and constant sourcing, reduced risks of viral, prion and mycoplasma contamination, and minimized risk of transmitting animal spongiform encephalopathy agents [78, 80].

For biopharmaceuticals, glycosylation is an important attribute for the pharmacokinetics and pharmacodynamics of drugs and patient safety. Glycosylation can also impact the mode of action of certain mAbs and Fc-fusion proteins [81]. CHO cells confer "human-like" glycans onto recombinant proteins, which is generally considered to be safe [43]. The attachment of two specific immunogenic epitopes (Galα1,3-Galβ1,4-GlcNAc and *N*-glycolylneuraminic acid (NeuGc)) is often regarded as a structural alert since it causes potential immunogenic reactions in humans [59, 82]. Compared to other mammalian cell systems (e.g., murine), CHO cells produce lower levels of both epitopes [59, 83], thus making them favorable production hosts.

Some issues associated with the use of CHO cells as production hosts remain. For example, CHO cells have relatively slow proliferation rates (doubling time of 20–24 h) compared to bacteria (doubling time of ~20 min) or yeast (doubling time of 1.3–2 h) [43], and exhibit high genetic instability rates that may result in a constant cell-to-cell variation in terms of functional attributes (e.g., transgene expression [84] protein quality [85], and proliferation rate [86]) and therefore in an unpredictable manufacturing performance of production clones. To prevent this "bottleneck," highly intensive and long-term studies are required to identify any signs of instability before selecting the final production clone [87]. Recent research into CHO host cell proteins have highlighted the challenges of

downstream processing of products manufactured in CHO [88]; however, this can be addressed by a rational purification design to remove these impurities.

The proven regulatory approval history (specifically by the US Food and Drug Administration (FDA) and European Medicines Agency (EMA) [89]) for producing therapeutic proteins and easy culturing technologies have made CHO cells the model system for biopharmaceutical manufacture. Their dominance is largely underscored by the huge amount of development work that went into improving the CHO cell bioprocess [77]. This has resulted in high titers in both stable and transient modes of expression [90, 91]. With continuous efforts being made to alleviate the bottlenecks in CHO production systems, CHO cells are set to remain the preferred host cell system for biotherapeutic production.

13.2.2
Alternative Mammalian Cell Hosts

Alternative mammalian cell hosts include baby hamster kidney (BHK) cells, mouse myeloma cells (NS0, Sp2/0-Ag14), and human lines such as HEK 293, CAP, HKB-11, PER-C6, and HT1080 [59, 89, 92]. Interestingly, HEK 293 is more efficient in producing specific carboxylation and hydroxylation modifications for a particular protein product as compared to CHO [93]. Additionally, PER.C6 has produced some promising human-like glycosylated proteins, although no product has achieved regulatory approval yet [94]. Similarly, the CAP cell line has shown to reach high product titers, having products in preclinical stages, whereas HT1080 cells have been utilized in the manufacture of four recombinant proteins [89]. Lack of approvals and pathogen propagation concerns hamper the utilization of human cell types. With regard to the rodent-derived cell lines, BHK hosts have been somewhat limited to the production of coagulant factors. Glycosylation genes in NS0 express high levels of two epitopes (Galα1,3-Galβ1,4-GlcNAc, and NeuGc) that are considered potentially immunogenic in humans [59]. Humans carry antibodies against these glycans, and therefore such glycoproteins could trigger potential immunogenic responses and alter pharmacokinetics [89, 95]. Currently, around 10 approved products are produced using mouse myeloma cells [58].

13.2.3
Non-mammalian Expression Systems

The first biopharmaceutical approved worldwide (recombinant insulin) was produced in an *Escherichia coli* (*E. coli*)-based system [96]. *E. coli* systems have been extensively used for the synthesis of simple proteins because of their fast and easy cultivation and low production costs. Currently, certain limitations deter industry's utilization of this host. These include their inability to produce complex PTMs (especially glycosylation), inefficient disulfide bond formation, and proteolytic degradation in the cytoplasm, coupled with their inability to secrete [96]. Engineering *E. coli* to secrete the protein product into the periplasm

is an attractive prospect, since the oxidizing environment of the periplasm aids the formation of disulfide bonds [92, 96]. Commercial products such as Fab fragments and full-length aglycosylated antibodies are produced by this engineered secretory pathway [96]. Following on from the successful incorporation of N-glycosylation machinery from *Campylobacter jejuni* [97–99], current efforts focus on producing more human-like glycans in *E. coli*. Nevertheless, *E. coli* is the second most utilized host type, contributing ~19% of all approvals since 1982 [58].

Yeast is known to be one of the simplest eukaryotes because of their unicellular nature. The two main species of yeast involved in biopharmaceutical production are *Saccharomyces cerevisiae* (*S. cerevisiae*) and *Pichia pastoris* (*P. pastoris*). *S. cerevisiae* is a GRAS (generally recognized as safe) food additive [100] and hence beneficial from a regulatory point of view [92]. However, their ability to produce complete and correct PTMs is somewhat limited. *P. pastoris* can be regarded superior to *S. cerevisiae* because of the high levels of secretion and low host cell protein accumulation [101]. Proteins from yeast systems embody a major issue of attached sugars that are high in mannose, increasing the likelihood of immune responses in humans and shortening the serum half-life [101]. Around 23 products (mainly vaccines) have been approved for therapeutic use worldwide (21 in *S. cerevisiae* and 2 in *P. pastoris*) [58]. The majority of proteins produced using yeast systems are small and easy to express (e.g., insulin analogs); however, one fusion protein produced in *S. cerevisiae* has been given regulatory approval [58]. Specific glycosylation gene introductions and knockouts have tackled the hyper-mannosylation issue and conferred the ability to produce more mammalian-like glycosylation [102], and the production of terminally sialylated recombinant erythropoietin has been reported [103].

Insect cells are mostly employed for the production of protein-based vaccines. Currently, there are three commercial protein vaccines that are produced by insect systems [58]. The protein production system is based on the infection of lepidopteron cell lines with a baculovirus, which can only propagate in invertebrates and thus eliminates any chances of infection in humans [104], to form the insect cell-baculovirus expression vector system (IC-BEVS) [105]. Lepidopteran host cells can perform relatively complex protein folding and assembly functions prior to protein secretion, but they generally exhibit a different glycosylation processing compared to that in humans [105]. This can lead to a high mannose content, impacting the formations of galactose and sialic acid residues [89]. The IC-BEVS is essentially a transient system, requiring cell lysis to obtain the product, and therefore hampers the quantity and quality of the product released [105]. The lytic nature of the process, along with the production of interfering virus particles in viral stocks, can lead to increased complications in downstream processing and low yields [105].

Apart from the above-mentioned systems, upcoming cell-free production systems have shown promise. They can provide a cost-effective and quick route to protein synthesis, because of the decreased need for production process development and downstream processing [106]. The system is based on supplying a

crude cell lysate with essential raw materials to generate protein products [106]. Cell lysate contains factors essential for metabolism, transcription, translation, and protein folding (i.e., ribosomes, foldases, metabolic enzymes/catalysts, etc.), while the substrates added to the mixture include the DNA or mRNA template (coding for the protein product), energy sources, amino acids, and nucleotides [106]. PTMs such as glycosylation are possible in insect, tobacco, and mammalian cell lysate systems because of the presence of endogenous microsomes, required for correct PTMs, in their lysates [107, 108]. Bacterial and wheat germ lysates have been shown to produce much higher yields than eukaryotic systems, but they are unable to produce PTMs because of the absence of microsomes [106, 107]. There are hurdles to be overcome with regard to cell-free expression, but these cell-free protein expression technologies may positively impact biotherapeutic production in the future.

13.3 Mammalian Cell Transfection

Transfection is the term given to the process of nucleic acid delivery into host cells, and it is used to facilitate the expression of exogenous products or the regulation of endogenous cellular processes. Indeed, it is the first step in generating a stable cell line or a population of transiently producing cells. Transfection methodologies can be categorized into three main types: biological, chemical, and physical. When considering what makes an effective transfection methodology, two main attributes must be reproducible: a high transfection efficiency (percentage of cells containing transfected material), and robust recovery post transfection, which can be assessed using cell viability and cell growth. Signs of cell toxicity can also be assessed via markers for apoptosis or changes in cell physiology, such as cell size [109, 110].

13.3.1 Methodologies

Biological transfection refers to virus-mediated transfection (transduction), which utilizes the inherent ability of viruses to introduce foreign DNA into a host by infection. Despite being an effective method, bioprocesses, generally, have moved away from these techniques, because of concerns over viral toxicity in the host cell, difficulty in preparation, and the limited size of the DNA vector to be delivered [78, 111, 112]. However, lentiviral transduction is still utilized in some cases because of its high efficiency and stability of gene expression [113, 114].

Chemical methods utilize a positively charged reagent that forms complexes with the negatively charged DNA. These complexes form electrostatic interactions with the host cell membrane, enter the cell via endocytosis, escape from endocytic vesicles, and dissociate, and then DNA is translocated into the nucleus where it

can be transcribed [115]. Calcium phosphate precipitation is a tried and tested chemical method in many cell types. However, in terms of biologics research and production, it is rarely utilized. This was initially due to relatively to low efficiencies and the need for serum-containing media, which are no longer used in standard bioprocess platforms. Despite optimization to improve efficiencies and the development of serum-free processes, other methods are preferred for their robustness and ease of use [116]. Lipofection using cationic lipids (such as lipofectamine) and polyfection using cationic polymers (such as polyethylenimine (PEI)) are the more commonly used chemical transfection methods because they are more efficient, do not require serum-containing media, and show relatively little toxicity to cells. PEI is the most commonly used of these because of its low cost, despite being outperformed in terms of transfection recovery and efficiency by some reagents [111, 116–118].

There are a number of physical transfection methods used for nucleic acid delivery. Mechanical methods, such as microinjection and particle bombardment, have been shown to be extremely efficient for single cells and tissue work, respectively. However, they are not deemed suitable for cell culture bioprocesses because of their laborious protocols, expense, and low throughput [119]. Electroporation is the most commonly used physical transfection method for biologic research and production. This method involves subjecting cells to an electric field pulse, which disrupts the transmembrane potential. As a result, pores are formed in the membrane for a short period before they reseal, and the cell recovers. DNA is thought to enter the cell via passive diffusion, through electrophoretic forces, and/or through a putative membrane-binding mechanism. Indeed, some reports suggest that membrane binding itself can facilitate pore formation. Electroporation is simple, inexpensive (small scale), and, when optimized, has high transfection efficiencies and relatively good post-transfection cell recovery, making it a popular transfection method [116, 120, 121].

13.3.2 Bioprocess Application

Generally, PEI-mediated transfection is reported to be the most common transfection utilized for gene delivery into mammalian cells [78]. However, this is only partly true for CHO cell bioprocesses. Predominantly, PEI-mediated (or other chemical) transfection is used for transient gene expression (TGE), and electroporation is used for stable gene expression (SGE) [90, 122, 123]. The main reason for this is likely the kinetics of the transfection process in either case. As already mentioned, PEI-mediated transfection involves the passage of the PEI–DNA complex through the endocytic transport system, meaning that there is a delay in nuclear uptake of plasmid DNA. On the other hand, electroporation, such as with Nucleofector technology, is thought to facilitate the direct transport of DNA into the nucleus [115, 119]. Moreover, DNA complexed with a cationic polymer protects it from degradation, which increases its half-life within the cell, whereas naked DNA molecules (in electroporation) will be degraded more

quickly [119]. Therefore, PEI-mediated transfection is more likely to yield a slow and steady level of gene expression that is relatively long lasting, which suits the needs of a TGE bioprocess that requires sustained expression. Subsequently, increases in titer are achieved through increased biomass over the production period. Alternatively, electroporation is likely to result in an immediate high level of gene expression that is relatively quick to dissipate, which is more suited to the SGE process that requires immediate high plasmid nuclear content to facilitate a fast selection process for recombinant cells [124]. Furthermore, electroporation typically has a more adverse effect on post-transfection cell recovery than PEI-mediated transfection [111]. This is not necessarily a problem for SGE because cells are given time to recover during the selection process. However, an ideal TGE platform would use a transfection method that immediately yields producing cells with normal growth characteristics, and so PEI-mediated transfection is likely to be more suitable in this case. Another reason why PEI-mediated transfection is desirable for TGE is its (potentially) limitless cell number to which DNA can be delivered, whereas standard electroporation is limited to the sample volume of an electroporation cuvette [111]. Recent advances in flow electroporation technology, such as MaxCyte electroporation, enable electroporation at larger scales and allow for longer-term expression, which has been shown to increase titers in TGE platforms [125, 126]. However, this methodology is yet to be implemented into standard biologic production platforms, presumably due to the expense of reagents and equipment. However, promising advances using cheaper alternatives may see flow electroporation increase in popularity [127].

13.3.3 Gene Targeting

In the generation of a stable cell line, transfection yields relatively few cells capable of high levels of recombinant gene expression. Optimal transfection conditions can help facilitate the integration of a larger number of plasmid molecules per cell, which is correlated with increased gene expression. However, the genomic environment surrounding the site of integration is also extremely influential. Indeed, only ~0.1% of the mammalian genome is transcriptionally active, and these active regions are highly variable in their levels of gene expression. Therefore, selection/amplification, cloning, and screening procedures are necessary to generate high-producing stable cell lines, which are time consuming and laborious [8, 70, 128–130]. Moreover, productivity in many cell lines is unstable, leading to dramatic reductions in cell line productivity over time. This is believed, in part, to be due to inherently unstable genomic environments, both genetically and epigenetically [84].

It is widely believed that targeted plasmid integration into transcriptionally active and stable environments (so-called hot spots) yields highly productive cell lines [128]. Site-specific integration has been notoriously difficult in CHO

cells, but recent developments have advanced this strategy as a useful cell line development tool [130]. Site-specific phage recombinases such as Cre/loxP [131], FLP/FRT [128], and phiC31 [132] have been applied successfully in CHO cells, resulting in increases in cell productivity in a shorter time than is usually taken for stable cell line generation. However, these recombinases require engineered cell lines, containing recombinase target sequences, and so may not be applicable in all cases because the creation of a cell line with an established target site is time consuming in itself. Moreover, these methods are also known to cause chromosomal aberrations, which is not desirable in an industry already concerned with genetic instability [84]. Engineered nucleases, such as clustered regularly interspaced short palindromic repeats (CRISPR)-associated (Cas) RNA-guided nucleases have also been shown to successfully facilitate site-specific plasmid integration, but without the need for engineered cell lines. As well as the development of these technologies, gene targeting strategies utilize information from the CHO reference genomes and other CHO sequence information, along with knowledge surrounding chromosomal instability, in order to determine desirable target loci [128–130].

13.4 Controlling Recombinant Gene Expression

13.4.1 Introduction

Recombinant gene expression is specifically controlled in mammalian cell factories by employing a combination of genetic elements that regulate the transcription and translation of the product gene. Utilizable elements fall into four distinct categories: promoters, untranslated regions (UTRs), epigenetic regulatory elements (EREs), and the protein-coding sequence. While some regulatory elements are specifically selected, or developed, for use in a single mammalian cell host, many exhibit similar functionality across diverse cell types. Accordingly, discrete genetic components are commonly used to control gene expression in multiple different host cells. Therefore, while this section will predominantly focus on examples from CHO cells, the control elements discussed are typically applicable to most mammalian cell factories. Each type of regulatory component will be discussed in turn, presenting the genetic parts that are currently employed in biomanufacturing and examples of recent technological advancements that may be incorporated into industrial vectors in the near future. We pay particular attention to promoters, as they (i) represent the largest source of characterized genetic parts available, (ii) have historically been associated with undesirable functionalities, and, therefore unsurprisingly, and (iii) have been the focus of much recent research aiming to identify novel "improved" components.

13.4.2 Promoters

Promoters are DNA sequences that function to specifically control the transcription rates of individual genes. They are comprised of two discrete structural components, namely the core and proximal regions, containing sequence-specific binding sites for general transcription factors (i.e., components of the pre-initiation complex (PIC)) and regulatory transcription factors (i.e., activators and repressors), respectively [133]. Transcription factors (TFs) bind at cognate sites (or transcription factor regulatory elements, TFREs) within promoters and mediate regulation via co-regulators that act to increase or decrease the rate of rate-limiting steps in the transcription process, including chromatin opening, PIC formation, initiation, promoter escape, elongation, and termination [134]. The nucleotide composition of a promoter therefore determines the frequency, intensity, and duration of transcription in order to generate a gene-specific pattern of mRNA synthesis. Accordingly, changing a gene's promoter is a simple, common, and effective method to rationally alter its expression level.

In order to achieve high levels of recombinant gene expression, the product gene is typically placed under the control of a highly active constitutive promoter that is either endogenous or of viral origin. With respect to the latter, multiple viral promoters have been utilized to drive recombinant gene expression in CHO cells, including the human cytomegalovirus immediate early (CMV-IE) 1, mouse CMV-IE1, rat CMV-IE1, mouse CMV-IE2, myeloproliferative sarcoma virus long terminal repeat (LTR), Rous sarcoma virus LTR, and simian virus 40 (SV40) early promoters [135–137]. Among these, the human CMV-IE1 (hCMV-IE1) promoter has been the most widely employed in industry for driving expression of the product gene. However, this highly active viral promoter has been associated with cellular stress induction (i.e., induction of downstream bottlenecks in translation and ER folding/assembly processes), cell-cycle dependence, and epigenetic silencing [84, 138]. Given these undesirable functionalities, there has been considerable recent interest in re-engineering the hCMV-IE1 promoter in order to improve its performance. For example, we recently determined the key functional regulators of hMCV-IE1 activity in CHO cells, identifying strategies to optimize and control its activity by engineering either the promoter's TFRE composition or the cell's repertoire of TFs [139, 140]. Further, Ferreira *et al*. utilized a random mutagenesis technique to introduce mutations throughout the hCMV-IE1 sequence and isolated variants exhibiting a 40-fold expression range [141]. Finally, Mariati *et al*. recently inserted a sequence element from the hamster adenine phosphoribosyltransferase promoter into hCMV-IE1 to create a hybrid promoter that exhibited enhanced expression stability in stable CHO cell clones and pools [142].

An example of an endogenous promoter that is currently utilized in biopharmaceutical production utilizes regulatory sequences from the highly expressed Chinese hamster elongation factor 1 alpha (CHEF1 α) gene [143]. Specifically evolved to function within the CHO cell background, promoters from constitutively expressed endogenous genes, such as CHEF1 α, typically exhibit high levels

of transcriptional activity that is relatively protected from epigenetic silencing. Although few other CHO endogenous promoters have been characterized to date, the current revolution in CHO cell genomics [21] and transcriptomics [75, 76, 144–146] will likely identify multiple promoters with useful functionality in bioproduction contexts. A major advantage of utilizing transcriptomic datasets to identify promoters for use in biopharmaceutical production is the ability to select elements with desirable levels of activity and/or expression dynamics. For example, using this approach, promoters have recently been identified that are preferentially active in late-stage culture and under hypothermic conditions [147, 148].

In the near future, endogenous and viral promoters may be replaced in industrial vectors with synthetic promoters that have been specifically designed for the purpose. Synthetic promoters can be built to exhibit the highly specific design criteria that are required in the context of biopharmaceutical production (e.g., long-term expression stability, predictable activity over several orders of magnitude, inducibility, coordination of promoter function with cellular and bioproduction processes, etc.; for a recent review, see [52]). For example, we recently identified CHO-active TFREs and utilized them to construct 140 synthetic promoters that exhibited variable activity over two orders of magnitude in various CHO cell lines, where the strongest promoters significantly exceeded the activity of hCMV-IE1 [51]. The precise control of transcription enabled by such synthetic promoter libraries may be utilized to (i) optimize expression of difficult-to-express proteins (e.g., bispecific antibodies, fusion proteins) by providing optimized protein-specific transcription activity kinetically coordinated with polypeptide-specific folding and assembly rates, or (ii) achieve mAb-specific light chain : heavy chain (LC:HC) expression ratios in order to optimize mAb production [149, 150]. Using an alternative strategy, multiple inducible expression systems have been built for use in mammalian cell factories by constructing synthetic TFs that are responsive to specific chemical stimuli [151]. In these systems, transcription of the product gene can be tuned "up" or "down" in response to the inducer in a dose-dependent manner, enabling optimal control of the timing of expression (e.g., to specifically switch on expression when cells are in the stationary phase). A number of recently constructed systems are particularly applicable to biomanufacturing, as they utilize inducers that are both low-cost and noncytotoxic [152–154].

13.4.3 Untranslated Regions, Epigenetic Regulatory Elements, and Protein-Coding Sequences

13.4.3.1 Untranslated Regions

UTRs are defined as the sequences upstream (5′ UTR) and downstream (3′ UTR) of the protein-coding region in mature mRNAs that function to regulate gene expression by (i) controlling transcription termination and (ii) modulating mRNA translation, stability, export, and localization. Regulatory function is mediated by both secondary structures and cis-regulatory elements that are recognized by

sequence-specific RNA binding proteins [155]. Similar to promoters, the UTRs employed in industrial vectors are generally either endogenous or of viral origin. Utilized 5′ UTRs typically share commonality in possessing two key sequence features: an optimized kozak sequence to enable efficient translation initiation [156], and an intron to facilitate efficient mRNA export and cytoplasmic localization (introns can also be included in the 3′ UTR or protein-coding sequence) [157, 158]. For example, the 5′ UTR sequence from the hCMV-IE1 gene contains both of these components and is commonly used in combination with the hCMV-IE1 promoter to ensure high levels of recombinant gene transcription and translation (i.e., a sequence spanning from approximately −600 to +945 bp relative to the transcriptional start site of the IE1 gene within the viral genome is utilized, which incorporates the hCMV-IE1 promoter, kozak sequence, and intron A) [159]. With respect to the 3′ UTR, commonly used elements in biomanufacturing include sequences from the SV40 genome and the bovine growth hormone gene. These components contain polyadenylation signals that are required for transcription termination and facilitate increased mRNA stability and translation [160].

Recent advancements in RNA synthetic biology have enabled the control of gene expression dynamics by engineering UTR sequences. RNA sequences that form secondary structures can be incorporated into UTRs to regulate gene expression by, for example, inducing premature termination of transcription, blocking access to the ribosome binding site to prevent translation initiation, or causing intramolecular RNA cleavage to induce mRNA degradation [161, 162]. Riboswitch expression systems can be designed by incorporating RNA elements whose secondary structure formation is regulated in response to small-molecule effectors [163]. This enables the construction of inducible expression systems that allow optimized spatiotemporal control of product gene expression, with minimal metabolic burden on the cell factory. Moreover, riboswitch-containing UTRs can be used in combination with synthetic TF-responsive promoters to facilitate sophisticated, precise control of product gene expression dynamics [164].

13.4.3.2 Epigenetic Regulatory Elements

Recombinant gene expression in CHO cells can be unstable as a result of epigenetic silencing caused by changes in the chromatin structure at the transgene integration site [165]. Formation of repressive chromatin conformations over a promoter prevents TFs from binding at target sites, effectively "switching off" product gene expression [166]. In order to prevent these negative positional effects, industrial vectors commonly include EREs that function to maintain integrated gene copies in transcriptionally active chromatin structures. EREs can promote the formation of open chromatin structures and/or prevent the spread of heterochromatin across the integrated transgene, and have been shown to (i) increase the occurrence of high-producing clones, (ii) enhance the productivity of top-performing clones, and (iii) facilitate long-term expression stability [167–169]. Multiple different EREs have been shown to be effective in CHO cells, including various matrix attachment regions (MARs) and ubiquitously

acting chromatin opening elements (recently comprehensively reviewed by [170]). However, the function of EREs can vary when used in combination with different promoters, exemplifying the need to select or design gene expression control elements with complementary functionality [171, 172]. Indeed, as studies continue to decipher how EREs function mechanistically, it may be possible in the near future to design synthetic EREs with optimal functionality in bioproduction contexts (e.g., by significantly reducing their size) [173].

13.4.3.3 Protein-Coding Sequences

Protein-coding sequences commonly contain features that negatively affect protein expression by, for example, decreasing mRNA stability, promoting premature transcription termination, inducing alternative splicing, or reducing translational efficiency [174]. Accordingly, in order to optimize biopharmaceutical production, product gene sequences are routinely redesigned using gene design software tools. These algorithms perform multiobjective optimization to remove sequence motifs that negatively regulate gene expression while also optimizing codon usage [175]. Sequence features that are eliminated include cryptic splice sites, polyadenylation sites, UpA-dinucleotides, problematic secondary structures, potential transcription start sites, and AU-rich elements [176]. While gene redesign has historically been used to maximize gene expression, as computational tools become more advanced, it may be possible to specifically tailor product gene expression levels by changing the protein-coding sequence. For example, optimal LC: HC ratios could potentially be achieved by simply altering the nucleotide sequence of both genes.

13.5 Selection and Amplification Systems

Selection and amplification systems enable the creation of engineered cell lines harboring one or more transcriptionally active copies of a recombinant DNA construct stably integrated in the host cell genome. Selection of stably transfected cells is typically achieved by the inclusion on the genetic vector, with the product GOI, of an additional gene that encodes a recombinant protein that either (i) supplements an inherent or engineered metabolic deficiency, or (ii) provides resistance against a toxin included in the growth medium. Amplification is achieved by increasing the stringency of externally imposed selection conditions in order to specifically select cells with a higher transcriptional output from the recombinant construct [177].

As discussed in Section 13.2, CHO cell biomanufacturing processes typically employ DHFR or GS selection systems, utilizing CHO cell lines that are deficient for the production of either DHFR (e.g., DUKX-B11) or GS (e.g., GS-CHO) [178]. Both systems share commonality in that (i) expression of the selection gene is under the control of a weak promoter (such as SV40 or herpes simplex virus thymidine kinase; HSV-tf) in order to prevent promoter–promoter interference

with the promoter driving transcription of the GOI [8], and (ii) selection and GOI amplification can be enhanced by the addition to the growth medium of chemical effectors that inhibit selection marker function. With respect to the latter, methotrexate (MTX) (DHFR system) and methionine sulfoximine (MSX) (GS system) are used to ensure that cell lines containing the integrated GOI are isolated [132, 179].

Strategies to improve selection systems (e.g., increase the occurrence of high producing clones) commonly focus on enhancing selection stringency by reducing expression of the selection marker. For example, at the transcriptional level, Fan *et al.* engineered the SV40 promoter in order to reduce its activity, facilitating a significant increase in the productivity of top-performing isolated clones [180]. At the translational level, increases in selection stringency have been achieved by engineering the selection marker coding sequence in order to reduce translational efficiency (e.g., by deoptimizing codon usage [181]) or mRNA stability (e.g., via incorporation of AU-rich elements and PEST (purine, glutamic acid, serine, and threonine rich) regions [182, 183]).

The IR/MAR amplification system utilizes the ability of plasmids bearing both a mammalian replication initiation region (IR) and a nuclear MAR to spontaneously amplify extra-chromosomally before inserting at multiple chromosomal locations, thereby increasing the rate of GOI insertion in to highly transcriptionally active genomic regions [184]. Use of this system has been shown to increase both GOI copy number, and enhance expression stability in CHO-DG44 cells over a period of several months [185]. Further, it has been demonstrated that fusion of the IR/MAR and DHFR systems in CHO-DXB11 and CHO-DG44 cells can increase the productivity of multiple recombinant proteins, as compared to the use of either the IR/MAR or DHFR system in isolation [186].

13.6 Transient Production Systems

TGE is the introduction of episomal recombinant DNA by transfection into a eukaryotic cell for (effectively immediate) expression of a GOI [187]. TGE has been used routinely as a small-scale, high-throughput research tool to investigate the impact of overexpression of specific genes within the transfected cell [188] or for drug candidate screen and selection during the drug discovery process [125]. More recently, polycation (e.g., polyethylenimine, PEI [189])-based methods to condense DNA prior to endocytotic uptake or direct flow electroporation are being predominantly utilized for larger scale production (>1 l, milligram to gram product yields) [190]. Mammalian TGE systems are consistently being developed as a reflection of continually increasing demand for an early supply of candidate recombinant protein for manufacturability assessment, preclinical testing, and analytical method development to support manufacturing process development [116]. TGE was initially performed in HEK293 cells, as it was a widely used

research cell line exhibiting high transfection efficiency and ease of maintenance [188]. To harness these advantageous features of HEK293 cells and boost expression titers, various process development and cell engineering manipulations have been performed. For example, titers of 1 g/l were recorded using a combination of an optimized transfection protocol, cell engineering (HEK293 cells stably expressing Epstein – Barr virus (EBV) nuclear antigen-1), vector engineering (including an OriP element to enable episomal plasmid replication), and various chemical additives (such as valproic acid (VPA)) [188, 191]. However, in recent years, industry has been more focused toward TGE using CHO cells to maintain comparability with stable production systems that employ CHO cells. For example, N-glycan processing in HEK293 cells may differ from that observed in CHO cells [192, 193]. Moreover, recent analyses comparing N-glycosylation of the same recombinant protein produced by both transient and stable production systems generally reveal a high degree of consistency in *N*-glycan processing between the two methods [90, 194]. For this reason, in the following we primarily focus on TGE in CHO cells (see Ref. [195] for a detailed discussion of TGE in other mammalian cell types).

13.6.1
CHO Cell Engineering for Increased Transient Production

A range of cell engineering strategies have been employed to improve TGE in CHO cells. Arguably the most successful approach has been the stable expression of viral elements such as the EBV nuclear antigen-1 (EBNA-1) [90] or polyomavirus (PyV) large-T antigen (PyLT) [196]. When a cell line coexpressing EBNA-1 and GS was combined with vector and production process engineering, the episomal-based expression system achieved a volumetric titer of recombinant mAb approaching 2 g/l [90]. Other recent approaches to increase transient productivity have reported engineering protein folding and assembly by coexpression of ER molecular chaperones and oxidoreductases [123], a combination of ER chaperones and chemical chaperones [197], or coexpression of anti-apoptotic genes such as Bcl-xL [198].

13.6.2
Recombinant DNA Delivery Mechanisms

Another approach to improve TGE is by optimization of episomal DNA delivery to and trafficking within the host cell. To date, large-scale TGE has primarily employed chemical polycation-based transfection methods including cationic liposomes, calcium phosphate precipitation, or cationic polymers such as PEI [118]. With respect to the latter, the most popular version is linear PEI, which is an extensively characterized, scalable, and cost-effective reagent [199]. Typically, production-system-specific optimization of the basic transfection parameters (i.e., rDNA:host cell concentration:PEI ratio) is necessary to obtain high transfection efficiency [200]. Alternatively, use of a PEI variant with the ideal

chemical characteristics such as low acylation and/or a higher molecular weight can improve transfection efficiency [199, 201]. Flow electroporation such as the MaxCyte STX has proven to be a good alternative DNA delivery mechanism for TGE [125]. Antibody titers of >1.2 g/l were observed from an unengineered CHO cell line when combined with process engineering. However, a drawback for this method is the sizeable investment in MaxCyte STX when compared with the cost of PEI.

13.6.3 Process and Media Optimization

Optimization of the cell culture process or environment has been shown to substantially increase the volumetric product titer. Most simply, reduction of culture temperature post transfection to 34 °C [90] or 32 °C [194] to arrest growth is effective in extending culture productivity. Recently, there have been developments in extending TGE by exchanging the media and repeating PEI-mediated transfection of HEK293 cells. This approach yielded a 4.3-fold increase in secreted GFP over an extended period of 10 days compared to classical TGE [202]. These results suggest that a similar process could be implemented in CHO cells as well.

Media supplementation with small-molecule effectors has also been utilized to improve TGE. For example, combining chemical chaperones like PBA and glycerol to increase cell-specific production has shown to increase the production of a difficult-to-express fusion protein by CHO cells over sixfold [197]. Alternatively, polar solvents such as *N*,*N*-dimethylacetamide (DMA) [193] and histone deacetylase inhibitors such as VPA have also increased product titer [203]. For instance, DMA is thought to extend mRNA's half-life during TGE and demonstrated increased antibody production by fourfold to 500 mg/l over 7 days in CHO-DG44 cells [193]. Similarly, VPA is known to suppress histone deacetylation and maintain transcription of extra-chromosomal DNA and extended TGE [191, 204]. Another form of supplementation to increase TGE titers is the addition of a proprietary feed that extends the duration of the transient culture. For example, using engineered CHO cell hosts, Rajendra *et al.* [193] and Daramola *et al.* [90] combined proprietary feeding regimes in combination with optimized transfection protocols to generate volumetric product titers of approximately 1 and 2 g/l, respectively.

13.7 Protein Purification

Current cell expression technologies are becoming increasingly capable of expressing high titers of product, but these advances in expression can be realized only if the product can be recovered with acceptable purity and yield and in a cost-effective way.

13.7.1 Clarification

Often overlooked as being part of the purification process, the clarification step removes a major impurity from the biotech process, namely cells and cellular debris. Selection of the clarification method depends greatly on the type of cells, mode of bioreactor operation, process scale, and characteristics of the product and cell culture fluid. Most traditional harvest methods use some form of centrifugation followed by depth and sterilizing-grade filtration [205, 206].

While fed-batch processes are still the most widely used production systems for mammalian cell cultures, perfusion is becoming increasingly popular and is used commercially for several products. Perfusion processes are those where the cells are retained in the bioreactor and are continuously or semicontinuously fed with fresh medium. The spent medium, containing the protein of interest, is collected and subsequently purified either in batch mode or continuously. Recent requirements to improve process performance and productivity have pushed the advancement in cell separation technologies, during fermentation, to enable economical and routine use of perfusion bioreactors [207]. Several methods for cell separation in perfusion are available, which are based on different physical principles. Since perfusion requires continuous cell removal over a period of several days to weeks, the selected harvest method must be able to ensure sterility during ongoing fermentation [208].

13.7.2 Chromatography

13.7.2.1 Affinity Chromatography

Once clarified, the soluble or solubilized recombinant protein will be in dilute form and in the presence of a vast array of other components including host cell proteins, host DNA, and other impurities. While ion exchange still remains a common and frequently implemented first step, such as is the case of the hugely successful drug Humira, the anti-TNFα antibody developed by Abbot Laboratories [209], if the protein of interest is tagged or increasingly if it is an antibody, the first chromatography step is typically affinity. A common example of an affinity step is protein A chromatography. The protein A ligand is highly specific for immunoglobulins including IgG1, IgG2, and IgG4 subclasses, and advances in ligand design, attachment chemistry, and matrix (e.g., MabSelect Sure, GE Healthcare Life Sciences) have made this approach viable for research, clinical, and commercial supply alike [210].

Other affinity strategies are based around fusion tags. Typically, amino acid sequences that are expressed and displayed on the recombinant protein have affinity for a chemical or biologic ligand immobilized on a chromatography column. A common example of this approach is the histidine (His) tag [211]. His tag is added to the C or N terminus of the recombinant sequence and is formed by the addition of up to 10 His residues. The His tag has high affinity for metal ions such as

nickel, zinc, or cobalt, which can be immobilized on metal affinity chromatography (IMAC) matrices which, under the right conditions, enable affinity capture of tagged proteins. Fusion tags provide an effective platform approach and enable multiple protein product candidates to be captured and purified in a similar way, providing a convenient strategy particularly in early research and development. A typical requirement to remove the fusion tag prior to clinical use complicates this strategy with the need for expensive proteases and additional purification steps [212].

Recent advances in combinatorial chemical libraries and *de novo* ligand design have made it possible to develop highly specific custom affinity approaches that remove the need for fusion tags [213]. While the technology is increasingly sophisticated, its high initial cost can inhibit its use until later stages of clinical supply once some of the uncertainty around early product candidates has been reduced.

13.7.2.2 Ion-Exchange Chromatography

The protein product can be separated from other biomolecules using ion-exchange chromatography (IEC), which isolates proteins based on differences in their net surface charge [214]. With a basic understanding of the physicochemical characteristics of the protein, buffer conditions (buffer type, pH, and salt strength) can be selected that induce a surface charge on the protein, which can enable charge differentiation with other molecules (proteins, DNA, etc.) and facilitate selective binding while other components are washed away. Alternatively, conditions (pH) can be altered so that the product charge is the same as that of the matrix, thus allowing it to flow though the column while other impurities are retained. The flow-through approach, in particular with more modern chromatography support substrates such as membranes, is becoming increasingly important to high-productivity processes where raw material and processing time can be significantly reduced [215].

There are two main types of IEC resin that have functional groups either positively charged (anion) or negatively charged (cation). Both types are commonly used in protein purification and often used together as a powerful, orthogonal, combination. A classic purification strategy is to use cation exchange to bind a positively charged protein product (i.e., when the pH of the buffer is below the isoelectric point of the protein), followed by anion exchange chromatography, at the same pH, in which case only negatively charged biomolecules such as deoxyribonucleic acid (DNA) and lipopolysaccharides (LPS) bind [216].

There is a vast array of IEC matrices commercially available, and while selection can be based, in part, by knowledge of the application, the only true way of determining the correct matrix with the right operating conditions is to evaluate it empirically. The use of high-throughput process development (HTPD) using robotics and ultra-scale down techniques makes it possible to readily screen matrix and conditions to select the most viable options [217].

13.7.2.3 Hydrophobic Interaction Chromatography

Hydrophobic interaction chromatography (HIC) resins separate biomolecules according to their degree of hydrophobicity. By modulating the salt levels in the protein solution, hydrophobicity and affinity to the HIC matrix of the product and impurities can be influenced. Because of the typical requirement for high levels of salt to be present in the protein load, HIC often follows IEC where the product would have been eluted in a buffer with a relatively high salt content [218]. Methods are being explored to determine a protein's hydrophobicity and correlate this with separation behavior on HIC [219]; however, so far in practice, the interaction of the protein with the hydrophobic ligands still requires large and empirical screening experiments to be conducted.

13.7.2.4 Mixed-Mode Chromatography

Mixed-mode chromatography covers an increasingly large array of chromatography resins utilizing a combination of electrostatic and hydrophobic interaction as well as hydrogen bonding to provide a platform that can be used to exploit unique characteristics of the protein product and enable exquisite separation from the surrounding biomolecules. The original mixed-mode resin, hydroxyapatite (HA), enables proteins to be bound via HA-phosphoryl (cation exchange) or HA-calcium residues (metal affinity). HA is extremely effective at reducing or eliminating aggregated antibodies, a common issue in monoclonal antibody processing [220]. A challenge to the use of mixed-mode chemistries has been the significant process development required to get optimal conditions established. The parameters that affect separation are more numerous than other simpler approaches, and consequently the use of multifactorial experimental design statistical software to develop key experimental approaches are necessary to fully understand the operational space.

The advent of more complex non-natural recombinants such as bispecific antibodies and fusion proteins poses a significant challenge to process development and manufacturing facilities alike. The need to separate the intact product from product-related impurities (aggregates and fragmented product) requires the development of often complex multistep purification strategies. The introduction of mixed-mode chemistries, which can simultaneously discriminate over several physicochemical properties of the product, enables a simpler process (fewer steps) to be developed. Furthermore, with an understanding of the right levers to modulate separation using the mixed mode, platform approaches can be potentially developed where the same matrices and process are reused for different protein candidates [221].

13.7.3 Membranes

Membranes for bioprocessing come in a variety of configurations. Ultrafiltration membranes with pore sizes that prevent the transmission of protein but allow

water and small molecules (e.g., salt) to pass through are extensively used in bioprocessing for protein concentration and to change the buffer composition of the protein product (diafiltration). Key issues during development of these types of process operation are the prevention of damage to the protein product through shear force and the optimization of the formulation strategy to minimize viscosity increases; both can impact membrane performance [222]. Nanofilters are another common addition to the purification process, as they enable robust adventitious virus removal by providing a tightly controlled pore size, typically 20 nm, which is sufficient to remove even the smallest viruses (e.g., parvo virus). Membranes with functional chemistries such as hydrophobic or ionic interaction are becoming increasingly common in purification. The open fibrous structure and configuration of these membranes allow purification to occur under very rapid conditions [222].

13.7.4 Economics

There are a number of fundamental components that affect process economics, including titer, scale, purification yield, time in plant, facility costs (depreciation, labor, utilities), raw materials, and process configuration. Recombinant protein titers vary widely depending on the expression system and the protein class. A well-established expression platform such as CHO expression of therapeutic antibodies can reach 10–15 g/l [223]. Other protein classes (e.g., blood factors) may be expressed at significantly lower levels.

The impact on cost of the goods manufactured per gram (COGM/g) by titer is very significant. The overall process yield is a function of the performance of each individual purification step. Improvements in step yield through process optimization and in some cases removal of "non-value-added" intermediate steps can increase the quantity of product per batch and thus reduce COGM. Typically, the more complex the process, the more the number of steps required to produce an appropriate quality product, and the lower the yield and the more expensive the final drug. For an established product type such as antibodies, process yields have risen in recent years from <50% to typically >70% [224].

Production of biologics requires the use of raw materials (chromatography resins, filters, buffer constituents, cell culture media, and water). The cost impact of raw material use is dependent on the scale and, in certain cases (resins and filters), whether the components are reused and, if reused, the number of times they can be reused. The decision to incorporate a disposable or reusable path is complex. While the first may lead to higher raw material costs, this is potentially offset to some extent by reduction of operating costs and other indirect costs such as cleaning validation [225, 226].

The choice of operating conditions, process performance, and the scale of manufacture will all impact COGM. Computer-based cost models have been developed to help rationally determine the optimum solution for process configuration and potential process development strategies [227]. The use of cost

models and other *in silco* process tools to evaluate and prioritize the impact of cost-related factors (e.g., titer, scale, process choice) with clinical data (potential dose, patient population) and manufacturing facilities is becoming fundamental to strategic and rational selection of process requirements and optimization goals [228].

13.7.5
Future Trends and Conclusions

The explosion in the number of therapeutic biomolecules moving through clinical trials and into commercial production has been dominated by mAbs. The effectiveness of protein A and subsequent polishing steps, which are almost agnostic to product and there only to remove known product and process impurities, inactivate and clear adventitious viruses, and deliver correctly the formulated drug substance in a "platform" process, enables large antibody-based portfolios to be readily supported. As protein therapeutics become increasingly complex, the requirement for purification strategies that are just as effective as the mAb platform will be needed. The days of developing a crude multistep process, utilizing exotic untested ligands in a laboratory process, are numbered. The future trend is for early product candidates to be developed with acceptable purification strategies built in so that commercially relevant scale-down models and analogs can be deployed, often as part of automated high-throughput strategies linked into sophisticated analytics, to enable early product candidates to be "screened" for manufacturability.

The purification process is now often referred to as the "bottleneck" of protein production. Recombinant protein titer is currently not capped by expression technology but by the practical scale of chromatography columns, buffer tanks, and mass-transfer limitations. New technologies such as functionalized membranes and continuous processing will help address some of these issues; however, continued exploration and development of new, efficient, and cost-effective purification strategies are still important challenges for the industry and academia alike.

13.8
CHO Cell Engineering for Enhanced Bioprocessing Properties

Cell engineering encompasses the manipulation of various features of protein expression with an overall aim of generating host cells with a higher capability of producing the recombinant product of interest. This could be achieved through maximizing the integral of viable cell density, increasing cell-specific productivity (Qp), or improving product quality. Cell engineering can be described as a powerful technique that has the potential to significantly enhance the efficiency of CHO-based bioprocessing. Genetic engineering, involving random integration of heterologous genes, is the most common method utilized to enhance CHO cells

as recombinant protein producers. A large variety of endogenous and exogenous genes to alter cellular processes have been upregulated through overexpression studies. Alternatively, disadvantageous genes have been repressed through gene knock-out or knock-down approaches [43].

13.8.1 Programmed Cell Death

There are many different aspects of the CHO cell host that can be manipulated. Engineering programmed cell death (PCD), including apoptosis and autophagy, is a clearly advantageous target. If PCD can be reduced, or even eliminated, culture durations and high viabilities could be prolonged, which, in turn, should increase the product yield. Additionally, reduced levels of proteases later on in culture are likely to be a consequence of anti-apoptotic engineering, which can result in a better quality product [43]. The potential of anti-apoptotic engineering is highlighted through the permanent disruption of genes, using ZFNs, encoding Bax and Bak proteins, which are essential for apoptotic activity. Resultant cells continue to grow normally but with resistance to apoptosis and a 2–5-fold enhancement in mAb yield [229]. Overexpression of proteins that modulate cellular apoptosis or autophagy, Bcl-xL, Bcl-2, Beclin1, and HSP27, all had a positive effect, albeit to varying degrees, on increasing cell growth or yielding higher levels of recombinant proteins [198, 230–232].

13.8.2 Folding and Assembly Machinery

Obvious choices for cell engineering targets are components of protein folding and assembly reactions within the endoplasmic reticulum (ER). In many cases, antibody mRNA levels and transgene copy numbers do not proportionally increase with recombinant production levels [233]. This suggests that translational or post-translational steps may often be those limiting recombinant protein production [234]. If engineering strategies can relieve these bottlenecks, higher levels of secretion are likely to be achievable. Chaperone proteins, for example, BiP (binding immunoglobulin protein) [150, 197, 235, 236], calnexin, calreticulin [237], and cyclophilin B [150, 197], have all been overexpressed and, in most cases, found to be beneficial to CHO cell productivity. Protein disulfide isomerase (PDI), which catalyzes the formation of disulfide bonds, essential in the formation of mAb structures [150, 235, 238, 239], and Ero1, which reoxidizes the active site of PDI [240], are the other frequently investigated genes, and often successful targets. It must be noted that for the majority of engineering strategies, different effects have been observed for different recombinant proteins, host cell lines, and types of expression (stable vs transient) [241]. PDI particularly has had mixed effects upon its effect on Qp. Specific reaction steps could be more of a bottleneck for one cell type or product than another. Product-specific solutions are likely to be optimal in most cases [150].

13.8.3 Unfolded Protein Response

The unfolded protein response (UPR) is a consequence of the accumulation of misfolded or unfolded proteins with the ER. It is a widespread, multicomponent response to overcome ER stress that occurs when protein synthesis surpasses the capacity of the ER [241]. Engineering components of the UPR has the potential benefit of regulating folding/secretory components in a more global manner. The three main UPR transducers, AFT4, PERK, and IRE1, induce a wide range of genetic pathways. Activating transcription factor 4 (ATF4) [242, 243], cleaved activating transcription factor 6 (ATF6) [150, 197], spliced X-box binding protein (XBP1) [244], and CHOP [236] are all UPR components that, when upregulated, have had a beneficial impact on recombinant protein titers. XBP1 is a central regulator of the UPR: it expands secretory compartments, induces secretory pathway genes, and increases the production capacity of the cell [244]. Upregulation of XBP1 has had different effects on productivity in different studies, and its effect is likely to depend on whether folding and assembly is a limitation with the production system [150]. A limitation of engineering the UPR is that it can negatively induce apoptosis. It has been highlighted that this limitation could be overcome through coexpression of an apoptosis inhibitor. For example, XIAP, a caspase inhibitor, and XBP1 co-overexpression improved Qp and CHO cell survival [245].

13.8.4 Secretory Pathway

Engineering the secretory pathway within CHO cells has also been a common target area of interest. As recombinant proteins generated within CHO cells are secreted out the cell, enhancing the secretory capabilities of the host cell should allow more protein to be processed and removed from the cell. Successful examples of engineering and enhancing the secretory capacity of CHO cells include the overexpression of human signaling receptor protein 4 [246], ceramide transfer protein [247], and the proteins involved in endocytosis Sly1 and Munc18c [248].

13.8.5 Glycosylation Pathways

Proper glycosylation is a critical parameter for the manufacture of glycoprotein therapeutics, as it can affect protein stability, bioactivity, pharmacokinetics, immunogenicity, and protein clearance in the circulation system [249]. For example, the removal of N-glycosylation sites on recombinant erythropoietin (EPO) was shown to significantly reduce its *in vivo* activity [250], which led to the hypothesis that additional carbohydrate content would be beneficial and the eventual discovery of darbepoetinalfa (Aranesp®) – a hyperglycosylated

EPO analog that exhibits a threefold increase in serum half-life and greater *in vivo* potency compared to recombinant human EPO [251]. In this regard, glycoengineering, including engineering of host cell lines, provides a versatile tool to obtain therapeutic glyco-products with novel/improved *in vivo* characteristics. Indeed, although CHO cells are effective cell factories, (e.g. possess the ability to produce *N*-glycans with branching and capping similar to what is typically produced in human cells), glycan structures are still limited by the cells' intrinsic glycosylation machinery, while the glycan processing exhibits significant heterogeneity that can compromise activity and safety. With respect to the former, some human tissue-specific terminal carbohydrate motifs are not synthesized by CHO cells, as they are devoid of the proper sugar-transferring enzymes such as α-2,6-sialyltransferases [252].

Considerable efforts have been devoted to CHO cell line engineering to control or expand the glycosylation repertoire and improve sialylation to avert lectin-mediated clearance. For example, studies suggest that the branch specificity of β-1,4-galactosyltransferase varies from one species to another, and recombinant mAb (IgG) produced in CHO cells are less galactosylated compared to mouse myeloma cells [253, 254]. Therefore, the overexpression of human β-1,4-galactosyltransferase in CHO cells has been shown to result in more consistent galactosylation patterns of TNK-tPA and TNFR-IgG fusion proteins [255] and less structural heterogeneity of human IFN-γ [256]. Moreover, the introduction of terminal human glycosyltransferases into rodent host cells can lead to protein products with human tissue-specific glycosylation. The coexpression of recombinant human α-2,6-sialyltransferase particularly has been demonstrated to improve the sialylation state of IFN-γ, thyrotropin, and IgG1 expressed in CHO cells [257–259]. However, because of the competition with the endogenous α-2,3-sialyltransferase, and as the enzyme has a preference for the Man-3 branch of oligosaccharides, the end products contain mixtures of α-2,3- and α-2,6-sialylated glycans. In this regard, the proportion of the α-2,6-sialylated oligosaccharides may be increased by coexpressing both α-2,6-sialyltransferase and β-1,4-galactosyltransferase [255, 260]. Conversely, most mammalian cells including CHO cells possess glycostructures containing potentially immunogenic epitopes such as Neu5Gc, which is not typically expressed in humans. As such, CHO cells have also been engineered using an antisense-RNA strategy to downregulate CMP-Neu5Ac hydroxylase activity and thus the Neu5Gc content [261].

In recent years, the biopharmaceutical industry has also focused on the elimination of core fucose from the *N*-glycan at Asn297 to produce therapeutic IgGs with substantially improved antibody-dependent cell cytotoxicity. The most common approach utilizes knocking out of the *fut8* gene, which controls α-6-fucosylation of the innermost *N*-acetylglucosamine residue of the chitobiose core, although the same outcome can also be obtained via overexpression of *N*-acetylglucosaminyltransferase III [262]. Such engineering approaches, however, require extensive characterization of glycosylation pathways and genes that code for glycosylation activities. To this end, Yang *et al.* recently

demonstrated a knock-out screen approach of glycosyltransferase genes controlling N-glycosylation with ZFNs, followed by the construction of a design matrix to facilitate the generation of the desired glycosylation such as the human α-2,6-linked sialic acid capping [263]. This engineering method not only enables the dissection of the *in vivo* functions of all expressed genes that encode isoenzymes but also allows distinct/homogenous glycoforms to be generated and comparative studies of their biological effects.

13.8.6 Gene Editing

There are specific gene editing tools available that will further contribute to the development of cell engineering of CHO cells. These include ZFNs and transcription activator-like effector nucleases (TALENs) [43]. A novel technology, which will progress CHO cell engineering, is a genetic editing tool known as CRISPR/Cas9 (reviewed in [32]). The CRISPR/Cas9 system allows specific DNA sites to be disrupted and modified. The tool allows for quick, easy, and effective engineering of mammalian genetic material. Singular genes can be targeted, or more widespread genome screens or regulation can be undertaken. The specific knockdown of a gene encoding tuberous sclerosis complex 2 (TSC2) was achieved using a CRISPR/Cas9 approach [264]. TSC2 is a major inhibitor protein of the mammalian target of rapamycin (mTOR), a central regulator of cellular metabolism. Knockdown of TSC2 led to cells having continuous mTOR activity, resulting in increased cell size, protein synthesis and, despite a detrimental effect on cell growth, a twofold improvement in specific productivity [264].

13.8.7 Directed Evolution Approach

A different approach to engineering CHO cells to improve their ability as biomanufacturing hosts is through a directed evolution methodology. Typically, this involves manipulating the whole cell population to adapt to particular conditions, with the aim of generating a population with a desired phenotype. The concept and application has been established for many years both within microbial and mammalian systems including myelomas and hybridomas [265]. More recent examples in CHO cell biomanufacturing involved evolving cells to cope with the stressful conditions present within a bioreactor setting. Cells were subjected to rounds of stressful conditions often present later on during culture, for example, nutrient limitation. The subsequent populations were found to have enhanced integral viable cell densities, leading to increased product titer [266, 267]. Directed evolution looks to be an alternative approach to genetic manipulation for cell engineering, but relying on natural mutation may not be a rapid enough tool within the industrial setting. Other means of introducing mutations, for example, through chemical methods, may increase the rate of genetic and phenotypic diversity. However, they have been shown to negatively

affect cellular performance, and evolved populations are typically less healthy and have limited short growth profiles (potentially due to the introduction of chromosomal abnormalities) [268, 269].

13.8.8 miRNAs – A Novel Cell Engineering Approach

The majority of published examples of cell engineering of CHO cells involve specific, singular manipulations, sometimes with subsequent selection for a clone displaying the most beneficial phenotype. Single gene overexpression may be limiting and not adequate to result in the desired change in phenotype. More physiological knowledge and implementation of "omic" tools and resources, to identify bottlenecks or characterize advantageous producer phenotypes, along with multigene engineering should aid progression to a more desirable CHO cell phenotype [234, 241]. With novel molecule formats, such as antibody fragments and artificial scaffolds, beginning to arise and be more difficult to express, the need for cell engineering to assist in generating sufficient product has never been greater.

miRNAs (microRNAs) are small noncoding RNAs able to interact with numerous mRNAs within a cell, resulting in widespread, even whole process, regulation, and are a developing tool for global gene engineering [270]. miRNAs can be suppressed using vectors encoding sponge decoys, which avert miRNAs from binding to their endogenous mRNA target. Using this method of suppression, miR-23, which plays a role within glutamate metabolism, was stably depleted. The resulting cell line had a threefold increase in specific productivity without any adverse effect on cell growth [35]. Stable overexpression of miR-17, attributed to play a role in G1/S-phase cell cycle and membrane trafficking, achieved the fairly rare but beneficial phenomenon of improving both CHO cell growth and specific productivity in parallel [38]. Lower culture temperatures are often established in later stage cultures to enhance productivity. In a differential miRNA investigation, miRNA-483 was found to be unregulated during this reduction in temperature. Subsequently, the introduction of miRNA-483 mimics were found to significantly enhanced mAb yields within CHO cells [271]. An advantage in using miRNAs as an engineering tool, over conventional genetic overexpression, is that miRNAs are noncoding elements and therefore do not themselves directly add to the translational burden of the cell [35, 272]. Further developments in the annotation and evaluation of miRNAs are needed to reach predictable phenotypic changes [43], but they are likely to be useful when single or multigene engineering is not sufficient to achieve the desired altered phenotype [273].

Abbreviations

ADCC	antibody-dependent cellular cytotoxicity
ATF4	activating transcription factor 4
BHK	baby hamster kidney cells

BiP	binding immunoglobulin protein
CHEF1 α	Chinese hamster elongation factor 1 alpha
CHO	Chinese hamster ovary
CMV-IE	cytomegalovirus immediate early
CRISPR	clustered regularly interspaced short palindromic repeats
DHFR	dihydrofolate reductase
DMA	*N*,*N*-dimethylacetamide
ER	endoplasmic reticulum
ERE	epigenetic regulatory element
EBNA-1	Epstein–Barr nuclear antigen-1
E. coli	*Escherichia coli*
EPO	erythropoietin
Fut8	α1,6-fucosyltransferase
GOI	gene of interest
GS	glutamine synthetase
HC	heavy chain
HEK	human embryonic kidney
IC-BEVS	insect cell-baculovirus expression vector system
IgG	immunoglobulin G
IR	initiation region
LC	light chain
LTR	long terminal repeat
mAb	monoclonal antibody
MAR	matrix attachment region
miRNA	microRNA
MSX	methionine sulfoximine
MTX	methotrexate
NeuGc	*N*-glycolylneuraminic acid
PCD	programmed cell death
PDI	protein disulphide isomerase
PEI	polyethylenimine
PEST	purine, glutamic acid, serine, and threonine rich
PIC	pre-initiation complex
P. pastoris	*Pichia pastoris*
PTM	post-translational modification
PyLT	Py large-T antigen
PyV	polyomavirus
QbD	quality by design
Qp	cell specific productivity
S. cerevisiae	*Saccharomyces cerevisiae*
SGE	stable gene expression
SV40	simian virus 40
TGE	transient gene expression
TF	transcription factors
TFRE	transcription factor regulatory element

tPA	tissue plasminogen activator
TSC2	tuberous sclerosis complex 2
UPR	unfolded protein response
UTR	untranslated regions
VPA	valproic acid
XBP1	X-box binding protein
ZFN	zinc finger nuclease

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14
Stability, Formulation, and Delivery of Biopharmaceuticals

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14.1
Introduction

Recombinantly produced proteins are increasingly used to treat severe diseases [1]. However, the development and manufacturing of proteins is challenging and complex. The manufacturing process can be generally categorized into the following:

1) Drug substance development and manufacturing
   a. Cell line engineering
   b. Upstream processing (fermentation) including harvest
   c. Downstream processing (purification)
2) Drug product development and manufacturing.
   a. Formulation/compounding
   b. Processing ("fill and finish").

The aim of this chapter is to discuss aspects and challenges related to stability, formulation, and delivery of biopharmaceuticals. While many of the stability elements are both applicable to drug substance and drug product unit operations, a specific focus on drug product development and drug product manufacture is provided.

14.2
Stability

Proteins pose specific challenges to their stability during manufacturing, storage, transportation, and/or administration [2, 3]. Stability liabilities include the following:

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1) Chemical instability, such as
   a. Oxidation
   b. Deamidation and succinimide formation
   c. Fragmentation/hydrolysis
   d. Disulfide shuffling.
2) Physical instability, such as
   a. Adsorption
   b. Denaturation
   c. Aggregation
   d. Precipitation (particle formation).

Chemical degradation is characterized by covalent changes to the primary sequence of the molecule at different amino acids of a protein. Further details are provided in comprehensive reviews [2]. Various amino acid motifs of the primary sequences have been found to be prone to chemical degradation (e.g., DG for deamidation). Besides the amino acid sequence, the structure and folding also play a role in determining the level and degree of instability. Specifically, surface solvent exposure of the amino acids is required in order to provide sufficient opportunity for degradants or reactants to attack at these sites. As an example, a buried Tryptophan (Trp) residue is less likely to be oxidized by reactive oxygen species compared to a surface-exposed Trp. Typically, different instability reactions can happen at the same time, although at different rates. Instability reactions may also be intertwined. For example, chemical instability such as oxidation can lead to physical changes such as aggregation [4]. Also, chemically modified proteins can lead to product-related degradants that may differ in their safety and/or efficacy compared to the actual target molecule. Deamidation and succinimide formation occurring in a binding region of a monoclonal antibody (mAb) have been reported to lead to impaired potency [5]. Sequence assessment for degradation hotspots thus provides significant opportunity to improve product stability. Firstly, *in silico* assessments monitoring known and previously described degradation hotspots may serve to evaluate the best of a series of possible molecule candidates [6]. Secondly, it may be valuable to engineer out these degradation hotspots to reduce the risk for product instabilities while maintaining the potency of the molecule (e.g., binding activity) and other relevant product characteristics (e.g., pharmacokinetics). Experimental *in vitro* data can support these assessments [7].

Physical instability reactions include changes in the structure of a protein. Proteins may interact with surfaces and bind to them non-covalently (see (2) above). Adsorption can occur to processing materials such as filters [8], as also glass and other primary packaging materials [9]. Unfolding (denaturation) of a protein may occur dependent on temperature or chemicals (e.g., guanidine). In theory, global or local unfolding leads to reduced binding. For example, thermal unfolding is one of the assessments that can support molecule selection (e.g., melting temperature, T_m), although it can also serve to rank thermal-based unfoldings and may

be unable to predict global stability [10]. In formulation development, unfolding is usually not characterized, given its poor relevance to the actual intended storage conditions, typically refrigeration (2–8 °C). Proteins can self-associate, aggregate, and/or precipitate, that is, they can form proteinaceous particles [4]. There are also individual cases in which extraneous material, such as silicone droplets, has been connected to generate aggregates or proteinaceous particles [11]. Aggregation and precipitation have been speculated to increase the risk of immunogenicity [12, 13]. However, clinical evidence in humans is missing and animal and *in vitro* data are in conflict (Singh *et al.*) given that these experiments usually included a variety of degradants and not only proteinaceous aggregates [14]. Transgenic mice data with actual purified protein particles or highly oxidized particles suggested that proteinaceous particles themselves do not lead to increased immunogenicity in mice, whereas heavily oxidized particles do [15].

14.3 Drug Product Development

Biologics are generally prepared as sterile dosage forms which are typically intended for parenteral use, that is, for injection, infusion, or implantation in humans or animals. Given the size and hydrophilicity of a protein, it has to be parenterally administered as it is poorly bioavailable after oral, nasal, or inhalative administration. Even when a certain degree of bioavailability after nonparenteral administration is found in humans (such as for smaller proteins), the degree of patient-to-patient variability, the required significantly high(er) doses due to low(er) bioavailability, and the related cost of goods for the patient treatment as well as some further safety considerations for chronic treatments often make non-parenteral administration prohibitive for biologics. The bioavailability of a protein depends typically on its size. Thus, in cases, where, for example, a small protein is used to obtain sufficient bioavailability, and where a sufficiently wide therapeutic window is present, and where cost of goods allow, nonparenteral administration could also play a role. It is certain that in cases where proteins are used for local disease treatment, for example, pulmonary administration of DNAse to treat patients suffering from cystic fibrosis, these routes of administration are medically warranted.

A protein drug product comprises of the protein and stabilizing excipients (the formulation) and of the container closure system (CCS) (primary packaging). It needs to be adequately designed and developed for drug product processing (fill and finish operations) as well as for product use. In case of drug/device combination products, this means the above elements needs to be co-developed very closely with the functional device (e.g., syringe, autoinjector, injection pump). None of these elements should be developed individually and in isolation.

14.3.1 Product Requirements

Drug products need to be sufficiently stable. Stability can be considered a relative term as an acceptable stability is defined by the specifications, which list analytical tests and related acceptance criteria, over a product shelf life at the intended storage condition. Typically, a shelf life of at least 18 months is required. All products undergo degradation. That means, a product is considered stable if the amount of degradation and related degradation products still render an acceptable product that is sufficiently efficacious and safe for human use over the shelf life.

Protein drug products for parenteral administration are sterile products and need to fulfill all relevant safety parameters for human administration (e.g., endotoxins and pyrogens), sterility [16–18], to comply to requirements related to subvisible particles [19–22], and to be essentially/practically free of visible particles [17, 18, 23] or "without visible particles, unless otherwise authorized and justified" [16], respectively. "Visible particles" are the number one reason for recall of parenteral products. It is an ongoing topic at facility inspections, and has been a topic of debate through the years, given that requirements such as "essentially free of visible particles" are not easily translated into an acceptance criterion of company procedures [24].

Parenteral drugs, including protein drug products, should preferably be euhydric (i.e., with physiological pH, pH 7.4) and isotonic (i.e., around 290 mOsm/kg) [25]. However, product requirements dictate and require a certain deviation from these targets. Not only solution pH but also buffer capacity, administered volume, and route of administration should be considered when developing formulations, the severity of disease and target indication, as well as the patient population [26]. The European Pharmacopeia also provides a framework to assess solution color and solution clarity (i.e., degree of opalescence) [27, 28]. The compendial monograph on "Monoclonal Antibodies for Human Use" mentions "slightly opalescent" and "slightly yellow" as likely targets for these endpoints, respectively. Opalescence (or "turbidity") can be related to many sources and is known to usually correlate with protein concentration. Opalescence can also be caused by protein aggregation, protein self-interaction [29], or can be a sign of phase-separation behavior [30]. The color can be related to aromatic amino acids of a given protein, it can relate to certain excipients (e.g., histidine), to instability reactions (e.g., Maillard reaction products with reducing sugars), or process residuals (e.g., vitamin B12). It can also relate to instabilities including modifications of aromatic amino acids of a protein [31]. Sometimes, companies also relate these various endpoints (color, clarity, etc.) into a specification endpoint called *appearance*. However, appearance can also relate to visual defects of a drug product, including, for example, defects of a stopper cap, under- or overfilling of a solution, or defects of a lyophilized product, such as fogging [32].

Besides stability and tolerability, the rheological behavior of a solution (e.g., viscosity and viscoelastic behavior) is of utmost importance for manufacturing and administration of the drug product [33, 34]. In addition, the compatibility with the

manufacturing equipment, with the administration set-up in case of IV administration (protein adsorption), as well as compatibility with the primary packaging material (e.g., for prefilled syringes) need to be adequately considered. A careful assessment and selection of processing parameters and manufacturing materials as well as administration materials such as syringe type, needle type, and needle length are critical to ensure product acceptability and functionality over the shelf life. These elements can also drive drug product development.

14.3.2 Container Closure System (CCS)

Every unit of a parenteral drug product consists of the liquid or solid protein formulation in its primary packaging, also called CCS. The CCS may most often relate to a vial, rubber stopper, and aluminum crimp cap, or in other cases, a syringe, cartridge, or blow-fill-seal (BFS) container. Typically, a container for protein drug product consists of type 1 glass (either expansion coefficient 33 or 52). In a few cases, plastic containers (plastic vials, syringes of BFS containers) have been suggested and evaluated. Challenges related to plastic containers may include inability to thermally sterilize, discoloration upon sterilization (e.g., irradiation), extractables/leachables, permeability (oxygen, water, excipient with low vapor pressure such as benzylalcohol), and possibly adsorption of the protein to plastic surfaces. Oxygen permeability can lead to protein oxidation, water permeability can lead to (apparent) increase in protein concentration, and excipient permeability may lead to critical product quality challenges.

The choice of the CCS is critical during drug product development and should be part of an initial target product profile (TPP). The combination of the primary packaging components must be adequately assessed and qualified [35]. In case the primary packaging also relates to functionality such as for a prefilled syringe, an injection pump, or an autoinjector, functionality needs to be assessed and ensured over the shelf life. In case of a prefilled syringe, for example, the selection of syringe attributes, such as homogeneity and degree of siliconization, needle diameter, needle length, stopper design, but also processing conditions such as method of stopper setting and subsequent air bubble size are critical for successful development of this drug/device combination product. Of note, these combination products pose specific documentation requirements.

14.3.2.1 Some Challenges with Container Closure Systems

A typical CCS consists of a glass vial, rubber stopper, and an aluminum crimp cap, or a syringe with its components, including glass barrel, rubber stopper, possibly needle or luer connector, rigid needle shield, and plunger rod.

A CCS needs to be adequately assessed prior to its use in humans as a drug product intended for human use. Assessments include suitability and integrity of the CCS in its combination, variations of dimensions, or extractables/leachables. This exercise can be called *CCS qualification* and is often independent of the actual product.

The container closure intergrity (CCI), especially, is of utmost importance and has gained significant attention recently. More sensitive tests have been suggested compared to dye ingress testing [36] and methods such as gas headspace analysis or helium leakage are evolving. Additionally, CCI testing needs to consider elements of a product life cycle, such as manufacture (e.g., capping), shipment (e.g., impact of pressure and temperature variances), and storage (e.g., storage condition). Especially, if considering frozen storage of a drug product, it may be suggested to evaluate CCI under frozen conditions as this may not be warranted, for example, due to glass transition temperature of the rubber stopper used for glass vials container [37].

The adequate selection of CCS is crucial as a number of challenges in drug product development relate to the interaction between the primary packaging container and the drug product formulation.

Glass delamination occurring in some drug products' glass containers for example, can be a safety risk for patients. Glass delamination is characterized by the formation of glass lamellae originating from the inner surface of the glass vial upon drug product storage. Glass delamination is dependent on the glass quality/glass composition, absence/presence of a coating, and terminal treatment of the drug product, for example, terminal heat sterilization; it is also dependent on the properties of the formulation (extreme pH, high ionic strength. and other formulation properties) [38, 39]. To investigate the propensity of glass delamination, different test methods are provided in the USP information chapter [USP 1660].

Glass contains a number of ions. These composites may leach into product solution as a function of time, formulation composition or temperature. One example of such a leachate is barium. In cases where sulfate ions are used in a formulation (e.g., as counterion in buffers), such glass leachates may lead to insoluble precipitants like barium sulfate over significant storage time [41]. These defects may be difficult to detect during stability testing, and levels may vary with glass vial type (expansion type 51 vs type 33, molding vs tubing) and glass batch to batch, given the variability of barium content in a given container.

For lyophilized drug products, the so-called "fogging" phenomenon is described in literature as originating from the interaction of the surface of the glass vial and the drug product. Owing to the Marangoni effect, which is based on a gradient in surface tension between the formulation (containing a surfactant) and a hydrophilic layer on the glass vial, the drug product solution creeps up the inner glass wall directly after filling. During the lyophilization process, the formulation freezes and dries yielding white drug product patches on the glass wall [32]. If the dried product is between the rubber stopper and glass vial, there is concern that this might lead to a critical defect due to possible liability of the container closure integrity. Usually, vials with fogging are classified as a having a cosmetic defect (especially if the dried solid is below the vial neck region). Yet, automated visual inspection becomes very challenging driving several and even manual/semimanual reinspections.

Prefilled syringes are siliconized by either baked on or sprayed on siliconization process to ensure functionality of the syringe by reduction of frictional forces

between the plunger and the syringe barrel. Thus, the drug product is in direct contact with the silicone layer over storage time. Especially upon injection, silicone droplets can migrate into the product solution. Perevozchikova *et al.* have shown that the formation of protein aggregates increased owing to interaction of protein and silicone oil droplets [42]. Yet, there are many examples where silicone oil did not cause protein aggregation or other instability reactions [43]. Thus, the drug product has to be evaluated if there is any concern related to silicone and the formulation may need to be developed in a way that drug product stability is maintained when stored in CCS with direct contact of the drug product solution with a silicone layer. Prefilled syringes are often delivered to the drug product fill-finish site with so-called staked-in needles, especially when these syringes should be ready for use by a patient or healthcare provider. The mounting hole for these needles is created during the syringe-making process by use of tungsten pins. During this process, tungsten oxide vapor deposits in the syringe funnel area and tungsten particles are occasionally shed. Precipitation of a protein as a result of interaction of tungsten with the drug product was shown by Bee *et al.* [44]. Many other proteins have shown acceptable stability in prefilled syringes over many years, despite sometimes significant residual amounts of tungsten in the pin region, thus, showing that not all proteins are susceptible to these residuals alike and that a case-by-case assessment is required.

14.3.3 Development of the Protein Formulation

14.3.3.1 Dosage Form

Parenteral drug products of biologics are typically either formulated as a liquid solution or suspension, or the liquid formulations are lyophilized in a final drug product manufacturing step depending on the stability at the intended storage temperature and TPP.

Liquid formulations can be combined easily with different injection devices (prefilled syringes, pens, and others) thus facilitating preparation/administration of the drug product by the healthcare professional. Moreover, use of injection devices foster self- and home-administration by the patient thus increasing patient convenience. Manufacturing of liquid formulations avoids the complex freeze-drying manufacturing step compared to the lyophilized (solid) dosage forms. This significantly reduces the cost of goods during manufacturing as well as resources during drug product development owing to development and transfer of complex freeze drying cycles.

Lyophilized formulations generally show increased stability compared to the corresponding liquid formulation due to removal of water during the freeze-drying process with the result of lower protein mobility. The protein is stabilized by lyo- and cryoprotectants during sublimation and desorption of the water during the lyophilization process. The lyophilization cycle has to be adequately designed on the basis of the formulation properties, vial design and fill volume, and the lyophilization equipment available. To keep cost of goods low, the

drying process should be developed as short as possible (drying close to the collapse temperature), yet as long as required in order to maintain elegant and stable drug product cakes. The capacity of the lyophilizer defines and limits the batch size. Although lyophilized formulations show an improved stability profile compared to their liquid formulations, lyophilized formulations have to be reconstituted before administration, which poses an additional (critical) handling step (aseptic handling) and is generally performed by a healthcare professional. The only currently available injection device for freeze-dried formulations are so-called "dual-chamber syringes," which allow for reconstitution and subsequent administration of the drug product with the same injection device.

Besides lyophilization, other drying technologies such as spray drying were investigated as alternative drying technology. This technology certainly has the advantage of a higher throughput compared to traditional lyophilization (batch process); however, it brings the challenge of subsequent powder filling for single-dose vials in a final manufacturing step. Thus, any powder-yielding process may be rather beneficial for bulk freezing/drying, for example, on a drug substance level. On the other hand, Gikanga *et al.* have shown the benefit of spray drying for the preparation of single-dose, highly concentrated protein solutions as an option to achieve higher protein concentrations [45]. In particular, they have elaborated on the challenge to find a balance of the sugar that is added to the formulation as stabilizer, as an increase in sugar concentration improves protein stability but decreases process efficiency at the same time.

14.3.3.2 Formulation Composition

A formulation of a protein for parenteral use typically contains the protein, which is dissolved in water for injection, buffer components, a surfactant, and one or more further excipients to ensure compliance and acceptability for human use. There are only few examples, where other (nonaqueous) solvents may be used, which are based on already approved products. However, these are commonly parenteral formulation of small molecules, which are poorly soluble, for example, Diazepam, which requires ethanol as organic/hydrophobic solvent or different formulation techniques (e.g., emulsion). A variety of excipients including solvents and solubility enhancers used in parenteral formulations have been reviewed by Nema and Brendel [46]. If used, the maximum amount needs to be carefully evaluated on the basis of their toxicological profile; the amount is also dependent on the route and volume of administration.

In the following section, typical excipients, their role in the formulation, and associated challenges are described. The section targets both liquid and lyophilized formulations as in some cases, dependent on the excipient concentration, liquid formulations are designed in a way that they can also be lyophilized (backup option). While buffer components and surfactants are equally used for both liquid and lyo formulations, these formulations can differ in type and concentration of stabilizing excipients.

Proteins are amphiphilic molecules. To maintain the pH during drug product manufacture and storage, buffer systems are often used in buffer strength suitable

to maintain the physiological buffer capacity of human blood (phosphate-, hemoglobin-, plasma protein-, carbonate-buffer) upon injection dependent on the injected volume. In the eye, even lower molarities are tolerated owing to the lack of proteins, which represents 7% of the total buffering capacity of the blood [47]. Commonly used buffer systems in protein formulations are for example histidine buffer ($pK_a = 6.0$), acetate ($pK_a = 4.8$), or citrate buffer ($pK_a = 3.1$; 4.8; 6.4). However, if a lyophilized formulation is considered, certain buffer systems should be chosen with care. Volatile buffer components such as carbonate or acetate buffers are usually avoided. If considered, the vacuum pump of the lyophilizer can be secured by a solvent trap. Yet, the homogeneity of buffer strength and thus pH, would still have to be evaluated across the batch and position in the lyophilizer.

Buffer systems can show crystallization of buffer components during the freezing step (e.g., sodium phosphate buffer, in some cases, citrate and succinate buffers) leading to a significant shift in pH during the freezing step, which, as a consequence, may potentially adversely impact protein stability. The choice of buffers is also critical from the patient perspective: the choice of buffer and pH should be designed in a way to minimize injection pain. Some buffers themselves have been reported to be connected to injection pain, such as citrate [48]. However, injection pain needs to be considered for many more elements than buffer or formulation, possibly including needle, injection volume, and others [49]. Sufficient low buffer strength may be warranted, and pH deviations from euhydric pH (pH 7.4) need to consider route of administration, injection volume, and buffer capacity.

Sugars and sugar alcohols are typically used to stabilize the protein in solution, during freezing/thawing or in the freeze dried state. These are, for example, sucrose or trehalose, acting as stabilizers and isotonizing agent at the same time if used in appropriate concentrations for the latter. Also, mannitol or sorbitol are often employed for freeze-dried formulations, yet, these excipients usually do not provide stabilizing effects on proteins rather than improving cake elegance, which will be elaborated for mannitol below. Excipients such as sucrose or trehalose are able to stabilize both liquid and lyo formulations under different conditions covering two different roles at the same time. These are (i) stabilizing the protein in the liquid phase and during freezing and thawing (applicable for both liquid and lyo formulation), and (ii) acting as cryo- and lyoprotectant, which is a prerequisite for stabilizing excipient used for lyophilization. However, these formulations may differ in their concentration of stabilizing excipient, as the protein:sugar ratio and the total solid content have to be considered, which is in most cases a compromise between (i) optimal stability (high sugar concentration/total solid content) and (ii) cake appearance/cake elegance (low sugar concentration/low total solid content) due to low glass transition-/collapse temperature. This requires the use of conservative lyophilization cycles, which directly increases the cost of goods as elaborated above (Section 14.3.3.1).

While all four mentioned stabilizing excipients are widely used, there are the following challenges associated with these excipients: Sucrose can invert to the reducing sugar fructose catalyzed at very acidic conditions. Reducing sugars, for

example, fructose or glucose, are rarely used to stabilize proteins due to the Maillard reaction. The carbonyl group of the reducing sugar and the amino group of the amino acids lysine, arginine, or the primary amine of the N-terminus lead to glycosylamine formation and thus change of color of the formulation (browning).

Trehalose and sorbitol were shown to lead to an increase in protein aggregation in the frozen state (e.g., ≥−20 °C) thus impacting frozen storage conditions of the drug substance. For trehalose formulations, the increases in high-molecular-weight species was shown to be a result of trehalose crystallization in the frozen state [50]. The crystallization of trehalose is only possible if formulations are stored above their glass transition temperature. Mitigation can be either to use slow cooling rates of the drug substance of <1 °C/min to allow the formation of solely the amorphous form of trehalose or to store the drug substance below the glass transition temperature of the formulations, for example, at −40 °C. In more detail, Connolly and colleagues have shown that trehalose crystallization is affected by the formulation composition as the phase distribution of amorphous and crystallized trehalose dihydrate in frozen solutions is dependent on the trehalose:protein ratio. They have identified an optimal range of trehalose–protein (w/w) ratio of 0.2–2.4 capable of physically stabilizing mAb formulations during long-term frozen storage.

Mannitol is often used in freeze-dried formulations acting as lyo- and cryoprotectant at the same time. However, for protein formulations, mannitol has in many cases only the task to form elegant cakes as it may crystallize upon freezing depending on the freezing parameters. Most protein formulations form amorphous cake structures as they usually freeze in an amorphous state. Thus, mannitol might not sufficiently stabilize the protein as required, but rather serve for cake formation. Other lyo- and cryoprotectants such as sucrose, in comparison, stabilize the amorphous form of the protein formulation by replacement of water (hydrogen bonds) and preferential hydration. Therefore, these excipients significantly improve stability of the protein formulation. However, as elaborated above for sucrose, the associated challenges are that the sugar concentration and sugar–protein ratios used are in most cases a compromise between optimal protein stability (high sugar content/high solid content) and cake appearance/cycle time. To add to this, mannitol does not solely crystallize when frozen, but forms different solid forms, that is, freezes in amorphous as well as crystalline forms. To avoid conversion from one to the other and to increase crystallization of mannitol to fasten primary drying, an annealing step is often recommended when using mannitol formulations. This is a temporary increase in temperature below the melting point of the formulation after initial freezing that allows for Ostwald ripening and ice crystal growth. Besides the formation of very elegant cakes by mannitol, the following point has to be considered in addition during formulation and cycle development when using mannitol: Mannitol may lead to glass breakage for certain formulations when frozen. Jiang *et al.* have shown that mannitol crystallization is fostered by high fill, fast freezing rates, and high mannitol concentration, and is dependent on target freezing temperature [51, 52]. Vial breakage can thus be mitigated by carefully evaluating the target freezing temperature and by adequate design of the freezing step.

Protein formulations are sensitive to interfacial stress given their poor physical stability. As amphiphilic molecules, they may interact at interfaces such as air/liquid or ice/liquid surfaces and aggregate or form particulates. To protect the protein from mechanical stress as well as from interfacial stress during freezing and thawing due to concentration, surfactants are often added to the formulation. These surfactants preferentially occupy interfaces competing with the protein, thus protecting it from exposure to the interface. Surfactant concentration above the CMC are commonly used to sufficiently stabilize the protein [53]. There are only few surfactants which are currently approved for parenteral use by the FDA and used in commercial products. These are polysorbate 20 and polysorbate 80 (Tween®), as well as (few products with) poloxamer 188 (Pluronic F 68®). Polysorbates, which are widely used were however shown to be prone to degradation either via the oxidative pathway or by hydrolysis. Hydrolysis has recently been suggested to be related to enzymes [54–56]. Oxidative degradation of polysorbate can occur in the final drug product as a result of the presence of reactive oxygen species. These radical species can reach into the product, for example, during drug product manufacturing if filling lines, isolators, or RABS (restricted access barrier systems) are decontaminated with vaporized hydrogen peroxide (VHP). Oxidative degradation can also occur in absence of oxidants introduced into the product, for example, iron cycling by Fenton reaction (residue in sugars) or impact of light, both of which can again lead to oxygen radicals that catalyze the oxidative degradation reaction. Degradation products of polysorbate consist, among thers, of fatty acids of different chain length and polyoxyethylene esters of fatty acids, which may have a lower solubility and precipitate/form particles upon storage [57] and thus be found as subvisible or visible particles. Oxidative degradation of polysorbate can often be mitigated by addition of a chelator, radical scavenger, or antioxidant, for example, methionine. Methionine can act as a stabilizer at the same time.

Acceptable injection forces (see Section 14.4) and manufacturability are among the requirements during formulation development of highly concentrated protein formulations. This requires low viscosity of the protein solution, which is a challenge at high protein concentration. Viscosity increases significantly (nonlinearily and mostly exponentially) when formulating at protein concentrations of 50–100 mg/ml and higher depending on the composition of excipients [58, 59]. Different chaotropic or cosmotropic salts are explored during formulation development to possibly reduce viscosity. Derivatives of arginine and arginine, especially, were shown to reduce viscosity most effectively for mAb formulations compared to other salts tested [60]. Recent investigations have shown that a variety of parameters contribute to viscosity and, in particular, the ones that charge interactions and zeta potential play a mechanistic role for excipients to modulate viscosity in protein formulations [61, 62]. To note, when salts are used as viscosity-reducing agents and, in particular, if chloride is used as counterion, corrosion of stainless steel containers during manufacturing and storage needs to be investigated, depending on the salt concentration used [63].

Parenterals are sterile products and are, in most cases, single-dose products. There are only few examples that allow for multiple dosing, for example,

the multidose version of Herceptin® 440 mg. Owing to the risk of microbial contamination although they may be handled aseptically, these products use preservatives, for example, benzylalcohol. Benzylalcohol is added, in this specific case, to the solution during reconstitution of the freeze-dried product.

For multidose products intended for parenteral use, cresol, phenol, benzylalcohol, or parabens are typically considered as preservatives for further evaluation. The efficacy of preservatives, especially, needs to be tested considering both the USP and Ph.Eur. requirements to ensure that bacteriostatic effects on the test germs that are evaluated. Of note, the preservatives used need to be carefully selected owing to potential safety considerations, especially considering specific patient groups (e.g., pediatric treatment). Benzylalcohol, for example, has been considered not acceptable for treating infants owing to safety concerns. Another relevant consideration is the compatibility with the actual product/formulation/other excipients from both the stability perspective of the protein [64] as well as the potential impact on the preservative efficacy. It has been found that in formulations containing the active (in this case, a peptide), a surfactant, and a preservative, the preservative efficacy is reduced [65]. A further consideration is the compatibility of preservatives with related manufacturing equipment. The authors' experience has provided evidence that benzylalcohol, for example, quickly evaporates across disposable tubings.

14.3.3.3 Stability Testing

Protein formulations are exposed to different stress conditions during formulation development. The stress conditions are chosen on the basis of different environmental conditions and settings that a drug product faces during its shelf life. These are, for example, (i) freezing and thawing, light exposure, oxidative stress from residual, reactive oxygen species from VHP decontamination during manufacturing,(ii) agitation during transport, (iii) different temperatures/humidities during storage, and (iv) shear stress during handling. Stress conditions need be chosen to be representative and relevant for the intended storage temperature and target manufacturing process with the aim to accelerate and/or force degradation along different degradation pathways. For example, using 50–60 °C in the development of a liquid mAb formulation may not be a good selection of the stress condition, as degradation pathways and instability reactions may be significantly different at the intended storage conditions (2–8 °C). In general, stress conditions close to or above the melting point of a protein are quite irrelevant to assess formulation stability given the (partial) thermal denaturation of the protein.

The use of stress conditions usually allows to detect liabilities early on. However, the direct extrapolation of drug product quality from accelerated or stress conditions to the intended storage or manufacturing conditions still remains difficult and may only give guidance for further development but cannot replace long-term stability testing at the intended storage conditions. Most important is the stress testing at different temperatures and relative humidities (rH). Guidance in this regard is given by the ICH guideline ICH Q1A and Q5C [66, 67]. The guideline

defines stability test conditions of drug products (DP) for long-term, intermediate, accelerated, and stress conditions for different storage temperatures as follows:

- Drug Product (DP) storage at room temperature (25 °C):
  - Long-term storage at 25 °C/60%rH
  - Intermediate storage at 30 °C/65%rH
  - Accelerated storage at 40 °C/75%rH
- DP storage in a refrigerator (5 °C):
  - Long-term storage at 5 °C
  - Accelerated storage at 25 °C/60%rH
- DP storage in a freezer (−20 °C):
  - Long-term storage at −20 °C

The complete data package for stability testing includes at least 6 months data from the test conditions enumerated above and is required for registration application and market approval for new drug products. In particular, the storage at refrigerated conditions, for example, requires data at the intended drug product storage temperature, but also accelerated data at 25 °C, which may detect instabilities relevant during the drug product manufacturing if at 25 °C. Additionally, temperature testing beyond 25 °C is suggested even for drug products intended to be refrigerated, in order to understand a product's degradation, as 25 ± 2 °C, can be easily exceeded during actual administration conditions. The shelf life of the drug product is assigned on the basis of formal stability data according to ICH Q1A [66].

Besides temperature stress testing, typical stress studies for biologics in formulation development include interfacial stress testing, such as mechanical stress testing (shaking) to ensure the product is robust against conditions observed, for example, during transportation/shipping [68, 69], and freeze/thaw studies to account for drug substance thawing and freezing during the compounding process. Forced degradation by light exposure (mandatory according to ICH Q1B [70]) or oxidative stress (forced oxidation by hydrogen peroxide, AAPH, or *t*-BHP) [71] is often performed only with the chosen formulation and not necessarily for all formulation combinations. Some of the selected stress tests and conditions, for example, light exposure, spiking of reactive oxygen species to simulate residual hydrogen peroxide after VHP decontamination, and extensive shaking, may be beneficial either for formulation differentiation to finally select the best one or to get a better understanding of the liabilities and the degradation pathways of the protein, yet, may reflect harsher conditions than the product will be exposed to throughout its shelf life even under worst-case conditions. Thus, these data need to be interpreted with care!

Table 14.1 presents the stress conditions and time points that are recommendable to be tested with the analytical panel specific for the drug product, considering the suggestions by ICH guideline Q1A and Q5C for a product intended to be stored at 5 °C [66, 67].

Following formulation development and accompanying stability testing, drug product development focuses on preclinical and clinical drug product supplies and related simulated administration (in-use) testing. In later stages, drug product

Table 14.1 Example of a stability program for sterile drug products intended to be stored in a refrigerator (5 °C).

Stress conditions		Time (months)									
		Initial	3	6	9	12	18	24	36	48	R
Long term	2–8 °C	x C	x	x	x	x O Y	x	x O Y	x O Y	x O Y	x O Y
Intermediate (optional)	15 °C	—	x	x	x	—	—	—	—	—	x
Accelerated	25 °C/60%rH	—	x	x	—	—	—	—	—	—	x
Stress (optional)	30 °C/65%rH or 40 °C/75%rH		x	x	—	—	—	—	—	—	—

The program (testing frequency) is applicable for products with shelf life request >1 year.
O = orientation testing. C = sterility and additionally possibly container closure integrity testing.
R = Backup sample.

process development is performed, which assesses the potential impact of each different unit operation of the drug product manufacturing process, and its related ranges (preliminary critical process parameter, pCPPs, and (pCPP) ranges) on drug product quality, specifically its critical quality attributes (CQAs). Unit operations that require thorough assessment include, for example, homogenization and mixing, filtration, filling, and lyophilization. During process characterization and validation, critical process parameters are tested and their impact on CQAs of the drug product (e.g., high-molecular-weight species or oxidation sites) is investigated. A quality-by-design approach for these studies may allow for flexibility in adjusting process parameters without additional regulatory approval, in a so-called design space. Within the design space, acceptable drug product quality is ensured by a robust manufacturing process [72].

14.3.3.4 Analytical Method Panel

The choice of the analytical method panel is essential for the successful development of stable parenteral drug products. Table 14.2 provides an overview of analytical methods used in protein formulation development to detect and/or quantify different instabilities and degradation products from different degradation pathways. This list is an extract of currently known instabilities and may be extended in the future.

Formulation development itself is recommended to use a combination of all relevant analytical methods covering the variety of instability reactions that can occur. However, using methods with inadequate precision, such as a bioassay or ELISA, or some methods used to assess particulates in the submicron range (e.g., NTA or RMM), which, in most cases cannot differentiate between the formulations, are typically only performed for confirmation only before formulation selection. In many cases, different methods provide different suggestions for a formulation selection. Thus, a formulation selection is usually a compromise of having all relevant data at hand. Choosing a formulation based on single analytical methods or endpoints is highly discouraged.

Table 14.2 Overview of analytical methods possibly to study drug product stability targeting different instabilities.

Category	Drug product quality attribute	Instability	Method	Comment
Protein purity	High- and low-molecular weight species	Aggregation and fragmentation/hydrolysis	SEC (size exclusion chromatography)	Size range: <100 nm; orthogonal methods, for example, AF4 [4]
			Capillary electrophoresis-SDS	High throughput: Microchip CE-SDS available
			SDS-PAGE	—[4]
	Charge variants (acidic and basic variants)	Chemical degradation (e.g., deamidation)	IEC (ion exchange chromatography)	—
			ICE (imaging capillary zone electrophoresis)	—
Protein content	Protein content	Protein adsorption	UV spectroscopy	—
			Others, for example, HPLC	—
Particle analysis	Visible particles	Aggregation, precipitation, denaturation	Black and white box, or equivalent	Size range: ≥100–150 µm [73]
	Subvisible particles		Light obscuration (quantification)	Size range: ≥2, ≥5, ≥10, ≥25 µm [19]
	Subvisible particles		Flow imaging (morphology)	>5/20 µm [74]
	Submicron particles		Coulter counter, NanoTracking, Archimedes, dynamic light scattering	Submicron (extended characterization) [74]
Physico-chemical solution properties	n.a.	Colloidal properties; can potentially indicate aggregation or phase separation	Turbidity	[27]
		Color of solution; can potentially indicate process residuals (e.g., vitamin B12) or changes in aromats (e.g., excipient degradation)	Color	[28]
		n.a.	Osmolality	[75]
			pH	—

(*continued overleaf*)

Table 14.2 (*continued*)

Category	Drug product quality attribute	Instability	Method	Comment
Critical excipients	Excipient content	Excipient degradation	(RP-)HPLC	
			Fluorescence micelle assay, mixed mode chromatography	Surfactant: polysorbate, poloxamer
	Excipient activity (as applicable)		Enzyme activity assay	Enzymes: rhuPH20 [76]
Potency	Potency	Binding properties	ELISA (enzyme-linked immunosorbent assay)	—
		Biological activity	Cell-based bioassay	—
Extended protein characterization	oxidation-, glycation-, deamidation-, isomerization-sites	Chemical degradation	LC–MS (liquid-chromatography–mass spectrometry)	[77, 78]
			Protein A chromatography	[77, 79]
			HIC (hydrophobic interaction chromatography)	[80, 81]
	n.a.	Change in higher-order structure	FTIR (Fourier-transformed infrared spectroscopy)	—
			CD (circular dichroism)	—
			Fluorescence spectroscopy	—
			ssHDX-MS	—

14.4 Handling and Administration Considerations

The common routes of parenteral administration include intravenous injection or infusion, intra-aterial and intrathecal injections, subcutaneous injection, intramuscular, intradermal, as well as intravitreal injections. The administration directly into the vitreous humor of the eye (intravitreal), especially, has been of increasing interest in recent years owing to an increase in ocular therapies in

development, for example, for neovascular age-related macular degeneration. In particular, for the treatment of chronic diseases, sustained-release formulations in combination with intravitreally administered implants have gained importance [82]. Formulations with controlled or sustained release of the protein in combination with a long half-life of the protein in the body would be most convenient for the patient to reduce dosing frequency and thus increasing patient adherence.

Drug products for intravenous injection are in many cases diluted before infusion in carrier solutions such as saline or dextrose or Ringer's lactate. Solutions used for infusion need to be thoroughly evaluated (from a pharmaceutical manufacturer perspective) and should always clearly follow the recommendations by the pharmaceutical manufacturer (from a user perspective). The products used, include large-volume IV bags, for example, 0.9% sodium chloride solution, Ringer's solution, Ringer's lactate solution, or 5% dextrane solution. During drug product development, compatibility with and stability in these solutions over a certain time (after preparation and before administration) has to be tested, as well as the compatibility with the administration setup comprising not only of the IV bag but also of the respective tubing, injection setup, and, in some cases, an in-line filter [83]. The adsorption of the proteins to different surfaces of the setup in use and the formation of visible particles as a result of, for example, insufficient, or a decrease in, surfactant concentration have to be investigated during drug product development. The IV bags are prepared in clinics by healthcare professionals and stored until use. The microbiological quality of the product has to be ensured and holding times have to be tested and defined during drug product development [84].

Intravenous infusion allows for administration of rather large volumes depending on the formulation composition (e.g., 250 ml). To reduce dosing frequency and to allow for home treatment by a healthcare professional or even self-administration by the patient, the subcutaneous route of administration may be considered instead.

Injection volumes for the subcutaneous space were recently still considered to be limited to a few (1–2) milliliters [33, 85]. Pain as well as damage of the tissue upon injection were discussed as limiting factors for injection volume. However, pain is also considered to be dependent on various other factors, including, but not limited to, injection speed, injection site, route of administration, device dimensions especially of the injection needle, formulation parameters (pH, osmolality, viscosity), the skills of the medical care person, and individual factors of the patient (disease status). Thus, the acceptability related to pain is usually investigated in patients for each drug product. Examples of marketed products with small injection volumes <2 ml intended for subcutaneous use are Enbrel® 1 ml, Humira® 0.8 ml, Cimzia® 1 ml, Actemra® 0.9 ml, or Prolia® 1 ml.

However, there are opportunities to push the boundaries of subcutaneous injection volume, which were recently reviewed by Mathaes *et al.* [49]. The recent market approval of Repatha® has shown that delivery of 3 ml into the subcutaneous space with one injection is feasible, tolerable, and acceptable for the patient. Moreover, there are other parenteral products already on the market,

for example, Firmagon® (deca-peptide) with a subcutaneous injection volume of 4 ml. This clearly shows that the boundaries for subcutaneous administration have to be revised – even beyond the currently approved injection volume. Another possibility to increase the applicable volume during subcutaneous injection is the addition of recombinant human hyaluronidase (rhupH20), an enzyme that transiently cleaves the hyaluronidase network in the subcutaneous tissue [85]. First products using this technique have already been developed, for example, trastuzumab for subcutaneous use [86] and approved and on the market, for example, MabThera® 1400 mg intended for subcutaneous use. Nevertheless, the advantage of larger injection volumes, which, in many cases, reduces dosing frequency thus directly increasing patient compliance and patient adherence needs to be carefully balanced against the physiological reactions and changes of the subcutaneous tissue during and after injection.

To conclude, injection volumes for intravitreal injections are even lower compared to intravenous or subcutaneous injections. They are usually limited to 50 or 100 µl owing to a potential increase in ocular pressure upon injection (closed compartment) [87].

For the limited injection volume for subcutaneous injections (and intravitreal injections), and considering the need to deliver equivalent doses as for IV administration, highly concentrated formulations need to be developed. Challenges of highly concentrated protein formulations during formulation development are, besides protein aggregation, the exponential increase in viscosity with increasing protein concentration: As elaborated in Section 14.3.3.2, the viscosity is directly related to the injection forces. Viscosity is also a function of temperature, thus, solution temperature prior to injection is key. As an example, it is crucial for a drug product which is, for example, stored at 5 °C that it is equilibrated to room temperature before administration as recommended in the packaging insert. At lower temperatures the viscosity of the drug product is much higher than at room temperature, which directly translates into higher injection forces. A second reason is that injection of an equilibrated sample is more convenient to a patient from the perspective of pain.

Acceptable injection forces are not universal and are highly dependent on the patient population, a given patient state, and their ability to use the product correctly. Anthropometric strength studies have shown that the force that a user can exert onto a syringe plunger is determined by multiple factors, including the health status and the strength of the individual, and the upper limb and hand posture required for injection [88]. These human factors also cover the personal preference and training of the individual. In reality, users moderate the injection force by adjusting their injection speed and may choose a slower injection resulting in a lower injection force to fit their capability or preference. Especially if self-administration is considered, the capability of the patient population to inject a specific drug product is usually tested in so-called "human-factor" studies.

Injection forces are directly related to viscosity as recently investigated and modeled for Newtonian as well as non-Newtonian solutions [89, 90]. It was shown that the most important factor is the inner needle diameter which

contributes to injection forces by the power of 4. Thus, the selection of the needle diameter (in combination with other device attributes) needs to be carefully made and injection forces evaluated. An open question for quite some time was the contribution of the tissue resistance during subcutaneous injection with injection rates in the range of 0.1 ml/s. Allmendinger *et al.*, who have recently shown the linear dependence of the tissue resistance on viscosity and injection rate, however also stated that the contribution is significant only at high viscosities [91].

Highly concentrated formulations intended for the subcutaneous use are often codeveloped with injection devices, such as prefilled syringes, autoinjectors, pens, or even more complex devices such as injection pumps. The use of these injection devices allows for home treatment or even self-administration, which is more convenient for the patient and adds to the overall quality of life. The design and engineering of these injection devices is another field of development on its own and is usually performed in parallel to the drug product development. The overall goal is to ensure drug product stability as well as device functionality over the shelf life and to design the most convenient and appropriate injection device for the specific patient population at the same time.

14.5 Summary and Conclusion

The development and manufacture of biologics drug products for parenteral administration is a science and an art. Drug products need to be adequately developed, considering product stability, fulfillment of regulatory and compendial requirements, but also need to be integrated with CCS development and adequate drug product manufacturing process design. Formulation development requires a comprehensive approach of using adequate stress conditions, acceptable excipients and combinations thereof, adequate analytical methods, and considering adequate primary packaging. The development of drug/device combination product provides additional challenges.

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Part V
Clinical Applications

15
Protein Therapeutics in Autoimmune and Inflammatory Diseases

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15.1
Introduction

Inflammation is a complex host response to tissue injury, pathogen exposure, and/or the activation of the immune system that inappropriately recognizes self-antigen as foreign agents. The signaling molecules that drive inflammation include cytokines released from immune cells, cells of stromal origin, and mediators released from dying or damaged cells. Over the last decade, there has been an increase in our understanding how the immune system can contribute to the pathogenesis of a number of disorders. In this chapter, we will discuss how our increased understanding has led to the development of new therapeutics to treat rheumatoid arthritis (RA), psoriasis, atopic dermatitis (AD), inflammatory bowel disease (IBD), and systemic lupus erythematosus (SLE).

15.2
Rheumatoid Arthritis

RA is an autoimmune disorder characterized by systemic inflammation – especially within the joints of articulating bones, and is frequently accompanied by the production of autoantibodies. RA affects multiple joints at a time and generally occurs bilaterally. Untreated, autoimmune attack on bone and cartilage may result in progressive joint destruction and, ultimately, disability. Roughly 40 out of 100 000 people or 0.5 – 1.0% of adults worldwide suffer from this autoimmune disorder [1]. The factors that trigger pathologic immune dysfunction in joint tissue are not well understood, but they include both genetic and environmental triggers [2]. Not surprisingly, disease associations with genes involved in T-cell repertoire selection (HLA-DRB) and T-cell receptor signaling (PTPN22), as well as with cytokine promoters, have been reported [3, 4]. Smoking, alcohol consumption,

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socioeconomic status, and region of birth are among the environmental factors correlating with the risk of developing RA [5].

The pathophysiologic mechanisms underlying RA are multifold, and involve dysregulation of innate and adaptive immunity. Macrophages, mast cells, and neutrophils are among the innate effector cell types present in elevated numbers in synovial tissue. Circulating autoantibodies against the Fc portion of human IgG (referred to as rheumatoid factor), keratin, and a range of citrullinated auto-antigens are commonly observed in RA patients [6, 7]. Joint destruction results from chronic inflammation mediated by several cell types including T cells, B cells, monocytes, macrophages, and osteoclasts. Clearly, pro-inflammatory cytokines are central to disease pathology. Because of redundancies and pleiotropic cell-cytokine signaling, however, it is not possible to attribute RA pathophysiology to a single cytokine node. RA disease progression is accompanied by an imbalance favoring pro-inflammatory Th1 cytokine signaling over Th2 [6, 7]. TNF-α, produced by activated macrophages and T cells, is clearly an apical player and paracrine inducer of additional pro-inflammatory cytokines including IL-1, IL-6, and IL-8 [8]. The IL-1 family cytokines (IL-1α, IL-1β, IL-18, and IL-33), which promote activation of leukocytes, endothelial cells, osteoclasts, and chondrocytes, are highly expressed in RA patients [7]. IL-6 is recognized as a driver of local synovial leukocyte activation, antibody production, and synovial fibroblast proliferation [8].

The term DMARDs, for disease-modifying anti-rheumatic drugs, was coined to refer to drugs used to treat RA. The most common DMARD is methotrexate (MTX), an anti-metabolite that interferes with DNA synthesis. While the precise mechanism of action through which MTX works in RA is unclear, it commonly relieves joint inflammation and pain, can delay joint destruction, and is frequently the first line of therapy for new RA patients [9]. Unfortunately, all patients do not respond to MTX, and many who respond initially go on to experience disease progression. Subsequent treatment strategies include switching to another DMARD, increasing the MTX dose, or moving to a biologic therapy (often while maintaining MTX). Fortunately, biologic therapies, some of which are highlighted in the following, have had a huge impact in the treatment of RA.

15.2.1 TNF-α Antagonists

TNF-α antagonists were the first biologic agents to be evaluated and approved for the treatment RA. TNF-α is a pro-inflammatory cytokine and is produced as a consequence of both microbial stimulation and tissue damage. TNF-α is produced from a variety of cell sources including T cells, monocytes/macrophages, and cells of non-hematopoietic origin including epithelial cells and fibroblasts. TNF-α is a homotrimeric cytokine and is either membrane-bound or can be cleaved and released as a soluble protein though the action of TACE. TNF-α exerts its biologic effects by acting on two receptors, namely TNFR1 and TNFR2. Early rationale supporting TNF-α as a therapeutic target came from the observation

that expression levels of TNF-α, among other cytokines, were elevated in synovial material isolated from patients suffering from RA [10]. Testing of antisera raised against TNF-α on *in vitro* synovial cell cultures reduced the production of IL-6, IL-8, and GM-CSF among other cytokines and suggested that TNF-α might be a master regulator of inflammatory cytokines. Consistent with such a role, mAbs against TNF-α resolved inflammation and slowed disease progression in collagen-induced arthritis mouse models [11].

Currently, five TNF-α antagonists have been approved for the treatment of RA: infliximab (Remicade™), a chimeric mAb; adalimumab (Humira™) and golimumab (Simponi™), both fully human mAb; certolizumab (Cimzia™), a humanized Fab fragment conjugated to polyethylene glycol (PEG); and etanercept (Enbrel™), a fusion protein wherein the Fc portion of IgG1 has been linked to two moieties derived from the TNF-α receptor extracellular domain. All these agents bind TNF-α with high affinity and prevent binding of the cytokine to the TNF-α receptor complex and/or prevent subsequent receptor complex signaling. TNF-α inhibitors are commonly used in combination with MTX and have comparable ACR response rates [12, 13]. Roughly 30% of patients suffering from RA are not responsive to anti-TNF therapy, and approximately 30% of patients who initially respond often develop resistance [14]. Varying frequencies of anti-drug antibody (ADA) have been reported for adalimumab, etanercept, and infliximab [15], a phenomenon that may contribute to the development of resistance. Interestingly, patients who relapse on one TNF-α therapy may go on to respond to a different TNF-α antagonist or to a distinct biological therapy [16, 17].

TNF-α antagonists have also been approved in other rheumatological indications including psoriatic arthritis (PsA) and ankylosing spondylitis (AS). All five anti-TNF-α antagonists have been approved for PsA and have dramatically improved the treatment of this disease, achieving improvements in the ACR20 between 50% and 60%. These therapies improve all the disease domains of PsA, including arthritis, spondylitis, and the associated skin manifestations. Similarly, in patients with AS, TNF-α antagonists have shown to be valuable therapeutics, with no additional safety concerns above those identified in patients with RA and are currently approved for use in both the EU and the United States.

15.2.2
Inhibition of Co-Stimulation

T-cell activation requires presentation of the antigen by the major histocompatibility complex II on antigen-presenting cells as well as a second "co-stimulatory" signal. The co-stimulatory signal occurs when the CD28 receptor on the T cell engages with CD80 (B7-1) or CD86 (B7-2) ligands on the APC. Cytotoxic T-lymphocyte-associated protein 4 (CD152/CTLA-4) also binds CD80 and CD86, but unlike the CD28 receptor, CTLA-4 engagement by CD80/86 delivers a negative signal to inhibit T-cell activation [18]. CTLA-4-Ig binds with high affinity to CD80/CD86 and hence prevents CD28-dependent co-stimulation. Abatacept (Orencia™) is a humanized fusion protein composed of the Fc

region of the immunoglobulin IgG1 (devoid of ADCC and complement fixation activities) fused to the extracellular domain of CTLA-4. Abatacept–MTX combination therapy led to meaningful and durable responses as defined by ACR (40% achieved ACR70) and DAS28 (CRP) criteria, (45% achieved DAS28 CRP-defined remission) and was well tolerated [19]. FDA approved the use of abatacept as a treatment for RA in 2005. While CD28 blockade of T cells is widely viewed as abatacept's primary mechanism of action, CD80/86 are also expressed on osteoclast progenitors, endothelial cells, myeloid dendritic cells, B cells, and macrophages. Thus modulation of signaling across these cell types cannot be excluded from contributing to abatacept's efficacy [20].

15.2.3
Anti-IL-1 Based Therapies

IL-1α and IL-1β are potent mediators of inflammation that signal via interaction with the IL-1 Type I receptor (IL-1RI). The effects of these cytokines are inhibited by an endogenous antagonist, interleukin-1 receptor antagonist (IL-RA). Anakinra (Kineret™) is a recombinant version of IL-1RA, wherein an additional methionine residue was inserted at the amino terminus. Anakinra was evaluated in numerous RA clinical trials as a monotherapy and in combination with MTX and found superior to placebo based on ACR20/50/70, health questionnaire assessment, and radiographic criteria [21]. It was approved by FDA in 2001 for patients with moderate to severe RA who failed at least one DMARD therapy. Anakinra is administered as a subcutaneous injection, but since its half-life is on the order of only 4–6 h, it is typically administered once daily, which has limited its utility in the treatment of RA. Additional IL-1 blockade therapies have been developed that include canakinumab (Ilaris™), an anti-IL-1β neutralizing mAb, and rilonacept (Arcalyst™), an IL-1 trap created by fusing an IgG1 FC domain with the extracellular domain of IL-1R1. However, the efficacy of these therapeutics is poorer than that of anti-TNF-α agents, which has limited their widespread use in RA. Canakinumab has, however, been approved both in the United States and the EU for the treatment of systemic juvenile idiopathic arthritis. In addition, canakinumab and rilonacept have also been approved for the treatment cryopyrin-associated periodic syndrome (CAPS), a condition caused by mutations in the NLRP-3 gene [22].

15.2.4
Anti-IL-6 Therapies

IL-6, a 26 kDa glycopeptide and pro-inflammatory cytokine, is produced predominantly by macrophages and fibroblasts. IL-6 levels are elevated in synovial fluid from the joints of patients with active RA versus healthy controls and correlate with other markers of disease and joint destruction [23, 24]. The biologic activities of IL-6 are consistent with an RA pathophysiologic role. IL-6 promotes inflammation through a variety of mechanisms including increasing neutrophil adherence

to fibroblasts, leukocyte recruitment, terminal differentiation of B cells, and stimulating autoantibody production in B cells. IL-6 also acts on bone metabolism and angiogenesis [25]. Further rationale for targeting IL-6 in RA comes from collagen-induced arthritis (CIA) studies in mice, where IL-6 blockade attenuated clinical and histologic manifestations of arthritis and induced the expansion of Treg cell populations [26, 27].

IL-6 signaling is triggered by two distinct mechanisms involving membrane-bound and soluble IL-6 receptor proteins. In the first, IL-6 binds the 80-kDa membrane-bound IL-6R (mIL-6R) followed by the association of the IL-6/mIL-6R complex with a non-ligand-binding signal transducer protein, gp130. In the second, IL-6 binds to a soluble IL-6R (sIL-6R), which lacks transmembrane and cytoplasmic domains, but which upon binding IL-6, can associate with gp130 and transduce a signal. In summary, both mIL-6R and sIL-6R play essential roles in IL-6 signaling [25]. Tocilizumab (TCZ) is a humanized IgG1 monoclonal antibody (mAb) that binds with high affinity to both soluble and membrane-bound forms of IL-6R and prevents binding to IL-6 cytokine [28]. More than a dozen RA patient trials have established that TCZ monotherapy and TCZ/MTX combination therapy are both effective in reducing the signs and symptoms of RA in patients with inadequate MTX responses [29]. Moreover, TCZ monotherapy is superior to MTX monotherapy, regardless of prior MTX exposure. TCZ was generally well tolerated. Common adverse events included infections, elevated liver enzymes, and elevated low-density lipoprotein [29]. In 2010, TCZ was approved as a second-line therapy for RA patients who failed other DMARDs.

15.2.5 B-Cell Depletion Therapies

Rituximab is a chimeric B-cell-depleting mAb directed against the B-lymphocyte antigen CD20. CD20 is expressed throughout B-cell development from the late pro-B cell through the memory cell lineages (but not on plasma cells or plasma blasts). Rituximab depletes B cells from the periphery and partially from the bone marrow and synovial tissue via a combination of antibody-dependent cellular cytotoxicity and complement-mediated cytotoxicity [30]. Administration of rituximab leads to transient but almost complete depletion of B cells in the peripheral blood and partial depletion of B cells in the bone marrow and synovial tissue. The Fc portion of rituximab mediates apoptosis of CD20+ B cells. Repopulation of the peripheral blood B cells usually occurs at 6–9 months after rituximab administration.

Rationale for B-cell targeting as a therapeutic approach in RA comes from several observations: plasma cells and activated B lymphocytes are abundant in RA synovium; circulating autoantibody titers are common in RA patients; B cells produce pro-inflammatory cytokines such as TNF-α and IL-6, which are known to contribute to RA pathophysiology [31].

The proof of principle that B lymphocytes were drivers of RA disease pathology came more than 15 years ago when rituximab was tested in the first RA patient

trial. Rituximab depleted B-lymphocyte counts to undetectable levels, significantly reduced circulating autoantibody titers, and improved American College of Rheumatology scores (ACR, a multiparameter disease index) by ≥50% [32]. In subsequent trials, B-cell depletion has been established as an efficacy biomarker [33]. Numerous clinical trials have established that rituximab in combination with MTX provides clinical benefit over either single agent, even in patients who are MTX-refractory or anti-TNF refractory [31]. Following the failure of a single anti-TNF-α therapy, Rituxan was proven to be more effective than implementing a different anti-TNF-α agent [34].

15.3 Psoriasis

Psoriasis is a chronic inflammatory skin disease affecting ~2.5% of the world's population [35]. Though the underlying pathogenesis still remains to be fully elucidated, it is clear that environmental factors – stress, chronic infections, smoking, and obesity, among others – and genetic factors (genome-wide association studies (GWAS) have implicated polymorphisms in the genes for the IL-23R, the human leukocyte antigen HLA-Cw6, and the promoter regions of the gene for TNF-α) contribute to a pathologic immune response mediated by multiple cytokines and chemokines [36]. The disharmony between immune cells and skin cells is thought to drive rapid proliferation of skin cells, ultimately resulting in excessive turnover of skin cells and the development of plaques of thickened scaling skin. While most patients suffer from a mild form of the disease, 20–30% of patients have moderate to severe disease.

Mechanistically, psoriasis appears to result from complex crosstalk involving distinct cell types. Perhaps at the root, keratinocytes, Langerhans cells, dermal dendritic cells, and macrophages in the skin produce elevated levels of IL-23. IL-23 stimulates Th17 cells to produce IL-17A, which is increased in the sera of psoriatic patients compared with healthy individuals. Increased numbers of IL-17A-positive CD4 and CD8 T cells are found in psoriatic lesions, where they are thought to drive increases in IFNγ, IL-17, IL-22, and TNF-α [37].

15.4 TNF-α Antagonist Therapy

TNF-α antagonists have dramatically improved outcomes for patients with moderate to severe psoriasis since their approval over a decade ago, and are extensively characterized. Etanercept, the first TNF antagonist, was approved by FDA in 2004 to treat plaque psoriasis. Infliximab was approved in 2006, and adalimumab in 2008. Certolizumab is currently being evaluated in Phase III studies, whereas golimumab, as noted earlier, is approved for multiple indications but not psoriasis. Despite the effectiveness of TNF antagonists in the treatment of psoriasis, similar

to patients with RA or IBD (see below), some never achieve an initial response to treatment (primary failure), whereas others lose their initial response over time (secondary failure). Switching to a different TNF antagonist, specifically after the secondary failure, is often successful in regaining a response.

15.5 Anti-IL-12/IL-23 Therapies

IL-12 and IL-23 are pro-inflammatory cytokines produced by activated antigen-producing cells, which bind to receptors on natural killer (NK) cells and CD4+ T cells. NK cell activation and Th1/Th17 differentiation, respectively, ensue. IL-12 and IL-23 consist of heterodimeric protein complexes (p40 and p35 for IL-12; p40 and p19 for IL-23) and share a common 40-kDa subunit, p40. IL-12 signals though the IL-12 RB1 and IL-12 RB2 receptors, whereas as IL-23 uses the shared IL-12 RB1 in combination with its own unique IL-23 receptor chain. The IL-12/IL-23 axis plays a central role in T-effector cell differentiation, with IL-12 driving, in the absence of IL-4, T-effector precursor cells to a Th1 phenotype. Moreover, IL-23, together with IL-6 and TGF-β, leads to the differentiation and expansion of Th17 cells. A role for p40 in mediating autoimmune disease was established through a series of mouse knock-out studies, wherein p40-deficient mice were found to be resistant to a range of experimentally induced autoimmune diseases [38]. Furthermore, intradermal injection of IL-23 into mouse skin led to the development of psoriatic sequelae in mice [39]. Genetically, polymorphisms within the gene encoding p40 (IL-12B) are associated with psoriasis [40], and a single nucleotide polymorphism (SNP) rs11209026 located in the IL-23R coding region confers a protective benefit from psoriasis [41]. Finally, IL-23 gene expression is increased in psoriatic lesions compared with normal uninvolved skin [42], further suggesting a pathophysiologic role.

Ustekinumab (Stelara™) is a human IgG1 mAb that recognizes the 40-kDa subunit shared by IL-12 and IL-23. Biophysical studies indicate that ustekinumab binds equally well to IL-12 and IL-23 [43]. Ustekinumab prevents p40 from binding the IL-12 receptor β 1 subunit, the receptor shared by the IL-12R and IL-23R complexes [43, 44]. Ustekinumab is unique among biologic therapies in its ability to simultaneously target NK cell activation and Th1 (IL-12)- and Th17 (IL-23)-mediated cellular responses.

The efficacy of ustekinumab monotherapy was evaluated in two placebo-controlled Phase III studies. Seventy five percent improvement to the Psoriasis Area Severity Index (PASI) (PASI-75) occurred in 65–75% of ustekinumab-treated cohorts versus 3–4% treated with placebo [38]. Ustekinumab has also shown benefit in patients with psoriatic arthritis, a disease characterized by stiffness and swollen joints occurring in ~24% or patients with plaque psoriasis [45].

Ustekinumab was evaluated head to head versus High-dose etanercept and found superior in reducing PASI scores in patients with psoriasis with an equivalent safety profile [46]. Ustekinumab and secukinumab were also evaluated

head to head in patients with plaque psoriasis. Secukinumab was found superior, with 79% versus 57% of patients experiencing PASI90 response in secukinumab and ustekinumab groups, respectively [47]. Ustekinumab has been approved for the treatment of plaque psoriasis in the United States, Europe, and Canada.

15.6 Anti-IL-17 Therapies

Interleukin-17 (IL-17) is produced by Th17 cells in response to IL-15 and IL-23. Large amounts of IL-17A are also secreted by CD8+ T cells, neutrophils, macrophages, and mast cells. IL-17A has two receptors, IL-17RA and IL-17RC, which are nearly ubiquitously expressed [48]. The binding of IL-17A to its cognate receptor triggers a pleiotropic release of pro-inflammatory cytokines and chemokines, which, unfettered, can result in destructive tissue damage.

IL-17 is a family of pro-inflammatory cytokines composed of six IL-17 family ligands: IL-17A (the founding member), IL-17B, IL-17C, IL-17D, IL-17E, and IL-17F [49, 50]. AA and FF homodimers, as well as AF heterodimers, have been implicated in autoimmune disease [50].

Evidence for IL-17's role in psoriasis and autoimmune disease is multifold. IL-17 mRNA levels accumulate in psoriatic lesions [37]. Knockout of IL-17 in mice slowed disease progression in CIA [51] and experimental autoimmune encephalitis models [52]. IL-17 levels are elevated in synovial fluid from RA patients [53].

Secukinumab (AIN457/Cosentyx) is a recombinant, fully human IgG1 mAb that is highly selective for IL-17A. Impressively, a single 3 mg/kg dose of AIN457 significantly reduced the mean PASI for up to 12 weeks and led to reductions in histomorphological signs of epidermal hyperplasia [54]. Skin samples from psoriasis plaques had significantly reduced levels of IL-17A, IL-1B, IL-6, and IL-12B (the p40 subunit of IL-12 and IL-23) among other cytokines and chemokines [54]. In Phase III studies, secukinumab was associated with a rapid reduction in psoriasis symptoms, eliciting significantly greater PASI 75 rates than placebo through 12 weeks and was associated with sustained high response rates in a majority of patients through 52 weeks. In head-to-head evaluation against the TNF-α inhibitor etanercept, secukinumab was found superior over a period of 52 weeks [55]. FDA approved secukinumab for the treatment of moderate to severe plaque psoriasis in 2015. Two additional IL-17 targeting agents are in late-stage development for psoriasis: brodalumab, an IL-17R targeting mAb, and ixekizumab, a humanized anti-IL-17 IgG4 antibody.

15.7 Atopic Dermatitis

AD is the most common inflammatory skin disease and is characterized by inflammation of the skin, which can present either acutely or chronically. As the

pathogenic drivers of the disease are poorly defined, the development of specific treatments has been elusive. Whereas psoriasis is increasingly considered to be a Th17-driven disease, the drivers of AD remain controversial. Interestingly, AD patients described more frequent and intense itch as compared with psoriatic patients. Associated sweating and heat sensation were also more common in AD versus psoriasis [56]. Pro-inflammatory cytokines, particularly of the Th2 variety, are strongly linked to acute AD disease pathology, whereas chronic disease may be more Th1-driven [57]. IL-4 and IL-13 have been referred to as the master inflammatory mediators of the Th2 response. Both cytokines bind to the IL-4 receptor α subunit, a component of the Type II IL-4 receptor complex. This complex is expressed on many cell types including macrophages, eosinophils, and fibroblasts. Additional rationale for targeting IL-4/IL-13 comes from the observation that allelic variations in the gene encoding IL-4Rα are linked with asthma and eczema, among other inflammatory disorders [58].

15.7.1
Anti-IL-4/IL-13 Therapies

Dupilumab is a fully human mAb directed against the IL-4Rα subunit. Thus, dupilumab blocks the signaling of two key drivers of Th2-mediated inflammation, IL-4 and IL-13. In placebo-controlled Phase IIb AD patient trials, dupilumab was evaluated as a monotherapy as well as in combination with topical glucocorticoids. Eighty-five percent of dupilumab-treated patients (vs 35% of placebo-treated patients) experienced a 50% reduction in the Eczema Area and Severity Index (EASI). Patients also reported clearing of skin lesions and reduced itching. In combination studies, 100% of patients in the steroid/dupilumab cohort (compared with 50% in the steroid-only cohort) met EASI50 criteria. Common adverse events included nasopharyngitis and injection-site reactions but they were not dose-limiting [59]. Dupilumab received breakthrough therapy designation from the FDA for use in patients with moderate to severe AD and is currently being evaluated as monotherapy in four Phase III clinical studies expected to read out in 2016. Dupilumab has also shown great promise in asthma patients with evidence of Th2-mediated pathology [60].

15.8
Inflammatory Bowel Disease (IBD)

Crohn's disease and ulcerative colitis are the two most predominant forms of inflammatory bowel disease. Crohn's disease is a relapsing, remitting systemic inflammatory disease, characterized by abdominal pain, abnormal stools, diarrhea, and complications including fibrostenosis and strictures that can often lead to surgical intervention. Similarly, ulcerative colitis is a chronic inflammatory disorder associated with ulcerations. While some of the existing treatments for IBD are effective in both ulcerative colitis and Crohn's disease, these disorders

are distinct pathologically, and often a biopsy of the gastrointestinal mucosa is required to differentially diagnose one from the other. Another distinguishing aspect of these two autoimmune disorders of the gastrointestinal tract is that ulcerative colitis affects only the colon and rectum whereas Crohn's disease can affect the entire gastrointestinal tract. The exact causes of both ulcerative colitis and Crohn's disease remain unknown, although there is a greater incidence of IBD in more developed countries, which is increasing. There also appears to be a genetic component to the disease, as the risk of inheritance increases when both parents have IBD. Research into the genetics of ulcerative colitis and Crohn's disease has led to the identification of more than 160 IBD-associated genetic loci. Interestingly, only approximately one-third of these genes overlap between Crohn's disease and ulcerative colitis, supporting the notion that these diseases have distinct etiologies [61].

15.8.1 Pathophysiology of IBD

There is growing evidence that Crohn's disease is driven at least in part by a dysregulation of the interaction between the intestinal microbiota and the host immune system [62], resulting in damage to the gastrointestinal mucosa. In addition, there appears to be a loss of barrier function of the intestinal epithelium, where the tight junctions between the polarized epithelial layer become leaky. A reduction in the mucin cover allows access of luminal antigens to the lamina propria [63]. As a result of increased microbial sensing through pattern recognition receptors including the Toll-like receptors (TLRs) and nucleotide binding domain-like receptors (NLRs), the innate immune system may become hyperactivated. In addition, inappropriate activation of the adaptive immune system is believed to play a central role in driving and perpetuating mucosal inflammation and injury. The activation and recruitment of effector T cells resulting in TNF-α, IL-17, IL-22, and IFN-γ secretion, together with the dysregulation of regulatory T cells that produce IL-10 and TGF-β, further contribute to the immune insult in the intestinal mucosa in Crohn's disease [64]. In contrast, ulcerative colitis has been described to be more associated with Th2 cytokines producing elevated levels of IL-5 and IL-13, but not IL-4 [65, 66]. While much research has focused on cytokines derived from effector T cells, in recent years, innate lymphoid cells (ILCs) have been suggested to play an important role in mucosal immunity. Although they represent a small fraction of the cells within the gastrointestinal mucosa, they produce a wide spectrum of cytokines upon innate activation [67]. Three distinct major subsets of ILCs have been identified: ILC1 express the transcription factor T-Bet and produce IFN-γ; ILC2 are GATA3+ and produce IL-5 and IL-13; and ILC3 are defined by RORγT and produce IL-17 and IL-22. A number of ILC subsets have been reported to be increased in the inflamed mucosal of patients with Crohn's disease most notably ILC1 [68]. The precise contribution of the interplay between the innate and the adaptive immune system to Crohn's disease remains to be further elucidated. There are no cures for IBD, but once a diagnosis

is made, the primary goals of therapy are to induce remission, provide a durable response (i.e., maintenance therapy in the absence of relapse), and improve symptomatology and quality of life for the patient. For patients with mild to moderate disease, oral immune suppressants, which include 5-amino salicylic acid (5-ASA), azathioprine, and corticosteroids, are commonly employed. More recently, a new class of small-molecule inhibitors of the JAK-STAT pathway has emerged and is under evaluation in both ulcerative colitis and Crohn's disease [69]. For patients with moderate to severe disease, biologics – some of which are discussed in the following – have now become commonplace treatment for induction and maintenance therapy.

15.8.2 Anti-TNF-α Therapies in IBD

There are currently five FDA-approved anti-TNF-α mAbs for the treatment of IBD, most of which (but not all) are approved in both Crohn's disease and ulcerative colitis. The mAbs differ in their affinity and specificity to membrane versus soluble TNF-α, their degree of humanization, and their ability to engage Fc receptors and hence induce ADCC and/or CDC. Despite these differences and their characteristics, it remains unclear which of these attributes contribute to their clinical efficacy.

Infliximab is a chimeric (25% murine, 75% human) IgG1 and, in 1998, was the first biologic approved for Crohn's disease in steroid-refractory patients. In 2006, infliximab was also approved for remission and maintenance therapy in patients with moderate to severe ulcerative colitis. Infliximab is administered as an intravenous infusion for patients who respond with maintenance therapy every 8 weeks. Subsequently, several other anti-TNF-α agents have been approved for Crohn's disease and/or ulcerative colitis. These include adalimumab, a fully humanized mAb that is administered subcutaneously (s.c.) and has a half-life of 10–20 days; certolizumab, which is a pegylated anti-TNF mAb with a prolonged half-life, allowing monthly sc dosing; and golimumab, another fully humanized anti-TNF mAb, which was approved for use in ulcerative colitis in 2012.

Paradoxically, while numerous anti-TNF-α mAbs are effective in the treatment of IBD, etanercept – a fusion protein containing the extracellular domain of the TNF-α receptor which was approved for the treatment of RA – was not more effective than placebo in patients with Crohn's disease. The difference between these two inflammatory disorders and in the efficacy of the mAb versus the Fc fusion protein remain unclear but suggests that mechanisms beyond solely TNF inhibition, including Fc receptor engagement and or depletion of cells expressing surface TNF-α, may contribute.

The introduction and adoption of anti TNF-α therapies have created a paradigm shift in the management of IBD. However, approximately one-third of patients are refractory to anti-TNF-α mAb treatment. Moreover, it is estimated that within 2 years, between 10% and 50% of patients will become nonresponsive to therapy. This lack of durability may be a consequence of immunogenicity with the

development of anti-drug antibodies, as discussed previously for patients with RA. Indeed, switching a patient who has failed one anti-TNF-α therapeutic to a second may result in improved clinical outcome. Nevertheless, despite these advances, there is still a considerable unmet medical need in both primary nonresponders and in secondary refractory patients, and alternative therapeutics are required for the effective management of these patients.

15.8.3 Integrin Inhibitors

Migration, trafficking, and tissue-specific homing of leukocytes is a multistep, complex process that occurs in the post-capillary venules and plays a central role in controlling immune surveillance. However, the inappropriate accumulation of leukocytes in the gut can also contribute to tissue inflammation and organ damage. Leukocyte extravasation involves selectin-dependent rolling, integrin-dependent adhesion, and chemokine-directed, tissue-specific cell migration. Integrins are heterodimers consisting of α and β subunits. There are 18 α subunits and 8 β subunits. α4β1, α4β7, and αEβ7 have been implicated in gut leukocyte trafficking. Natalizumab was the first anti-integrin agent approved for use in patients. Natalizumab is a fully humanized IgG4 anti α4 integrin inhibitor that blocks both α4β1 and α4β7 interactions with mucosal vascular addressin cell adhesion molecular-1 (MAdCAM-1) and with vascular cell adhesion protein 1 (VCAM-1) [70]. In a number of placebo-controlled trials, natalizumab achieved ~25% placebo controlled remission [71]. However, most likely due to the expression of VCAM-1 on vascular endothelium outside the GI tract, patients treated with natalizumab have a higher likelihood of developing progressive multifocal leukoencephalopathy (PML). PML occurs when latent JC virus becomes reactivated as a result of impaired immune surveillance in the central nervous system (CNS) [72]. While the incidence of PML in patients with Crohn's disease is less than in patients with MS, this has limited the use of natalizumab outside specialized centers. Vedolizumab is a fully humanized anti-α4/β7 integrin mAb that prevents the interactions of α4/β7, but not α4/β1 with its ligand, MAdCAM-1. As MAdCAM-1 is found exclusively on venules within the intestine, no vedolizumab-related abnormalities have been observed with respect to CNS immune surveillance [73, 74]. A 15% clinical remission rate (compared to 7% with placebo) in patients with moderate to severe Crohn's disease or ulcerative colitis earned vedolizumab FDA approval in 2015 [75, 76]. More recent meta-analyses comparing the relative efficacy of natalizumab and vedolizumab demonstrated comparable efficacy between the two agents, with the absence of PML with vedolizumab and equivalent responses in anti-TNF naïve and anti-TNF experienced patients [77].

A number of other integrin inhibitors are at various stages of clinical development. Etrolizumab is a fully humanized mAb that targets the β7 subunit of both α4β7 and αEβ7 and prevents binding to MAdCAM-1 and E-cadherin, respectively [78]. αEβ7 – E cadherin interactions are thought to promote T-cell retention in the

gastrointestinal tract, suggesting potential additional benefits compared to α4β7 blockade alone. Based on encouraging Phase II studies, Phase III studies are under way in patients with ulcerative colitis who are TNF-α-naïve or intolerant to anti-TNF-α mAbs [79, 80], although the potential of this molecule in Crohn's disease remains unclear. An alternative approach to inhibition of the integrin heterodimer is to target the mucosal addressin. PF-00547659 is a fully human IgG2 monoclonal that binds to MAdCAM-1 and prevents interactions with α4β7 [81]. A Phase II study is currently under way to evaluate this agent in patients with ulcerative colitis who have failed or are intolerant to anti-TNF inhibitors.

15.8.4
IL-12/IL-23 Therapies

Preclinical data demonstrating that inhibition of IL-12/IL-23 inhibits colitis in murine models, together with human genetic association data, provided sufficient rationale for the evaluation of inhibitors of this pathway in IBD. As discussed previously, ustekinumab is a fully humanized IgG1k mAb that binds to the shared p40 subunit of IL-12/IL-23 and prevents binding to IL-12RB1. In the Phase III study, ustekinumab induced clinical remission in patients with moderate to severe Crohn's disease who failed steroids and/or immunosuppressive therapy and most of whom were anti-TNF-α-naive.

15.9
Systemic Lupus Erythematosus

Systemic lupus erythematosus (SLE) is a highly heterogeneous chronic multisystem autoimmune disorder that involves dysregulated cytokine signaling, B-cell activation, autoantibody production, complement activation, and immune complex deposition. Kidney pathology, musculoskeletal involvement, and disorders of the joints and the central nervous system are hallmarks of SLE. Humoral autoimmunity is a distinctive feature of SLE. Many patients have circulating autoantibodies directed against double-stranded DNA (anti-ds-DNA) and/or small nuclear RNA-binding proteins (such as anti-Ro, anti-La, anti-Sm, and anti-RNP). The prevalence of SLE is higher in women than men and higher in African Americans and Hispanics than in Caucasians. The underlying etiology of SLE is unknown, although there appears to be a genetic component to the disease. GWAS have been performed in patients with SLE across various ethnic populations and more than 40 common risk loci identified [82, 83]. Active disease is managed primarily with broad immunosuppressant drugs including azathioprine, cyclophosphamide, cyclosporine, methotrexate, and mycophenolate mofetil. Newer, more specific biologic-based therapeutics have recently emerged as our understanding of SLE pathogenesis has evolved. Particular attention has focused on B-cell-based therapies given the association of autoantibodies with the disease [84]. B cells can be

selectively targeted for depletion either via direct killing by monoclonal antibodies recognizing B-cell surface molecules or by blockade of B-cell survival factors.

15.9.1 B-Cell-Directed Therapies

15.9.1.1 Rituximab

Two randomized controlled trials have evaluated the use of rituximab, an mAb recognizing the B-cell surface marker CD20, in patients with SLE. In the placebo-controlled EXPLORER (the exploratory Phase II/III LE evaluation of rituximab) trial, 257 patients with moderate to severe SLE were randomized to rituximab or placebo combined with immunosuppressive agents and corticosteroids. While rituximab reduced complement levels and serological markers including autoantibodies, there was no significant reduction in clinical activity [85, 86]. The second key trial was the Lupus Nephritis Assessment with Rituximab (LUNAR) trial, which evaluated the efficacy of rituximab in 144 patients with lupus nephritis [87]. However, despite successful depletion of B cells, there was no significant difference in overall renal response rates. While the results of these trials were disappointing, the trial design may not have been optimal with regard to patient selection, disease severity, and background immunosupressants [88, 89]. Ocrelizumab, another anti-CD20 mAb, has also been evaluated in patients with lupus nephritis. Although the clinical efficacy of this study was more compelling than studies with rituximab, the trial was suspended because of severe infections in the ocrelizumab-treated group [90]. At this stage, it appears unlikely that CD20 mAbs will have a place in the management of SLE – the role of B cells in SLE pathology remains an open question on the basis of clinical studies to date.

15.9.1.2 Epratuzumab

Epratuzumab is a fully humanized mAb against the B-cell antigen CD22 [91]. CD22 is an inhibitory receptor and part of the siglec family of molecules (siglec-2). Upon B-cell stimulation, CD22 colocalizes to the BCR/CD19 signaling complex, where it is thought to counter-regulate BCR-mediated activation. CD22 engagement by epratuzumab results in the inhibition of BCR and TLR activation, reduced B-cell proliferation, and cytokine production [92]. Two epratuzumab studies suggested clinical improvement in patients with active disease without severe adverse events [93, 94]. A larger 12-week, Phase IIb multicenter, randomized, controlled study also suggested significant efficacy with commensurate steroid reduction; however, there was no clear dose–response relationship [95, 96]. In 2015, data was released in a Phase III trial, which failed to show efficacy of epratuzumab, and further development of this compound was discontinued.

15.9.1.3 Belimumab

B-cell survival is dependent on a variety of cytokines. Among these, BAFF, or BLys, is the most important. BAFF and APRIL are two key B-cell-stimulatory cytokines. BAFF is a transmembrane protein belonging to the tumor necrosis factor ligand superfamily and is present on the surface of macrophages, monocytes, and dendritic cells and activated T cells. BAFF is a growth factor required for B-cell maturation, activation, and survival, and acts by binding to three distinct receptors, namely BCMA (B-cell maturation antigen), TACI (transmembrane activator and calcium modulator and cyclophilin interactor), and BR3 (BLyS/B-cell activating factor receptor). Preclinical data in murine models has demonstrated that mice overexpressing BAFF develop lupus-like symptoms, whereas mice on an autoimmune background have reduced disease severity.

Belimumab is an IgG1 mAb that binds to and antagonizes BAFF. The efficacy and safety of belimumab was evaluated in two pioneering multicenter studies: BLISS-52 and BLISS-76 included more than 1500 lupus patients with moderate to severe disease activity. Patients with renal and CNS involvement were excluded from the study. Belimumab demonstrated a significant clinical benefit as measured by reduced SLE-related flares, normalized C3 levels, and reduced steroid usage [97, 98]. Taken together, this result provided sufficient evidence of benefit to lupus patients, and in 2011 belimumab was approved by the US FDA and EMA. This was noteworthy, as it was the first drug approved for SLE in over 50 years. In subsequent long-term follow-up studies, the data remained positive with maintained reduction of corticosteroid usage and a significant reduction in autoantibodies. Taken together, targeting BAFF with belimumab can provide significant clinical benefit to SLE patients. However, it is important to highlight that ~60% of patients did not respond to the therapy; thus SLE remains a considerable unmet medical need.

15.9.1.4 Other Regulators of B Cells Survival

With the success of belimumab, other molecules that regulate B-cell survival are currently in clinical development. Blisibimod is a peptibody antagonist of BAFF fused to human IgG1 and is currently in Phase II studies [99, 100]. Tabalumab is another fully humanized anti BAFF mAb that may bind to both membrane and secreted BAFF. While Phase II data was encouraging, the development of tabalumab [101] was halted following completion of a Phase III study because of lack of efficacy over placebo. Whether the successful launch and approval for beliminmab as compared to tabalumab relates to the potential differences between the pharmacology of two molecules, or whether it was based on study design, end points, or patient selection, remains unknown. Atacicept is a recombinant TACI receptor fusion protein that binds not only BAFF but also APRIL. In early clinical studies, the efficacy of this molecule was suggestive based on a reduction in mature B cells and dose-dependent decreases in autoantibody levels [102–104]. However, despite these early encouraging results, further development was halted because of an increase in adverse events.

15.9.2 Type I Interferons and SLE

Type I interferons (IFNs) are a family of cytokines expressed from at least 17 functional genes including genes encoding for multiple IFN-α proteins as well as genes encoding for IFN-β, IFN-τ, IFN-κ, and IFN-ω. Type I IFNs signal through receptors composed of two chains: IFNAR1 and IFNAR2. IFNAR2 binds to IFN with high affinity and recruits IFNAR1, which leads to the activation of the JAK-STAT pathway with subsequent induction of a large number of IFN inducible genes. Characterization of IFN activity had previously focused primarily on the antiviral properties of these molecules. However, in recent years, the role of Type I IFNs in immune homeostasis has become apparent. In particular, there is a growing body of evidence to suggest that Type I IFNs, including IFN-α, have a role in autoimmune diseases. In SLE, it has been shown that IFN-α levels are elevated in the serum from patients and appear to promote antigen presentation by dendritic cells. IFN gene "signatures" are also prominent in SLE, as reflected by elevations in the expression of IFN response genes [105]. Patients with high anti-DNA antibody titers, lupus nephritis, and skin rashes have high Type I IFN activity in their serum. *In vivo* models of autoimmune disease show that IFN-α induces glomerulonephritis in normal mice and accelerates the onset of the spontaneous autoimmune disease of NZB/W mice. More direct evidence that Type I IFNs play a role in the pathogenesis of SLE is supported by the observation that autoimmune-predisposed mice deficient in the IFNα/β receptor have significantly reduced anti-erythrocyte autoantibodies, hemolytic anemia, anti-DNA autoantibodies, kidney disease, and mortality. These preclinical data provide additional evidence that IFN-α plays an important role in the pathogenesis of SLE and suggest that inhibition of IFN-α may provide therapeutic benefits in the treatment of SLE. Based on these data, two anti IFN-α mAbs have been developed and evaluated in Phase II studies. Sifalimumab is a fully human mAb derived from transgenic mice. While sifalimumab binds to the majority of IFN-α subtypes, it is more potent against some IFN-α subtypes than others. Similarly, rontalizumab is a fully humanized IgG1 that inhibits the majority of IFN-α [106]. In early clinical studies, both of these mAbs were effective in inhibiting the IFN gene signature by ~40% and demonstrated significant clinical improvement in patients with mild to moderate disease. Somewhat paradoxically, rontalizumab was more effective in patients with a low interferon signature [107, 108]. The observation that these mAbs only partially inhibited the IFN signature suggests that other IFNs may also contribute, and additional approaches are currently in clinical development. Anifrolumab is a fully humanized mAb to IFNAR1 and inhibits not just IFN-α but all type I IFNs, including IFNβ. Intravenous administration of anifrolumab resulted in an almost complete inhibition of the IFN signature as well as marked improvements in clinical outcome with an acceptable safety profile. Anifrolumab is currently in Phase III trials for the treatment of moderate to severe lupus.

15.10 Conclusions

An understanding of the basic immunological mechanisms that drive diseases such as RA and SLE has led to the development of new transformative biologic-based therapeutics that have provided new alternatives to patients with these diseases, moving from broad-based immunosuppression to new more targeted therapies. However, there is still a significant need for many of these patients. It remains unclear why, for example, only ~50% of RA patients respond in a clinically meaningful way to anti-TNF-α therapies. It is anticipated that in the coming decade we will be able to tailor these drugs to select the right drug for the right patient population to improve clinical outcomes for these diseases that still have high unmet medical need.

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16
Antibody-Based Therapeutics in Oncology

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16.1
Introduction

The last two decades have witnessed monoclonal antibody (mAb) therapy revolutionize the treatment of cancer with diverse mAb-based therapeutic modalities approved and many more in clinical development. The introduction of rituximab, a chimeric anti-CD20 mAb for the treatment of non-Hodgkin lymphoma (NHL), marked the first U.S. Food and Drug Administration (FDA)-approved mAb therapy for a cancer indication in 1997, while most recently FDA approval has been gained for two independent mAbs targeting multiple myeloma: daratumumab (HuMax-CD38), a human mAb targeting CD38, and elotuzumab, a humanized mAb targeting SLAMF7 (also called CS1). Pivotal to the mechanism of action of these three hematological malignancy therapies is the targeted recognition of cell-surface antigens abundantly expressed on the targeted malignant cell population through the Fv region of the antibody coupled with recruitment of host immune-mediated effector functions through Fc interactions [1–3]. Utilization of mAbs as a delivery vehicle for cytototoxics has likewise ushered in a wave of antibody-based conjugates (i.e., ADC, radioimmune conjugates, immune conjugates) as described in Chapter 9. The targeting selectivity of mAbs has also paved the utility of mAb-based functional intervention of oncogenic signaling and intracellular networks including tumor immune evasion. Indeed, direct mAb targeting and blockade of the immune check-point inhibitors CTLA4 and PD-1 has resulted in unprecedented response rates in cancer. In this chapter, we describe the application of mAb-based selectivity in the context of cell-surface receptor targeting of solid tumors and immune cell–tumor cell interactions through blockade of T-cell inhibitory pathways or activation of co-stimulation pathways. Leveraging of bispecific antibody (BsAb)-based platforms to fully

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exploit targeting of such networks will also be discussed. Focus throughout will be on antibody-based therapeutics that have reached the stage of clinical evaluation.

16.2 Targeting Cell-Surface Signaling Pathways in Solid Tumors

Cell-surface receptors are transmembrane proteins that relay the extracellular signals, such as those provided by growth factors, cytokines, or adhesion molecules, to the cytoplasm through the intracellular enzymatic domain. The activated enzymatic domain then initiates a cascade of downstream signaling events that ultimately control many aspects of cellular activities such as proliferation, survival, differentiation, metabolism, and homeostasis. Unsurprisingly, the aberrant upregulation or activation of the cell-surface receptor is observed in many types of cancer. Over the past decades, numerous attempts have been made to perturb the aberrant function of the cell-surface receptor by RNA interferences, domain-negative mutant expression, kinase-specific inhibitors, and mAbs. However, only the latter two modalities have yielded significant clinical benefit to patients. In this section, we focus on mAbs targeting cell-surface receptors and antigens expressed on solid tumors (Table 16.1), with a brief summary of those already approved or having reached the stage of clinical evaluation. Regarding mAb-based therapeutics targeting cell-surface antigens in hematological cancers, readers are referred to recent in-depth review articles covering lymphoma, leukemia, and myeloma [4–7].

16.2.1 Antibody Targeting of Receptor Tyrosine Kinases (RTKs) Pathways

Receptor tyrosine kinases (RTKs), including but not limited to the families of ErbB, insulin, platelet-derived growth factor receptor (PDGFR), proto-oncogene c-Kit (KIT), vascular endothelial growth factor receptor (VEGFR), fibroblast growth factor receptor (FGFR), hepatocyte growth factor receptor (HGFR), and erythropoietin-producing human hepatocellular receptor (EPHR) represent the major growth factor pathways targeted by antibody therapeutics for solid cancer in the past two decades. By targeting the extracellular domain of the RTK, an antibody can potentially block ligand-dependent/independent receptor activation, prevent receptor oligomerization, or lock the receptor in an inactivated conformation, thereby shutting down the intracellular signaling pathways that are responsible for aberrant cellular activities and/or promote immune cell effector functions [8, 9].

16.2.1.1 ErbB Family

The ErbB family contains four structurally similar members: ErbB1 (epidermal growth factor receptor, EGFR), ErbB2 (human epidermal growth factor

Table 16.1 Solid Tumor Cell-Surface Targeting Antibodies in Clinical Development.

Target antigen	Mechanism of action	Antibody format	Name(s)	Indications	Stage[a)]
EGFR	Blockade of ligand binding, receptor dimerization and activation, ADCC	Chimeric IgG1κ	Cetuximab (Erbitux®, IMC-225)	CRC, SCCHN	Approved
		Human IgG2κ	Panitumumab (Vectibix®, ABX-EGF)	CRC	Approved
		Human IgG1κ	Necitumumab (Portrazza™, IMC-11F8, LY3012211)	NSCLC	Approved
		Humanized IgG1κ	Nimotuzumab (TheraCIM®, Theraloc, hR3)	SCCHN, glioma, nasopharyngeal, pancreatic	Approved (other than US and EU); Orphan (US and EU); Phase III
		Human IgG1κ	Zalutumumab (HuMax-EGFR™)	SCCHN	Phase III
		Humanized IgG1	Matuzumab (EMD72000, H425)	CRC, NSCLC, gastroesophageal	Phase II
		Humanized IgG1κ Fc-optimized	Imgatuzumab (GA201, RG7160, RO5083945)	CRC, NSCLC, SCCHN	Phase II
		Oligoclonal chimeric IgG1 mAbs	Sym004	Glioma, SCCHN, NSCLC, CRC	Phase II
		Oligoclonal human mAbs	MM-151	CRC, SCCHN, NSCLC, breast	Phase I
		Humanized IgG1	ABT-806	Gastroesophageal, advanced tumors	Phase I
HER2	Blockade of receptor dimerization and activation, ADCC	Humanized IgG1κ	Trastuzumab (Herceptin®, 4D5V8)	Breast, gastric	Approved
		Humanized IgG1κ	Pertuzumab (Perjeta®, Omnitarg™, rhuMAb 2C4)	Breast	Approved
		Chimeric IgG1κ Fc optimized	Margetuximab (MGAH22)	Breast, gastroesophageal	Phase III

(*continued overleaf*)

Table 16.1 (*continued*)

Target antigen	Mechanism of action	Antibody format	Name(s)	Indications	Stage[a)]
HER3	Blockade of ligand binding, receptor dimerization and activation, ADCC	Human IgG1κ	Patritumab (AMG888, U3-1287)	NSCLC, breast, SCCHN	Phase III
		Human IgG2λ	Seribantumab (MM-121, SAR256212)	CRC, SCCHN, NSCLC, gynecologic, breast	Phase II
		Human IgG1κ	Elgemtumab (LJM716, NVS201010)	SCHNN, breast, gastric, esophageal	Phase II
		Humanized IgG1κ Fc optimized	Lumretuzumab (RG7116, RO5479599)	Breast, NSCLC	Phase I
		Humanized IgG1	AV-203	Advanced tumors	Phase I
		Human mAb	REGN1400	NSCLC, CRC, SCCHN	Phase I
		Chimeric IgG1	GSK2849330	Advanced tumors	Phase I
		Human IgG1	KTN3379	SCCHN, thyroid, advanced tumors	Phase I
IGF-1R	Blockade of ligand binding and receptor activation	Human IgG2κ	Figitumumab (CP-751871)	CRC, lung, breast, sarcomas, prostate, SCCHN	Phase III
		Humanized IgG1κ	Dalotuzumab (MK-0646)	Ovarian, NSCLC, CRC, neuroendocrine, breast, pancreatic	Phase II/III
		Human IgG1λ	Cixutumumab (IMC-A12, LY3012217)	Sarcomas, NSCLC, SCL, breast, gastroesophageal, pancreatic, prostate, hepatic, eye melanoma, CRC, mesothelioma, thymoma, SCCHN	Phase II

		Human IgG1κ	Robatumumab (19D12, SCH717454)	CRC, RCC, neuroblastoma, sarcomas	Phase II
		Humanized IgG1	AVE1642	Breast, hepatic	Phase II
		Human IgG1κ	Ganitumab (AMG479)	Pancreatic, CRC, gastrointestinal, prostate, sarcomas, ovarian, breast, melanoma, carcinoid	Phase II
		Human IgG1	Teprotumumab (R1507, RG1507, RO4858696, RV001)	NSCLC, sarcomas, breast	Phase II
PDGFRα	Blockade of ligand binding and receptor activation	Human IgG1κ	Olaratumab (Lartruvo™, IMC-3G3, LY3012207)	Sarcoma	Approved (US); Orphan (EU)
		Human IgG2κ	Tovetumab (MEDI-575)	NSCLC, glioma	Phase II
CSF1R	Blockade of ligand binding and receptor activation	Human IgG1	IMC-CS4 (LY3022855)	Breast, prostate, advanced tumors	Phase I
		Humanized IgG1κ	Emactuzumab (RG7155, RO5509554)	Advanced tumors	Phase I
		Humanized IgG4κ	Cabiralizumab (FPA008)	NSCLC, melanoma, SCCHN, pancreatic, CRC, glioma	Phase I; Phase I/II (orphan)
KIT	Blockade of receptor activation	Humanized IgG1	KTN0158	GIST, advanced tumors	Phase I
VEGFR1	Blockade of ligand binding and receptor activation	Human IgG1κ	Icrucumab (IMC-18F1, LY3012212)	CRC, breast, urethral cancers	Phase II
VEGFR2	Blockade of ligand binding and receptor activation	Human IgG1κ	Ramucirumab (Cyramza™, IMC-1121B, LY3009806)	CRC, NSCLC, gastroesophageal	Approved
		Human IgG1	Tanibirumab (TTAC-0001)	Advanced and metastatic tumors	Phase IIa (Australia); Phase I
VEGFR3	Blockade of ligand binding and receptor activation	Human IgG1	IMC-3C5 (LY3022856)	Advanced tumors	Phase I

(*continued overleaf*)

Table 16.1 (*continued*)

Target antigen	Mechanism of action	Antibody format	Name(s)	Indications	Stage[a)]
FGFR2	Blockade of ligands binding and receptor activation, induce receptor internalization	Human IgG1	BAY1179470	Advanced tumors	Phase I
FGFR2b	Blockade of ligand binding and receptor activation, ADCC	Humanized IgG1 Fc optimized	FPA144	Gastroesophageal, advanced tumors	Phase I; Phase I (orphan)
FGFR3	Blockade of ligand binding and receptor activation	Human IgG1	MFGR1877S (RG7444)	Bladder, advanced tumors	Phase I
MET	Blockade of ligand binding and receptor activation, ADCC	Humanized Fab-IgG1κ	Onartuzumab (MetMAb, OA-5D5, PRO143966)	Breast, NSCLC, CRC, gastric, glioma, HCC	Phase III
		Humanized IgG4κ	Emibetuzumab (LA480, LY2875358)	Gastric, RCC, NSCLC, HCC	Phase II
		Humanized IgG1 Fc optimized	ABT-700	Advanced tumors	Phase Ib
		Chimeric Llama-Human IgG1 Fc optimized	ARGX-111	Advanced tumors	Phase Ib
RON	Blockade of ligands binding and receptor activation	Human IgG1κ	Narnatumab (IMC-RON8)	Advanced tumors	Phase I
EPHA2	Blockade of receptor activation, ADCC	Humanized IgG1 Fc optimized	DS-8895a	Advanced tumors	Phase 1
DLL4	Blockade of receptor interaction	Humanized IgG2κ	Demcizumab (OMP-21M18)	Pancreatic, NSCLC, ovarian	Phase II
		Human IgG1κ	Enoticumab (REGN421, SAR153192)	Advanced tumors	Phase I

Notch1	Blockade of ligand binding and receptor activation	Humanized IgG2λ	Brontictuzumab (OMP-52M51)	Adenoid cystic carcinoma, advanced tumors	Phase Ia
Notch2/3	Blockade of ligand binding and receptor activation	Human IgG2κ	Tarextumab (OMP-59R5)	Pancreatic, SCLC	Phase II
FZD7	Blockade of ligand binding and receptor activation	Human IgG2λ	Vantictumab (OMP-18R5)	Breast, NSCLC, pancreatic	Phase 1b
TRAILR1	Induces apoptosis	Human IgG1	Mapatumumab (HGS-ETR1, TRM-1)	NHL, NSCLC, HCC, cervical	Phase II
TRAILR2	Induces apoptosis	Human IgG1λ	Drozitumab (PRO95780, Apomab, rhuMAb DR5)	CRC, NSCLC	Phase II
		Human IgG1κ	Conatumumab (AMG655, TRAIL-R2mAb, XG1-048)	Sarcoma, CRC, pancreatic, carcinoid, NSCLC, ovarian	Phase II
		Humanized IgG1	Tigatuzumab (CS-1008, TRA-8)	Breast, CRC, ovarian, HCC, pancreatic, NSCLC	Phase II
		Human IgG1	KMTR2 (HGS-TR2J)	Advanced tumors	Phase I (Canada)
		Human IgG1λ	Lexatumumab (HGS-ETR2)	Sarcomas	Phase I
		Chimeric IgG1	LBY135	Mesothelioma, CRC, breast	Phase I (EU)
Endoglin	Blockade of receptor function, ADCC	Chimeric IgG1κ	TRC105 (c-SN6j)	NSCLC, glioma, urothelial, prostate, breast, RCC, HCC, gynecologic, choriocarcinoma, sarcoma	Phase II; Phase IIa (orphan)
GD2	ADCC, CDC	Chimeric IgG1κ	Dinutuximab (Unituxin®, ch14.18)	Pediatric neuroblastoma	Approved (US); Orphan (EU)
CLDN6	ADCC, CDC	Chimeric IgG1	IMAB027	Ovarian	Phase I/II
CLDN18.2	ADCC, CDC	Chimeric IgG1	Claudiximab (IMAB362)	Gastroesophageal, pancreatic	Orphan (US and EU); Phase II

(*continued overleaf*)

Table 16.1 (*continued*)

Target antigen	Mechanism of action	Antibody format	Name(s)	Indications	Stage[a)]
EpCAM	Blockade of receptor function, ADCC, CDC	Human IgG1	Adecatumumab (MT201)	Liver metastases	Phase II
		Mouse IgG2aκ	Edrecolomab (Panorex®, 17-1A)	CRC	Phase III
Mesothelin	ADCC	Chimeric IgG1κ	Amatuximab (MORAb-009)	Mesothelioma, pancreatic, ovarian, NSCLC	Phase II
Vimentin	Blockade of receptor function	Human IgG1	Pritumumab (CLNG11)	Brain	Phase II (Japan); Orphan (US)
Integrin α5β1	Blockade of ligand binding and receptor activation	Chimeric IgG4	Volociximab (M200)	Melanoma, NSCLC, ovarian, peritoneal, RCC, pancreatic	Phase II
Integrin αV	Blockade of ligand binding and receptor activation	Human IgG1κ	Intetumumab (CNTO-095, CNTO-95)	Melanoma, prostate	Phase II
		Humanized IgG2κ	Abituzumab (DI17E6, EMD525797)	Hepatic, bone, prostate, CRC	Phase II
Integrin αVβ3	Blockade of ligand binding and receptor activation	Humanized IgG1	Etaracizumab (Vitaxin®, Abegrin™, MEDI-522, hLM60)	CRC, prostate, melanoma, kidney	Phase II

a) The most advanced stage of clinical testing.
Source: Information derived and supplemented from clinicaltrials.gov, www.imgt.org, www.pubmed.gov, and websites of individual pharmaceutical companies.

receptor 2, HER2), ErbB3 (human epidermal growth factor receptor 3, HER3), and ErbB4 (human epidermal growth factor receptor 4, HER4), of which all the ErbB members except HER3 contain the intracellular kinase domain for signal transduction. Eleven natural ligands have been identified for the ErbB members, although none directly binds to HER2, which serves as a signaling component. Upon ligand binding, the ErbB members assemble into homo/hetero/oligodimer, activate the intracellular kinase, and initiate downstream signaling pathways such as MAPK and PI3K/AKT. Receptor overexpression, mutation, and aberrant activation are routinely identified in colorectal, head and neck, lung, glioma, pancreatic, breast, gastric, and other epithelial cancers. Receptor crosstalk, modification of receptor structure, and utilization of alternative downstream signaling pathways are the main resistance pathways to compensate for the loss of receptor functions in cancer cells [10, 11]. The understanding of the biology of ErbB members, their interactions, and resistance mechanisms in cancers provides new perspective in targeting the ErbB family with antibody besides receptor antagonistic activity. Collectively, the ErbB family harbors the most approved antibodies for different cancer indications with four EGFR-targeting antibodies (cetuximab, panitumumab, necitumumab, and nimotuzumab) and two HER2-targeting antibodies (trastuzumab and pertuzumab). Numerous additional ErbB-targeting antibodies are being evaluated in the clinic, although notably there are no HER4-targeting antibodies in development [10].

16.2.1.2 **EGFR**

Cetuximab, a chimeric IgG1 antibody, binds to the extracellular domain of EFGR, and inhibits the ligand-dependent receptor activation and dimerization. Being the first antibody approved for targeting EGFR-overexpressing cancer, cetuximab sets the benchmark for future EGFR-targeting antibodies. Cetuximab has been approved for squamous cell carcinoma of the head and neck (SCCHN) and metastatic colorectal cancer (CRC). The blockade of EGFR signaling in cancer cells by cetuximab was shown to decrease growth, metastasis, and angiogenesis, while it can also elicit immune effector functions, such as antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC), and synergize with chemotherapy and radiotherapy [10]. Panitumumab, a fully human IgG2 antibody, was approved for CRC with disease progression despite prior treatment. While its anticancer mechanisms are similar to those of cetuximab, panitumumab blocks both EGF and tumor-growth factor (TGF)α binding to EGFR but does not support immune effector functions. Panitumumab also synergizes with chemotherapy in cancer regression [10]. Necitumumab, a human IgG1 antibody, approved for use with gemcitabine and cisplatin in previously untreated metastatic squamous non-small cell lung cancer (NSCLC) but not for non-squamous NSCLC. The anticancer potency of necitumumab is similar to that of cetuximab. Nimotuzumab, a humanized IgG1 antibody, was approved for SCHNN, glioma, and nasopharyngeal cancer in countries other than the EU and the United States, and it was granted the orphan designation for glioma and pancreatic cancer in the latter two, respectively. Nimotuzumab has

similar anticancer potency as cetuximab without causing the most common side effect of other EGFR-targeting antibodies, namely skin rashes. It is hypothesized that nimotuzumab preferably binds to cancer cells with moderate to high EGFR expression rather than normal cells with relatively low EGFR expression [12]. The primary and acquired resistance mechanism for EGFR-targeting antibody is KRAS mutation and/or amplification. KRAS encodes a G-protein in the EGFR signaling pathway, and its constitutive activation initiates downstream signaling independent of ligand-dependent EGFR activation. As a result, regulatory authorities have restricted the use of EGFR-targeting antibodies to patients not bearing KRAS mutation [13]. EGFR-targeting antibodies attempting to improve patient outcome but failed because of unmet clinical end points include matuzumab, a humanized IgG1 antibody; zalutumumab, a human IgG1 antibody; imgatuzumab, a humanized IgG1 with Fc domain glycoengineered for increased binding to CD16A on immune effector cells for enhanced ADCC. Despite this, several antibodies with novel EGFR-targeting strategies are being tested in the clinic, including targeting a tumor-associated EGFR epitope with ABT-806 and maximizing EGFR clustering through multiple epitope engagement with oligoclonal antibodies such as Sym004 and MM-151. In a Phase 1 clinical trial, ABT-806, a humanized IgG1 antibody that selectively targets EGFRvIII, which is the most common deletion mutant in tumors that lacks the ligand-binding domain but with constitutive kinase activity [14], was shown to lack normal tissue uptake or toxicity in patients [15]. Sym004, a mixture of two chimeric IgG1 antibodies (futuximab and zatuximab), and MM-151, a mixture of three human antibodies, were shown to block ligand-dependent receptor activation, accelerate receptor degradation by internalization and ADCC, and overcome acquired resistance to cetuximab [16, 17].

16.2.1.3 **HER2**

Trastuzumab (anti-HER2) was the first approved antibody targeting RTK-overexpressing cancer and provided proof of concept to support the development of additional RTK-targeting antibodies. Trastuzumab, a humanized IgG1 antibody that binds to the domain IV of HER2, has been approved for HER2-positive breast cancer and gastric cancer. The anticancer mechanism of trastuzumab remains unclear, but the antibody inhibits intracellular signaling, downregulates HER2 by internalization, and supports ADCC. These combined activities reduced cancer proliferation, angiogenesis and metastasis. In patients treated with trastuzumab, primary and acquired resistance was developed as a result of the upregulation of other ErbB members to compensate for the loss of HER2 signaling. Taken together with the fact that HER2 heterodimerizes with other ErbB members, this led to the development of another HER2-targeting antibody, pertuzumab. Pertuzumab, a humanized IgG1 antibody, is the first approved "HER-dimerization inhibitor" that binds to the dimerization domain of HER2, thereby blocking the ligand-dependent/independent HER2 heterodimerization and signaling. Among all permutations of HER2 heterodimers, HER2-HER3 was

shown to be the predominant target for pertuzumab [18]. Clinical studies have demonstrated benefit by combining trastuzumab and pertuzumab in the same treatment with the engagement of distinct HER2 epitopes, resulting in more potent anticancer activity than with one antibody alone [19]. Additional means to improve the anticancer activity of a HER2-targeting antibody include antibody-drug conjugate (ADC) strategies or enhancement of immune effector function through modifications to the Fc region. Trastuzumab emtansine, an ADC version of trastuzumab, was approved for metastatic breast cancer (Chapter 9). Margetuximab, which incorporates the same anti-HER2 specificity as trastuzumab and preserves direct antitumor activity, incorporates an optimized Fc domain engineered for increased binding to activating FcγRIIIA (CD16A) and reduced binding to inhibitory FcγRIIB (CD32B) on immune effector cells. Notably, genetic analyses have revealed that patients who express the weak binding allele (F) at position 158 of FcγRIIIA are less responsive to trastuzumab than those carrying the high binding allele (158V), demonstrating the importance of Fc-mediated responses for therapeutic response and supporting the rationale to enhance CD16A binding through Fc optimization to overcome limitations in trastuzumab response [20, 21]. Margetuximab therefore has the potential to be effective in a broader population than is currently treated with trastuzumab by overcoming resistance in populations that do not respond or respond poorly to trastuzumab [22].

16.2.1.4 HER3

HER3 lacks an intracellular kinase domain, with its ligand-dependent/independent activation requiring heterodimerization with other ErbB members such as EGFR and HER2. Recent studies revealed that HER3 plays an important role in drug resistance to EGFR- and HER2-targeting therapies, and its overexpression is associated with poor prognosis in breast, ovarian, lung, and colon cancers [23]. Clinical-stage HER3-targeting antibodies designed to block HER3 signaling either through blockade of heterodimerization or association with ligand(s) include patritumab, a human IgG1 antibody; seribantumab (MM-121), a human IgG2 antibody; AV-203, a humanized IgG1 antibody; REGN1400, a human antibody. There are other HER3-targeting antibodies that can lock the receptor in an inactive conformation through the binding of a unique epitope on HER3, such as elgemtumab, a human IgG1 antibody, and KTN3379, a human IgG1 antibody with domain engineered for better serum half-life. Other HER3-targeting antibodies are glycoengineered for enhanced ADCC and CDC with immune effector cells, such as lumretuzumab, a humanized IgG1 antibody, and GSK2849330, a chimeric IgG1 antibody [24, 25]. Unfortunately, HER3-targeting antibodies have thus far proven largely inefficacious as monotherapy in various cancer indications including CRC, NSCLC, SCCHN, and breast and gastric cancers. It is hoped, however, that the therapeutic index can be further improved by combining with other ErbB-targeting antibody such as cetuximab [26], MM-151 [27], and trastuzumab [28, 29].

16.2.1.5 Insulin-Like Growth Factor 1 Receptor (IGF-1R)

IGF-1R is a tetrameric transmembrane protein made up of two disulfide-linked heterodimers. Each heterodimer contains its ligand-binding domain in the extracellular domain and an intracellular kinase domain. IGF1, IGF2, and insulin are the natural ligands for IGF-1R. Similar to EGFR signaling pathway, ligand binding activates the intracellular kinase and subsequently triggers downstream signaling pathways such as MAPK and PI3K/AKT. The interplay of these signaling cascades allows IGF-1R to mediate normal cellular activities. Overexpression of IGF-1R is frequently associated with many cancers, including, but not limited to, gastrointestinal, colorectal, breast, ovarian, lung, prostate, and sarcoma. Indeed, upregulation of IGF-1R signaling enables oncogenic transformation and suppresses apoptosis, while its downregulation inhibits tumorigenesis and metastasis [30, 31]. All the IGF-1R targeting antibodies in the clinic share a common anticancer mechanism by blocking ligand-dependent receptor activation and downstream signaling in cancer cells. However, the efficacy of such antibodies in patients was shown to be modest as monotherapy or in combination with chemotherapy [32–34]. While generally, the IGF-1R targeting antibodies are well tolerated in patients, and the most common toxicity is hyperglycemia [34], it should be noted that clinical development of figitumumab was terminated as a result of serious adverse events including treatment related deaths in patients with NSCLC [35]. To improve the overall efficacy and potential therapeutic window of IGF-1R targeting antibodies, studies have explored the signaling crosstalk of IGF-1R/insulin receptor and IGF-1R/EGFR [33], while there is also a Phase II clinical trial for CRC patients receiving both ganitumab and conatumumab, a human IgG1 antibody targeting TRAILR2, based on the hypothesis that the dual targeting of separate pathway by these antibodies should inhibit survival (ganitumab) and induce apoptosis (conatumumab). However, while the antibody combination was well tolerated, no objective responses in patients were observed [36].

16.2.1.6 PDGFR – PDGFRα

A functional PDGFR consists of a homo/heterodimer of PDGFRα and PDGFRβ. There are four inactive PDGF monomers identified, and they form a mature PDGF ligand as homodimer of AA, BB, CC, DD, and a heterodimer of AB. The five PDGF ligands selectively interact and activate PDGFRs through the MAPK, PI3K/AKT, and JAK/STAT pathways for similar but not identical cellular activities. For example, PDGF-AA only binds to PDGFRαα; PDGF-BB binds to all three variants of PDGFRs; PDGF-AB/-CC bind to PDGFRαα/αβ but not PDGFRββ; PDGF-DD binds to PDGFRββ with higher affinity than PDGFRαβ. PDGFR signaling is crucial for early development and wound healing, and unsurprisingly, its dysfunction has been shown in cancer growth and angiogenesis. Recently, PDGFRα has gained much attraction as a potential cancer target due to its dual expression on cancer cells and cancer stromal cells [37]. To improve the toxicity profile in patients, PDGFR-targeting antibodies are designed not to affect PDGFRβ signaling because the dual inhibition of both PDGFRs can lead to extravascular fluid accumulation [38]. Two PDGFRα-targeting antibodies

have reached clinical stage testing. Olaratumab, a human IgG1 antibody, binds to PDGFRα and blocks ligand-dependent receptor activation by PDGF-AA, PDGF-BB, and PDGF-CC. FDA recently approved olaratumab for soft-tissue sarcoma in combination with doxorubicin, and the antibody is being fast-tracked for conditional approval in the EU for rare soft tissue sarcoma. Tovetumab, a humanized IgG2 antibody, also binds to PDGFRα [39, 40] but its development was suspended after a Phase II clinical trial in patients with glioblastoma due to undisclosed reasons [41].

16.2.1.7 KIT Family – Colony-Stimulating Factor 1 Receptor (CSF1R) and KIT

The KIT family contains three structurally similar members: KIT, CSF1R, and FLT3. Unlike other RTKs, the KIT receptors are predominantly expressed on stem cells and immune cells, such as macrophages and mast cells, and play a crucial role in modulating the cancer microenvironment. Upon ligand activation, the KIT receptors initiate downstream signaling cascades through the MAPK, PI3K/AKT, and JAK/STAT pathways. Aberrant KIT family signaling promotes cancer cell growth, invasion, metastasis, and angiogenesis, while CSF1R is the control node for macrophage differentiation and survival and also potentiates tumor-associated macrophages (TAMs) through binding of its ligands CSF1 and IL34 [42, 43]. The anticancer mechanism of the three CSF1R-targeting antibodies, namely IMC-CS4, a human IgG1 antibody, emactuzumab, a humanized IgG1 antibody, and cabiralizumab, a humanized IgG4 antibody, in the clinic is to block ligand-dependent activation of CSF1R on TAMs, thereby decreasing their survival and its effect on cancer cells [44]. Based on this hypothesis, clinical trials were designed to test CSF1R-targeting antibody in combination with immune check-point inhibitory antibodies for various cancer indications such as NSCLC, SCHNN, CRC, melanoma, glioma, and pancreatic cancer. Examples of this approach include IMC-CS4 with durvalumab, a human IgG1 PD-L1-targeting antibody, or with tremelimumab, a human IgG2 CTLA4-targeting antibody [NT02718911]; emactuzumab with atezolizumab, a human IgG1 PD-L1-targeting antibody [NCT02323191]; cabiralizumab with nivolumab, a human IgG4 PD-1-targeting antibody [NCT02526017]. There is only one KIT-targeting antibody testing in the clinic for GIST and other KIT-expressing tumors such as SCLC and melanoma. KTN0158, a humanized IgG1 antibody, blocks ligand-dependent receptor dimerization and activation on cancers and the immunosuppressive mast cells and myeloid cells in the cancer microenvironment [45].

16.2.1.8 VEGFR Family – VEGFR1, VEGFR2, and VEGFR3

The three members of VEGFR family, VEGFR1, VEGFR2, and VEGFR3, are predominately expressed on endothelial cells and tumor vasculature, and play important roles in angiogenesis, endothelial cell functions, cancer growth, and metastasis. VEGFRs can be expressed as membrane-bound or as a soluble receptor by alternative splicing. There are six ligands identified for VEGFRs: VEGF-A binds to VEGFR1 and VEGFR2; VEGF-B and PIGF bind to VEGFR1

only; VEGF-C and VEGF-D bind to VEGFR2 and VEGFR3; VEGF-E binds to VEGFR2 only. Similar to other RTKs, VEGFRs signal through pathways such as MAPK, PI3K/AKT, JAK/STAT, and FAK. Bevacizumab, a humanized IgG1 antibody targeting VEGF-A, was the first approved angiogenesis inhibitor for metastatic CRC, NSCLC, RCCs, ovarian cancer, and glioblastoma, but not for breast cancer, when used in combination with chemotherapy. Bevacizumab approval has led to the active development of therapeutics interfering with different aspects of VEGF-VEGFR signaling such as antagonistic antibodies to VEGFRs and VEGF trap. These are the VEGFR-targeting antibodies in the clinic: VEGFR1 (icrucumab), VEGFR2 (ramucirumab, approved; tanibirumab), and VEGFR3 (IMC-3C5). The recent identification of two VEGFR coreceptors, namely Neuropilin-1 and Neuropilin-2, may provide new insight for developing the next generation of VEGFR-targeting antibodies [46–48]. Apart from this, aflibercept, a human IgG1 Fc-fusion protein of the extracellular domains of VEGFR1 and VEGFR2 that binds VEGF-A, VEGF-B, and PIGF, was also approved for metastatic CRC in combination with chemotherapy.

16.2.1.9 **VEGFR1**

While the function and downstream signaling of VEGFR1 remain poorly understood, effective signaling is known to require VEGFR1-VEGFR2 heterodimerization, in which VEGFR1 serves as the high-affinity receptor for VEGFs and the tyrosine kinase of VEGFR2 helps to propagate the signals. VEGFR1 also acts as decoy receptor for sequestering VEGFs from VEGFR2 binding. Icrucumab, a human IgG1 antibody, is the only VEGFR1-targeting antibody in the clinic and is undergoing evaluation in patients with CRC, breast, and urothelial cancers. The antibody blocks ligand-dependent receptor activation and downstream signaling initiated by VEGF-A, VEGF-B, and PlGF [49].

16.2.1.10 **VEGFR2**

Angiogenesis is primarily carried out through VEGFR2 signaling. Ramucirumab, a human IgG1 antibody, blocks ligand-dependent receptor activation [50, 51]. It was approved by FDA for patients with gastroesophageal cancer, CRC, and NSCLC as monotherapy or in combination with chemotherapy [52], but failed to meet the primary endpoints for metastatic breast and hepatic cancer in Phase III clinic trials [50]. The antibody is now being tested in another Phase III clinic trial for urothelial cancer [NCT02426125]. Tanibirumab, a human antibody, blocks ligand-dependent receptor activation by VEGF-A, VEGF-C, and VEGF-D [53]. The antibody was well tolerated [54] and is now in a Phase IIa clinic trial in patients with recurrent glioblastoma in Australia [ACTRN12615001156572].

16.2.1.11 **VEGFR3**

VEGFR3 signaling has been implicated in cancer lymphangiogenesis and metastasis. IMC-3C5, a human antibody, blocks ligand-dependent receptor activation by VEGF-C and VEGF-D. The antibody was well tolerated but was inefficacious as monotherapy in CRC [55]. Based on the identification of

VEGFR2-VEGFR3 heterodimer, the dual blockade of VEGFR2 by IMC-3C5 and other VEGFR2-targeting agent may improve the clinical outcome.

16.2.1.12 FGFR Family – FGFR2, FGFR2b, and FGFR3

FGF-FGFR signaling is both multifactorial and complex. There are five members identified for the family, and each of them displays different ligand-binding affinities and tissue expression profile. FGFR1-FGFR4 are functional receptors comprising ligand-binding and tyrosine kinase domains, while FGFR5 lacks the tyrosine kinase domain. FGFR1-FGFR3 can produce over 48 different isoforms through alternative splicing. There are 18 FGF ligands identified for FGFRs, which can be classified into two families: hormone-like FGFs (FGF15/19, FGF21, and FGF23) and the canonical FGFs (FGF1-10, FGF16-18, and FGF20). It is noted that FGF11-FGF14 do not interact with any FGFRs and have functions unrelated to other FGFs. The bioavailability of FGFs is controlled by heparan sulfate proteoglycans (HPSGs) or specific FGF-binding proteins. The binding of FGF induced the oligomerization of the FGF-FGFR-HPSG complex, tyrosine kinase activation, and signal transduction through pathways such as MAPK, PI3K/AKT, and STAT. FGF-FGFR signaling is critical for various cellular activities, particularly for angiogenesis, and its dysregulation, through overexpression or gene mutations, has been shown to promote cancer growth, invasiveness, metastasis, and angiogenesis [56]. There are two antibodies targeting FGFR2: BAY1179470 for FGFR2 and FPA144 for FGFR2b. BAY1179470, a human IgG1 antibody, binds to all four isoforms of FGFR2, blocks ligand-dependent receptor activation, and induces receptor internalization [57]. A Phase I clinical trial with BAY1179470 in patients with advanced refractory solid tumors was completed, but data has not been posted [NCT01881217]. BAY1187982, an ADC version of BAY1179470 [57], was also developed but its Phase I clinical trial was terminated [NCT02368951]. FPA144, a humanized IgG1 antibody, binds specifically to FGFR2b and prevents ligand-dependent receptor activation by FGF7, FGF10, and FGF22. The antibody also has a glycoengineered Fc domain for enhanced ADCC with natural killer (NK) cells. FPA144 is well tolerated and displayed objective response in Phase I clinical trial for gastric cancer [58]. MFGR1877S, a human IgG1 antibody, binds to and inhibits FGFR3 activation [59] but was removed from Phase I clinical trial for unspecified reason [60]. Additional modalities to block FGF-FGFR signaling in clinical development include LY3076226, a DM4-conjugated FGFR3-targeting human IgG1 ADC [NCT02529553], and FP1039, a human IgG1 Fc-fusion protein of the extracellular domain of FGFR1, which binds and neutralizes the binding of multiple FGFs to FGFR1 [61, 62].

16.2.1.13 HGFR Family – Proto-Oncogenec-MET (MET) and Recepteur d'Origine Nantais (RON)

Proto-oncogene c-MET (MET) and Recepteur d'Origine Nantais (RON) are the only two members of the HGFR family. HGF and its isoforms (NK1 as agonist and NK2 as antagonist) are the ligands of MET, and HGFL is the ligand of RON. HGFRs are predominantly expressed on the epithelial cells, while the ligands are mainly

restricted to mesenchymal cells for MET or hepatocytes for RON. The ligand binding leads to receptor dimerization, activation of tyrosine kinase domain, and initiation of downstream signaling pathways of MAPK, PI3K/AKT, JAK/STAT, and β-catenin, which subsequently regulate various cellular activities, particularly migration, scattering, epithelial-mesenchymal transition (EMT), and morphogenesis. HGFRs were shown to complex with and may potentially trigger signal crosstalk through other membrane proteins such as EGFR, IGF-1R, CD44, CD151, Fas, integrin α6β4, plexins B1-B3, and the MET-RON heterodimer. Furthermore, HGFR activation was implied in upregulating the expression of AXL, PDGFR, EGF, and Delta. The dysfunction of HGFRs expression and signaling has been observed in cancers of epithelial, mesenchymal, and hematological origin, making HGFRs attractive therapeutic targets [63, 64].

There are four MET-targeting antibodies at different stages of clinical trials: onartuzumab, a humanized Fab-IgG1 antibody; emibetuzumab, a humanized IgG4 antibody; ABT-700, a humanized IgG1 antibody; ARGX-111, a chimeric IgG1 antibody. These MET-targeting antibodies are capable to block ligand-dependent and ligand-independent receptor activation, downregulate receptor expression by internalization and degradation, and elicit ADCC on MET-expressing cancer cells. Unique among other RTK-targeting antibodies described here, onartuzumab is a monovalent humanized antibody engineered for receptor antagonism. However in a Phase III clinical trial performed in patient with NSCLC, onartuzumab in combination with erlotinib, a EGFR inhibitor, failed to demonstrate clinically meaningful efficacy [65]. Tivantinib, a MET inhibitor, also failed in a separate clinical trial with NSCLC patients, suggesting that MET may not be a viable target for lung cancer [65, 66]. Presently, emibetuzumab, which recognizes an epitope on the HGF binding site but poses no agnostic activity [67], is being evaluated in a Phase II clinic trial in patients with NSCLC and advanced gastric cancer. Furthermore, ABT-700 and ARGX-111, both antibodies containing an Fc-domain glycoengineered for enhanced ADCC, are well tolerated and have demonstrated anticancer activity in patients in Phase I clinical trial [68–70].

Regarding the targeting of RON, narnatumab, a human IgG1 antibody, was shown to block ligand-dependent receptor activation and downstream signaling and downregulate receptor expression, but was unable to affect proliferation or apoptosis of cancer cells in preclinical studies [71]. Unfortunately, the results of the clinical trial are not available publicly.

16.2.1.14 **EPHR Family**

Sixteen EPHR have been identified in human, which are divided into two groups, designated EPHA (1-8;10) and EPHB (1-4;6), based on their ligand specificities. The EPHR ligands (Ephrin) contains five Group A (EphrinA1-5 as GPI-linked proteins) and three Group B (EphrinB1-3 as transmembrane proteins), which can differentially interact with both types of EPHRs regulating diverse cellular activities, particularly for embryonic development [72]. Generally, EPHRs and Ephrins display reciprocal expression on cells in a microenvironment. In contrast to other

RTKs, Ephrin-EPHR signaling requires the trans-activation of ligand/receptor on the juxtaposed cell, and such interaction allows bidirectional cell-cell signaling. The aberrant Ephrin-EPHR expression and activity have been shown to promote cancer growth, angiogenesis, invasiveness, and metastasis [73, 74]. Presently, DS-8895a, a humanized Fc-optimized EPHA2-targeting antibody, is the sole EPH-targeting antibody in clinical trial for advanced solid tumors [75]. Considering that previous clinical development of an anti-EPHA2 ADC (MEDI-547) was thwarted because of safety concerns [76], it will be of interest to determine whether DS-8895a can overcome such limitation through targeting of an alternate EPHA2 and incorporation of an alternate mechanism of mediating cell cytotoxicity.

16.2.2 Targeting of Additional Signaling Pathways and Cell-Surface Antigens

16.2.2.1 Notch Signaling Pathway

There are four Notch (Notch1, Notch2, Notch3, and Notch4), three Delta-like Notch ligands (DLL1, DLL3, and DLL4), and two Jagged Notch ligands (JAG1 and JAG2) identified for the pathway. While Notch is trans-activated by cell-surface ligand expressed on the neighboring cell, it does not transduce signal via the phosphorylation cascade; rather, upon ligand binding, Notch induces a self-proteolytic cleavage, releasing the Notch intracellular domain (NICD) to enter the nucleus and modulate gene expression. The Notch pathway regulates cell fate determination in embryogenesis, neurogenesis, angiogenesis, and endocrine system development. Notch has been shown to crosstalk with other signaling pathways such as EGFR, VEGFR, PDGFR, FGFR, HGFR, EPHR, and Wnt. Therefore, it is not surprising that many types of cancer display functional Notch signaling [77]. Presently, there are two antibodies targeting DLL4 in clinical trials: demcizumab, a humanized IgG2 antibody, and enoticumab, a human IgG1 antibody. The potential anticancer mechanisms of Notch-DLL4 blockade are inhibition of cancer stem cell growth and angiogenesis, promotion of cell differentiation, and enhancement of immune response to the cancer cells. Demcizumab demonstrated activity in Phase I clinical trials in patients with NSCLC and pancreatic cancer as monotherapy and in combination with chemotherapy [78]. Enoticumab was tolerated and showed initial activity in patients with ovarian cancer and other solid tumors in a Phase I clinical trial [79]. Regarding DLL3, rovalpituzumab tesirine, a humanized DLL3-targeting IgG1 ADC, is currently in Phase II clinical trials for patients with SCLC and other advance solid tumors [80]. Regarding direct targeting of the Notch, there are two antibodies in clinical development. Brontictuzumab, a humanized IgG2 Notch1-targeting antibody that blocks Notch1 signaling in cancer stem cells, is being evaluated as monotherapy in Phase Ia clinical trials in patients with advanced solid tumors and hematologic malignancies [81]. Tarextumab, a human IgG2 Notch2/3-targeting antibody, blocks both Notch2 and Notch3 signaling in cancer stem cells. A Phase 1b clinical trial demonstrated

that tarextumab is well tolerated in combination with chemotherapy, and also demonstrated dose-dependent and biomarker-driven activity in patients with SCLC [82].

16.2.2.2 Wnt–FZD Pathway

A family of 10 Frizzled receptors (FZD1-10) and 19 secretory Wnt ligands comprise the FZD-Wnt signaling pathway. Depending upon the type of FZD-Wnt interaction, FZD can transduce signal through the canonical Wnt/β-catenin pathway, β-catenin independent noncanonical Wnt pathway, and β-catenin independent noncanonical Wnt/calcium pathway. FZD-Wnt signaling has been shown to crosstalk with other pathways such as EGFR family, TGFβ, BMP, Cadherins, and Notch. This interwoven web of FZD-Wnt signaling governs diverse biological activities such as embryogenesis, morphogenesis, angiogenesis, stem cell biology, glucose metabolism, and aging [83]. The dysfunction of the FZD-Wnt signaling has been implicated in various cancers such as breast, CRC, lung, prostate, melanoma, and glioblastoma [84]. Vantictumab, a human IgG2 FZD7-targeting antibody, binds to FZD7, while also potentially interacting with FZD1, FZD2, FZD5, and FZD8 through a conserved epitope in the extracellular domain, and blocks canonical Wnt/β-catenin signaling induced by multiple Wnts in cancer stem cells. In a Phase Ia clinical trial, vantictumab demonstrated Wnt pathway modulation and potential activity as monotherapy manifested by prolonged stable disease [85], leading to multiple clinical trials in combination with chemotherapy in patients with NSCLC and breast and pancreatic cancer.

16.2.2.3 Death Receptors – TRAILR1 and TRAILR2

In contrast to the approach of disrupting RTK functions by antagonistic antibody, effective TRAILR-targeting requires agnostic antibody for inducing apoptosis in cancer cells. There are four TRAILRs expressed on the cell surface: TRAILR1 (DR4), TRAILR2 (DR5), TRAILR3 (DcR1), and TRAILR4 (DcR2). TRAILR1 and 2 are capable of inducing apoptosis through the activation of the intracellular death domain, while TRAILR3, a GPI-linked protein, and TRAILR4, a transmembrane protein with a truncated death domain, are decoy receptors and are incapable of doing so. TRAIL and OPG are identified as the agonistic and the antagonistic ligand, respectively, for the TRAILRs. The binding of TRAIL induces receptor trimerization, activates the death domains, and subsequently initiates apoptosis through caspase- and mitochondrion-mediated pathways. The interplay between the functional and decoy ligands/receptors may confer protection for normal cells or evasion for cancer cells from apoptosis. A number of therapeutic agents are designed to target the extrinsic and the intrinsic pathways of the TRAILRs, such as agonistic antibodies, multivalent/BsAb molecules, multimeric TRAIL constructs, inhibitors for prosurvival proteins, and modulators for protein synthesis and degradation. Among death receptor-targeting antibodies

evaluated in clinic trials for various cancer indications such as CRC, HCC, NSCLC, breast, ovarian, pancreatic, and sarcomas are both the DR4-targeting antibody mapatumumab, a human IgG1 antibody, and DR5-targeting antibodies drozitumab, a human IgG1 antibody; conatumumab, a human IgG1 antibody; tigatuzumab, a humanized IgG1 antibody; KMTR2, a human IgG1 antibody; lexatumumab, a human IgG1 antibody; LBY135 a chimeric IgG1 antibody. Although these antibodies were well tolerated and provided encouraging results in Phase I clinical trials, their developments are stalled in Phase II trials because of the disappointing clinical outcomes as monotherapy, in combination of chemotherapy, or with other antibody therapeutics. The lack of clinical efficacy for some of the antibodies can be explained in part by their requirement of cross-linking to achieve maximum receptor clustering, a condition that is largely inadequate in the cancer microenvironment of the patients. The clinical efficacy of the agonistic antibody should be improved with FcγR-dependent or -independent engineering: Fc-glycoengineered for improved FcγR cross-linking and ADCC, and multivalent engagement of the TRAILR such as TAS266 and RG7386, a FAP-DR5 BsAb [NCT02558140], which is discussed in the section on BsAbs [86–88].

16.2.2.4 Additional Cell-Surface Antigens

Other than targeting RTKs or other cell signaling pathways, additional "naked" antibodies targeting cell-surface proteins expressed on cancer cells have been clinically evaluated including those targeting cell adhesion molecules such as Claudins (CLDN6 and 18.1) and EpCAM; mesothelin and vimentin (members of the integrin superfamily), and endoglin (Table 16.1). Endoglin (CD105), a co-receptor of TGFβ receptor, modulates TGFβ signaling that is upregulated on endothelial cells upon neoangiogenesis and is crucial for tumor growth, survival, and metastasis. Preclinical studies have shown that endoglin is a complementary target to VEGF for angiogenesis inhibition. TRC105, a chimeric IgG1 endoglin-targeting antibody, has been tested in patients with advanced solid tumors in multiple clinical trials as monotherapy or in combination with bevacizumab and other VEGF inhibitors [89]. In addition to disrupting cancer cell biology and progression through perturbation of biological responses, mAbs targeting cancer cell-surface antigens can also mediate immune effector functions via interaction of their Fc domain with FcγR expressed on NK cells, macrophages, and dendritic cells. These Fc-domain-mediated interactions result in ADCC, manifesting in tumor cell death and adaptive tumor immunity through enhanced antigen presentation. Indeed, dinutuximab, a chimeric IgG1 antibody targeting the glycolipid disialoganglioside (GD2) and recently approved for treatment of pediatric patients with high-risk neuroblastoma in combination with multimodality therapy of GMCSF, IL2, and 13-*cis*-retinoic acid, is believed to primarily mediate cell lysis of GD2-expressing cells through ADCC and CDC.

16.3 Targeting of Immune Modulators

16.3.1 Tumor Immunology

As indicated in the previous section, the development of mAbs to treat cancer has traditionally targeted tumor cells either through the antigens they overexpress or by the signaling pathways they subvert to enable their growth, survival, and adhesion/dissemination. While tumor targeting/cell signaling remains an attractive approach, an alternative approach that has gained tremendous momentum and traction is the ability of mAbs to harness and leverage the immune system to generate antitumor immunity. Indeed, the generation of antitumor immunity, and the effectiveness of cancer immunotherapy in general, relies on the ability of the immune system to recognize, respond, and eliminate cancer cells while sparing normal cells.

The immune system, which is divided into the innate and adaptive immune systems, is exquisitely designed to recognize self-proteins from non-self (altered self or foreign) proteins through the generation of specialized lymphocytes such as NK cells and T cells that can elicit potent cytolytic effector function. Evolutionarily older, the innate immune system, which includes mast cells, phagocytic cells, granulocytes and NK cells, is particularly effective at eliminating infections and pathogens in a generic way (i.e., complement activation, opsonization, and/or inflammation) without conferring long-lasting immunity (immunologic memory). As part of the innate immune response and a major cellular player, NK cells rapidly recognize and eliminate stressed or transformed cells by their altered expression of MHC-class I molecules [90]. Indeed, NK cells were known initially to reject MHC-class I deficient tumors via a mechanism initially proposed as the "missing-self hypothesis," which subsequently and mechanistically was described as a complex network of both activating and inhibiting receptors, such as killer-cell immunoglobulin-like receptors (KIRs). These receptors can effectively determine whether NK cells become cytotoxic, and have stimulated the development of antibodies that target NK receptors for the generation of antitumor activity. Indeed, lirilumab, a pan anti-KIR mAb, is actively being investigated in combination with rituximab (anti-CD20) and nivolumab (anti-PD-1) [91] in several cancer indications.

In contrast, the more modern branch of the immune system, the adaptive immune system, which includes both T cells, B cells, and antigen presenting cells (APCs), is designed to exquisitely recognize and eliminate pathogens while generating protective immunological memory against future challenges or infections. As a major player of the adaptive immune response, T cells recognize cancer cells by expression of tumor antigens (proteins specifically appearing in tumor cells and presented in the context of HLA complex) [92]. As a result of mutations or genome-wide gene deregulation, tumors express two broad classes of such antigens [93, 94]. The first class of tumor antigens encompasses normal

(non-mutated) proteins that are poorly immunogenic mainly due to antigen sequestration at immune-privileged sites, to restricted tissue and/or developmental stage expression, or to their production in small quantities. These are often processed fragments of intracellular antigens that are released upon tumor cell lysis and subsequently presented in the context of human leukocyte antigens (HLAs) expressed on APCs [92]. Taken together, this interaction serves as the signal to activate naïve T cells but is usually insufficient alone without a second signal termed co-stimulatory [95]. The second class of antigens encompasses mutated tumor antigens, termed neoantigens [96], that the immune system had not seen previously. In theory, neoantigens should be ideal targets for antitumor response, since they are inherently immunogenic and absolutely specific, and indeed targeting such antigens in patients has been shown to generate potent antitumor immune responses, in particular against melanoma [97, 98]. Unlike the cell-surface receptors reviewed earlier, most of tumor antigens recognized by T cells are derived from intracellular products inaccessible for conventional antibody targeting. Thus, generating tumor-eradicating activity against these antigens relies on patient's own T cells and involves manipulation of two sets of immune receptors expressed by T cells: check-point molecules, which dampen T-cell responses, and co-stimulatory receptors, which potentiate T-cell responses [99, 100]. This choice is based on our understanding of T-cell biology in cancer [101]. During early stages of immune recognition, T cells require co-stimulation signals such as CD28, CD27, OX40, or 4-1BB activation to propagate and acquire cytotoxic potential. Later, however, activity of these cells decreases as a result of repeated signals from inhibitory receptors such as CTLA-4, PD-1, TIM-3, LAG-3, or TIGIT, servings as natural breaks of immune response evolved to prevent collateral tissue damage and autoimmunity. Many advanced tumors take advantage of this tight and coordinated regulation, preventing T cells from receiving co-stimulation signals and actively engaging inhibitory receptors. Animal tumor model systems, and more recently clinical experience, have demonstrated that strategies blocking the inhibitory pathways and providing co-stimulation to tumor-antigen-specific T cells have significant antitumor effect [102]. The most important benefit of tumor immune therapy realized is that, once achieved, clinical responses tend to persist for a very long time because of a phenomenon called immune memory or the maintenance of antitumor immune cells long after the last treatment. The development of mAbs as immune modulators to elicit potent cancer immunotherapy broadly fits into two classes: co-stimulatory agonists (i.e., against OX40, 4-1BB, GITR targets) and check-point inhibitory antagonists (i.e., against CTLA-4, PD-1, PD-L1, LAG-3, and TIM-3) [103]. Intrinsic to both classes is the concept that T cells express both co-stimulatory and inhibitory molecules that form potent immunomodulatory axes that can be harnessed to generate anttumor immunity, as discussed below. Selective targeting of these axes through antagonistic and agonistic antibodies, often in combination with other modalities such as cancer vaccines, soluble receptor/ligands, and chemotherapeutic/radiotherapeutic approaches, is the focus of intense and fervent activity.

16.3.2 Check-point Inhibitors

The critical role of co-inhibitory pathways in mediating tumor immune evasion is evident from the clinical efficacy of antibodies that block either the CTLA-4 or the PD-1/PD-L1 pathways. Here we describe these antibodies in addition to those directed against additional putative immune inhibitors preventing antitumor immunity (see listing in Table 16.2).

16.3.2.1 Cytotoxic T-Lymphocyte Antigen-4 (CTLA-4)

Cytotoxic T-lymphocyte antigen-4 (CTLA-4) was the first immune checkpoint successfully targeted therapeutically by a mAb. CTLA-4 expression is induced on naïve T cells from intracellular vesicles within 24–48 h following activation and binds to the same B7 ligands: CD80 and CD86 expressed on APCs that are recognized by the CD28 co-stimulatory receptor – but with 10-fold higher affinity. Contrary to CD28 stimulation, which induces NF-κB signaling and stabilizes IL-2 mRNA, CTLA-4 does not appear to induce opposing inhibitory signaling pathways following CD28 signaling. Rather, CTLA-4 competes for B7 ligands by excluding CD28 recruitment to the immunological synapse [104] and is believed to attenuate the effector function triggered by CD28-mediated T-cell signaling including activation, proliferation, IL-2 production, and survival. While CTLA-4 is induced upon activation in naive T cells, CTLA-4 is expressed constitutively and at higher levels in T-regulatory cells. Taken together, CTLA-4 expression is hypothesized to serve two functions: (i) minimize autoimmunity following activation in peripheral tissues such as the lymph node, and (ii) induce a state of anergy or "unresponsiveness" by maintaining peripheral tolerance either through stripping B7 ligands on APCs and/or exerting T-regulatory control through the secretion of suppressive factors that control T-cell responsiveness. Thus, the exquisite control of T-cell activation and inhibition runs sequentially, ultimately determining the level of T-cell effector function. While CTLA-4 blockade through the use of antibodies has been shown to enhance T-cell responses *in vitro* and elicit tumor-eradicating immunity *in vivo* [105], clinically the exact mechanism of how CTLA-4 functions *in vivo* is unclear. Rather than just potentiate cytotoxic antitumor responses by "releasing the brakes" on immune responses as supported by animal studies, anti-CTLA-4 has been postulated clinically, at least in the case of melanoma, to remove the inhibitory responses of T-regulatory cells, which overexpress CTLA-4 through their selected depletion at the tumor site by FcγRIV-positive macrophages [106].

Two mAbs against CTLA-4 have been evaluated clinically: the fully human IgG1 ipilimumab and the humanized IgG2 tremelimumab. Both antibodies bind to CTLA-4 and block B7–ligand interactions, leading to increased T-cell activation and proliferation [107, 108]. Numerous studies have supported durable, long-lasting responses following the administration of ipilimumab, particularly in the pivotal Phase III melanoma trial [109], where neoantigens are likely present at higher frequency. Taken together, ipilimumab (Yervoy®), a human IgG1κ

Table 16.2 Check-point blockade antibodies in clinical development.

Target antigen	Mechanism of action	Antibody format	Name(s)	Indications	Stage[a)]
CTLA-4	Blockade of T-cell inhibition, T-regulatory depletion	Human IgG1	Ipilimumab (Yervoy®)	Melanoma	Approved
		Human IgG2	Tremelimumab	Advanced melanoma, malignant mesothelioma	Phase III[b)], Phase IIb[b)]
PD-1	Immune check-point blockade, restoration of T-cell responses	Human IgG4	Nivolumab (Opdivo®)	[A]Melanoma, non-small cell lung cancer, classical Hodgkin lymphoma (cHL) that has relapsed or progressed after autologous HSCT, urothelial carcinoma [BTDD] Squamous cell carcinoma of head and neck, advanced renal cell carcinoma	Approved
		Humanized IgG4	Pembrolizumab (Keytruda®)	[A]Melanoma, non-small-cell lung carcinoma, renal cell carcinoma, metastatic head and neck squamous cell carcinoma (+ platinum-containing chemotherapy) [BTDD] Classical Hodgkin lymphoma	Approved
		Human IgG4	REGN2810	Advanced malignancies, cutaneous squamous cell carcinoma	Phase II
		Not disclosed	TSR-042	Advanced or metastatic solid tumors	Phase I
		Humanized IgG1 Fc-null	BGB-A317	Advanced solid tumors	Phase I
		Humanized IgG1	Pidilizumab	Multiple myeloma	Phase II
		Not disclosed	PDR001 (Novartis)	Nasopharyngeal	Phase II
		Humanized IgG4	MEDI0680	Select advanced malignancies, given in combination with MEDI4736	Phase I/II
PD-L1	Unmasking tumor cells, tumor targeting, check-point blockade, and restoration of T-cell responses	Humanized IgG1 aglycosyl	Atezolizumab (Tecentriq®) (MPDL3280A)	[A] Bladder Cancer (urothelial carcinoma), [BTDD] non-small cell lung cancer	Approved
		Human IgG1 Fc-null	Durvalumab (MEDI4736)	[BTDD] Urothelial bladder cancer	Phase III
		Human IgG1	Avelumab (MSB0010718C)	[BTDD] Metastatic Merkel cell carcinoma	Phase III
		Human IgG4	BMS-936559 (MDX-1105)	Advanced melanoma, hematologic malignancy, several Phase 1 studies withdrawn	Phase 1

(*continued*)

Table 16.2 *Continued.*

Target antigen	Mechanism of action	Antibody format	Name(s)	Indications	Stage[a)]
LAG-3	Immune check-point blockade	Human IgG4	BMS-986016	Advanced lung cancer and in combination with Nivo and Dasatinib	Phase I/II
		Humanized IgG4	LAG-525 (Novartis)	Advanced malignancies and in combination with PDR001 (anti-PD-1)	Phase I/II
TIM-3	Immune check-point blockade, reversal of exhaustion, killing of cancer cells	Humanized IgG4	TSR-022	Advanced solid tumors	Phase 1[a)]
		Not disclosed	MGB453 (Novartis)	Advanced malignancies and in combination with PDR001 (anti-PD-1)	Phase I/II
TIGIT	T-regulatory depletion, reversal of T-cell exhaustion	Human	MTIG7192A	Locally advanced or metastatic tumors	Phase I
B7-H3	Killing of cancer cells	Humanized IgG1 – Fc optimized	Enoblituzumab (MGA271)	Advanced solid tumors	Phase I
VISTA	Killing of cancer cells	Human IgG1	JNJ-61610588	Advanced cancer	Phase I

[A] = Approval, [BTDD] = breathrough designation disclosure.

a) Not-yet recruiting.

b) Failed to meet end points.

isotype antibody, was approved by the FDA and the European Medicines Agency (EMA) in 2011 for the treatment of metastatic melanoma, and is administered in four doses every 3 weeks at 3 mg/kg administered intravenously over 90 min. Severe adverse reactions, in particular enterocolitis, hepatitis, and dermatitis, are commonly observed during treatment, but in general are managed with corticosteroid therapy [110]. Thus far, tremelimumab has failed to demonstrate comparable activity to ipilimumab in a Phase III randomized clinical trial in melanoma and to gain FDA approval. This may be due to isotype differences, IgG2 (tremelimumab) versus IgG1 (ipilimumab), that facilitate T-regulatory cell depletion by monocyte/macrophages through CD16-dependent mechanism of action [111] as observed with ipilimumab and the inclusion/exclusion criteria proposed for tremelimumab/control-arm study [112, 113]. The approval of ipilimumab to treat melanoma and later additional indications, coupled with the preclinical observations in animal models that combination of CTLA-4 with other immune checkpoints in particular anti-PD-1 can generate enhanced anti-tumor activity [105], has initiated additional studies to evaluate which checkpoints could be combined to elicit potent tumor activity while maximizing safety and minimizing off-target effects [114, 115]. Both ipilimumab and temelimumab are actively being evaluated in combination-based approaches with other checkpoints, including PD-1/PD-L1 inhibitors, as described in the following.

16.3.2.2 Programmed Death-1 (PD-1) and PD-1 Ligand

The programmed death-1 (PD-1) pathway comprises the second immune check-point axis to be successfully targeted clinically via antibody-based therapy. Although PD-1, like CTLA-4, is expressed on T cells, binds to multiple ligands, and plays a major role in negatively regulating T-cell activation, exhaustion, and tolerance [116], PD-1 expression is spatially and temporally distinct and differentially regulated from CTLA-4, indicating that the normal homeostatic mechanisms/pathways involved in negative signaling are mutually exclusive [117–119]. Within the tumor microenvironment (TME), the expression of PD-1 and CTLA-4 may be segregated on CD8 tumor infiltrating lymphocytes (TILs) and T-regulatory cells, respectively, or co-expressed together on a subpopulation of $CD8^+$ TILs [120], suggesting that strategies that target both axes may provide greater efficacy than targeting a single check-point axis alone albeit through different mechanisms [116].

PD-1 is a transmembrane protein receptor for two ligands: programmed death-ligand 1 (PD-L1; also known as CD274 and B7-H1) and programmed death-ligand 2 (PD-L2, also known as CD273 and B7-DC). Expression of PD-1 appears within 24 h of T-cell activation and then declines with clearance of antigen. Upon engagement with its ligands, PD-1 inhibits T-cell activation and cytokine production via signaling through SHP-2 recruitment and reduced phosphorylation of TCR signaling molecules. Upon repetitive antigen stimulation (e.g., chronic infection or cancer), PD-1 expression remains high, resulting in epigenetic modifications that culminate in T cells acquiring a state of exhaustion [121]. The ligands for PD-1 are expressed on APCs and other cell types. PD-L1 is more broadly expressed

on epithelial cells and stromal cells and in many tumor cells [122, 123], while PD-L2 expression is more restricted to APCs, and their expression is induced by pro-inflammatory cytokines (i.e., IFNα, IFNγ, TNFα, and VEGF) in the case of PD-L1, and by myeloid differentiating factors (i.e., IL-4 and GM-CSF) in the case of PD-L2.

The complexity of PD-1/PD-L1/PD-L2 binding partners have orchestrated the development of strategies that completely block PD-L1 and PD-L2 interactions, as in the case of the generation of antagonizing PD-1 mAbs, or leave PD-L2 activity intact, as in the case of antagonizing PD-L1 mAbs. Specifically, while antagonist PD-1 mAbs block both PD-L1 and PD-L2 interactions, PD-L1 antagonists block both PD-1 and CD80 interactions. Taken together, such differences have been proposed to elicit antitumor immunity via distinct mechanisms of action. When evaluated in preclinical cancer models, antitumor efficacy could be achieved using anti-PD-1 mAbs [124–128] or anti-PD-L1 mAbs [129–131], albeit through overlapping and/or distinct mechanisms. Blockade of the PD-1/PD-L1 axis using anti-PD-1 mAbs appears primarily to reverse T-cell exhaustion or restore T-cell function [132], while blockade using anti-PD-L1 antibodies appears to enhance DC function, limit T-cell anergy, and control T-regulatory function [133–135]. Although limited studies exist, blockade using anti-PD-L2 mAbs has not elicited the tumor-eradicating immunity observed with anti-PD-L1 mAbs, and consequently led to enhanced tumor growth [136, 137]. Taken together, these studies have provided strong rationale for clinical evaluation for anti-PD-1 and anti-PD-L1 mAbs in cancer.

The general mechanism of action with regard to the development of antibodies that block PD-1/PD-L1 interactions is likely to reverse the state of immunosuppression observed in the TME through disrupting the interactions of tumor cells, which express PD-L1, against other immune cells – primarily T cells, which express PD-1. These include inhibiting T-regulatory cell induction and IL-10 production, reversing T-cell anergy and exhaustion, limiting T-cell apoptosis and resistance to CTL lysis, controlling DC suppression, and altering myeloid subsets [116]. While the mechanism may be varied, studies employing an MC38 colorectal tumor model have indicated that anti-PD-1 and anti-PD-L1 mAbs differ in their FcγRs requirements to elicit antitumor activity. In the case of anti-PD-1 mAb rat clones, optimal tumor activity could be achieved in the absence of Fc–FcγR engagement (IgG1-D265A null mutation), and could be diminished following the introduction of inhibitory FcγR-binding capabilities (IgG1 or IgG2a). In the case of anti-PD-L1 mAbs, optimal activity could be achieved only following the engagement of activating FcγRs by altering the myeloid subset present in the TME [138].

Thus far, two anti-PD-1 mAbs, namely pembrolizumab and nivolumab, have received FDA (2014) and EMA (2015) approval initially for the treatment of melanoma with the label extended in the United States for the treatment of non-small cell lung cancer (NSCLC) and advanced squamous cell carcinoma of the head and neck (SCCNH). Recently, the FDA granted approval to nivolumab for the treatment of prior anti-angiogenic treated renal cell carcinoma, relapsed classical Hodgkin lymphoma (cHL), and urothelial carcinoma. As single agents,

both anti-PD-1 mAbs' dosing regimens are similar: Pembrolizumab is administered 2 mg/kg every 3 weeks as an intravenous solution over 30 min, while nivolumab is administered 3 mg/kg every 2 weeks as an intravenous solution over 60 min. Pneumonitis, colitis, and hepatitis are the most common adverse events observed with anti-PD-1 mAbs; however, the incidence and severity of adverse events were significantly less than that observed with ipilimumab (anti-CTLA-4) [115, 139].

Concurrent with PD-1 mAb clinical development, four PD-L1 mAbs are under clinical evaluation [140–143]. Atezolizumab, a fully humanized anti-PD-L1 IgG1 antibody containing a single mutation in the Fc domain, was the first to achieve FDA approval for the treatment of metastatic urothelial bladder cancer and was recently approved for NSCLC. Durvalumab, a humanized anti-PD-L1 IgG1 antibody containing the triple mutation in the Fc domain, is in late-stage clinical development for advanced solid tumors and NSCLC, while avelumab, a fully human anti-PD-L1 wild-type IgG1 antibody, is under evaluation in solid tumors and Merkel cell carcinoma. Both atezolizumab and durvalumab are similar in that the Fc domain has been modified to restrict FcγR binding and thus minimize antibody dependent cell-mediated cytotoxicity (ADCC). [144]. In contrast, avelumab was designed to retain FcγR binding and induce ADCC. The fully human BMS-936559, which is of the wild-type IgG4 isotype, until recently was under evaluation for hematological malignancy but has since been withdrawn (Table 16.2).

While both PD-1- and PD-L1-targeting mAbs are designed to antagonize the PD-1/PD-L1 checkpoint, the fact that anti-PD-1 mAb blocks both the interaction of both PD-L1 and PD-L2 while anti-PD-L1 blockade leaves PD-1 and PD-L2 interactions intact suggests that their antitumor responses could be driven by unique or overlapping mechanisms. This is further supported in view of anti-PD-L1 mAbs preferentially modulating myeloid DCs and T-regulatory activity while anti-PD-1 mAbs reverse cell exhaustion [132–135]. As example, in NSCLC, PD-1 overexpression is observed on CD8-positive TILs, and PD-L1 overexpression has been observed on NSCLC tumors. Thus, blockade of PD-L1 might be more effective in targeting both T-cell exhaustion and tumor shielding, yet still leave PD-1/PD-L2 activity intact, an important consideration in promoting PD-L2-mediated respiratory tolerance [145] and adverse events that include airway hypersensitivity [146].

The growing arsenal of antibodies against CTLA-4, PD-1, and PD-L1, as well as their success in eliciting tumor responses when used as a monotherapy, has prompted studies to evaluate whether antibodies against immune checkpoints can yield greater efficacy when given in combination. Using the RECIST criteria, the pivotal CheckMate 067 Phase III trial combining ipilimumab with nivolumab demonstrated a higher percentages of objective response rates (ORRs) and progression-free survival (PFS) when compared to single-arm monotherapies alone [139], leading the FDA to grant the first approval for the treatment of advanced melanoma using a combination of two immune checkpoints. While enhanced clinical efficacy could be achieved in some patients, administering nivolumab in combination with ipilimumab increased the incidence of adverse

events, even when given at a lower dose (1 mg/kg nivolumab, given in combination, compared to 3 mg/kg given as a single agent). Thus, the ability to combine immune checkpoints successfully may not be as simple as administering combinations in a 1 : 1 ratio and requires a tempered approach by managing the increased incidence and severity of the associated adverse events. Striking the right balance with safety and efficacy will thus decide which potential combinations are best suited to obtain the optimal clinical benefit. Nevertheless, combination approaches are quickly gaining momentum. More recently, the anti-CTLA-4 antagonist tremelimumab, which failed to generate approval as a monotherapy, is currently under evaluation with durvalumab (anti-PD-L1) and has shown clinical activity in NSCLC [147]. Interestingly, a Phase I clinical trial is recruiting patients to evaluate a dual-blockade approach of the PD-1/PD-L1 axis by combining duvuralmab (anti-PD-L1) with an anti-PD-1 mAb (MED-0680/AMP-514) in select advanced malignancies [NCT02118337]. Whether the combination of anti-PD-1 and anti-PD-L1 will have enhanced efficacy compared to either antibody alone will ultimately be determined clinically, it does suggest that there may be value outside lung cancer and in additional cancer indications for combination-type approaches.

Considering the proven clinical efficacy of targeting PD-1, additional PD-1 mAb programs are going on (Table 16.2), while stalled PD-1 mAb programs, such as CT-011 [148] pidilizumab [149] and lambrolizumab [150], may be rejuvenated. As of September 2016, more than 125 proposed clinical trials have been registered on https://clinicaltrials.gov evaluating various anti-PD-1 mAbs across cancer indications and pharmaceutical sponsors. The driving force behind this is the opportunity for approvals in additional indications and the potential for pharmaceutical companies to matrix additional efficacy off their proprietary assets in combination with anti-PD-1 mAb; indeed, more than 75 studies have been registered on https://clinicaltrials.gov that list anti-PD-1 mAbs in combination with other cancer-targeting therapeutics.

16.3.2.3 Targeting of Additional Putative Check-Point Inhibitor Pathways

Several additional potential negative modulators of tumor immune responses, in particular LAG-3, TIM-3, B7-H3, and TIGIT, are actively being pursued clinically with the notion that they may provide more exquisite lymphoid, anatomic, a functional specification, and a more attractive safety profile with greater synergy than the current FDA-approved ipilimumab and nivolumab combination [151].

LAG-3 or Lymphocyte Activation Gene-3 is a higher affinity homolog of CD4 that is an inhibitory molecule expressed on activated T cells, T regulatory cells, DCs, and NK cells that binds to MHC/HLA-class II molecules [152–154]. LAG-3 appears to negatively regulate CD4+ and CD8+ T-cell proliferation, function, and homeostasis through a unique cytoplasmic domain KIEELE motif [155, 156] whose downstream signaling apparatus is as yet unresolved but distinct from PD-1. Lending further support to LAG-3's inhibitory role is the observation that, within the autoimmune setting, anti-LAG-3 blocking antibodies can accelerate

diabetes onset in the NOD (non-obese diabetic) mouse model [157]. While LAG-3-deficient mice exhibit no spontaneous autoimmunity, mice deficient for both LAG-3 and PD-1, similar to CTLA-4-deficient mice, develop lethal systemic autoimmunity, underscoring a synergy with check-point molecules in regulating T-cell tolerance [158]. In the context of tumor immunology, studies in mouse tumor models have indicated that PD-1 and LAG-3 can synergize to generate potent tumor-eradicating immunity. Specifically, combined blockade of PD-1 and LAG-3 in pre-existing MC38 or SA1N tumors demonstrated enhanced antitumor immunity sufficient to induce remission in ~80% of mice, compared to 0–15% of the mice treated with either single blocker alone [159]. Furthermore, translational studies using TILs from patients with ovarian cancer showed that NY-ESO-1 antigen-specific LAG3+/PD-1+ CD8+ T cells were impaired in their ability to respond to antigen stimulation, but following combined LAG-3 and PD-1 blockade, T-cell responsiveness could be restored to a greater extent than a single-agent blockade [160]. Together, these data suggest that in tumors where LAG-3 and PD-1 are coexpressed on TILs, dual therapy may increase response rates and/or effectiveness of immunotherapy [161]. Toward this end, Novartis is developing an anti-LAG-3 mAb (LAG525) as a single agent and in combination with their anti-PD-1 mAb (PDR001) [NCT02460224] in solid tumors, while BMS is developing an anti-LAG-3 mAb (BMS-986016) mono in hematologic neoplasms [NCT02061761] and glioblastoma [NCT02658981] and as a single agent or in combination with nivolumab across solid tumor indications [NCT01968109].

TIM-3 or T-Cell Immunoglobulin and Mucin Protein 3 TIM-3 or T-cell immunoglobulin and mucin protein 3 is another negative immune receptor expressed on CD4 T-helper 1 cells, cytotoxic CD8 T cells, T-regulatory cells, as well as the innate immune cells NK cells, DCs, and monocytes [151]. TIM-3 has been reported to be capable of binding at least three ligands: phosphatidylserine, galectin-9 (GAL9), and the high-mobility group protein B1 (HMGB1), and was initially investigated within the context of animal models of autoimmunity, where the development of blocking TIM-3 antibodies exacerbated spontaneous autoimmunty [162–164]. Subsequent investigation within the context of PD-1 co-expression in chronic viral infections and TILs from melanoma patients [165, 166] suggested a negative role for TIM-3 in T-cell dysfunction and exhaustion [151]. TIM-3 expression is known to be regulated by the Th-1 transcription factor T-bet [167], but lacks any specific negative signaling motif. TIM-3 seems to require carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1) heterodimerization in order to deliver inhibitory activity [168]. Preclinical tumor models employing both anti-PD-1 and anti-TIM-3 and the phenotypic characterization of temporal and spatial expression patterns of TIM-3 have suggested a potential synergistic role between TIM-3 and PD-1 [128, 151, 165, 169, 170], although questions remains as to when it is clinically relevant to administer anti-TIM-3. The observation that adaptive resistance to PD-1 blockade upregulates additional immune checkpoints like TIM-3 and LAG-3 [171] suggests that there may be some value

in considering a sequential, rather than concurrent, administration strategy with PD-1, an approach that failed when nivolumab was given in sequential combination with ipilimumab [172]. Indeed, at least two TIM-3 antibodies, namely Novartis' MGB453 [NCT02608268] and Tesaro's TSR-022 NCT02817633, are in early phase clinical development for advanced malignancies or solid tumors as either a single agent or in combination with a PD-1 antibody.

B7-H3 (CD276) is an additional member of the B7 ligand family, displaying structural homology to PD-L1 (B7-H1, CD274). Though initially identified as comprising an extracellular domain of single V-like and C-like Ig domains [173], subsequent studies revealed the dominant human form (4Ig-B7-H3) to contain tandemly duplicated V-like and C-like domains [174] consequent to an evolutionary exon duplication [175]. Notably, while expression of human B7-H3 protein is limited in normal tissues, it is overexpressed on a broad spectrum of solid tumors with expression inversely correlating with patient outcome and T-cell infiltrate (reviewed in [176]). Although no receptor has been conclusively determined for B7-H3, functional data indicate a role in modulating T-cell responses with increasing evidence of negative regulation, supported by resolution of the crystal structure of mouse B7-H3 that led to the identification of the subregion of the extracellular domain as pivotal in mediating T-cell inhibition [177]. In addition to its role in modulating immune-mediated responses, B7-H3 also appears to play a direct role in mediating tumor cell migration [178], chemosensitivity [179], and cell metabolism through promotion of the Warburg effect [180]. Preclinically, the therapeutic benefit of targeting of B7-H3-expressing xenografts was demonstrated with enoblituzumab (MGA271), a humanized anti-B7H3 mAb comprising an Fc modified for enhanced effector activity [181]. Interim Phase I studies have revealed enoblituzumab to be well tolerated (up to 15 mg/kg), with antitumor activity observed in patients with melanoma as well as prostate and bladder cancer, and evidence of T-cell modulation demonstrated by increased clonality of the peripheral T-cell repertoire following enoblituzumab treatment [182]. The complementary benefit of targeting B7-H3 together with CTLA4 or PD-1 is also being clinically explored in combination studies of enoblituzumab with ipilimumab or pembrolizumab [183, 184].

VISTA (V-domain Ig suppressor of T-cell activation) is an Ig superfamily member comprising a single Ig V domain in its extracellular domain bearing distant homology to PD-L1 and PD-L2 [185]. Expression of VISTA appears to be restricted to hematopoietic cells, with expression detected on both CD4 and CD8 T cells, monocytes, dendritic cells, and neutrophils [186]. While the receptor for VISTA has not been identified, *in vitro* studies with immobilized VISTA-Fc fusion protein showed that it mediates the inhibition of T-cell proliferation and cytokine production. Demonstration of exacerbated autoimmune responses and T-cell responses in VISTA-deficient mice further supports a role for VISTA as a negative regulator of T-cell responses [187]. In a mouse melanoma model, administration of anti-VISTA antibody increased CD8 T-cell infiltrate and the effector function of TILs while dampening Treg function, suggesting that VISTA supports tumor immune evasion [188]. In some mouse tumor models,

anti-VISTA also decreased the immune-suppressive MDSC population in the TME while increasing the presence of activated dendritic cells, suggesting that VISTA also plays a role in myeloid tumor responses. Notably, the VISTA pathway of immune inhibition appears to be nonredundant with the PD-1/PD-L1 pathway, as co-administration of anti-VISTA and anti-PD-L1 mAbs synergized to inhibit mouse CT26 syngeneic tumor progression and tumor-specific CD8 T-cell responses [189]. Based on these encouraging preclinical observations for potential monotherapy or combination with PD-1 blockade, clinical studies have recently been initiated to evaluate the VISTA-targeting antibody JNJ-61610588 in patients with advanced cancer such as NSCLC [NCT02671955].

TIGIT (T-cell immunoreceptor with immunoglobulin and ITIM domains) is an Ig superfamily member expressed on CD8 T cells, CD4 T-regulatory cells, and NK cells following immune activation [190, 191]. TIGIT forms part of a complex series of both receptor/ligand and receptor/receptor engagements, which ultimately determines how NK cells and T cells receive and integrate both inhibitory and activation signals. TIGIT is one of three ligands (TIGIT, CD96, CD226) capable of binding to the high-affinity CD155 receptor [192] and to the low-affinity CD112 and CD113 receptors [193], which comprise a family of nectin-like/polio virus receptor (PVR) molecules induced on immune cells and tumor cells following inflammation that mediate epithelial interactions [190, 194]. With regard to CD155 binding, both CD96 and TIGIT act as a negative regulators, delivering signals via and immunoreceptor tyrosine-based inhibitory (ITIM) motifs, and TIGIT possesses a second immunoglobulin tail tyrosine (ITT). In contrast, CD226 can deliver a counterbalancing activating signal [191, 195]. Curiously, CD96 also contains an intracellular YXXM motif that can lead to PI3K activation, suggesting that CD96 may possess both activating and inhibitory activity, via the aforementioned ITIM motif. Additional complexity and further signal modulation is possible given that TIGIT, the higher affinity receptor for CD155, and CD226, the lower affinity receptor for CD155, can form both heterodimers or homodimers with TIGIT/TIGIT homodimers and TIGIT/CD226 heterodimers delivering a negative signal, while CD226/CD226 homodimers delivering a positive signal [195]. While it remains unclear how these signals interplay with each other, the net activation of inhibition of lymphocyte signaling is fine-tuned through the integration of CD96, CD226, and TIGIT signaling, and likely depends on the overall abundance of the individual receptors and the signal strength delivered by hetero and homo-dimerization [196]. Consistent with its role as a negative immune regulator, blockade of TIGIT through mAbs promotes cytotoxicity and human T-cell effector function, in particular IFN-γ secretion [197], while TIGIT-deficient mice showed delayed tumor growth and reduced suppressive function of T-regulatory cells [198]. The clinical observations that TIGIT is coexpressed with additional immune checkpoints such as PD-1 [199], PD-L1 [197], and TIM-3 [198] on T cells or T-regulatory cells in the tumor setting and enhances T-cell effector function suggest that TIGIT would make an attractive target when given in combination. Taken together, both TIGIT and CD96 have been proposed for targeting in the context of cancer immunotherapy,

with TIGIT perceived as more advanced, given the validation of coexpression with other immune checkpoints on exhausted T cells [151, 196]. Indeed, Genentech has proposed evaluating clinically their anti-TIGIT mAb MTIG7192A both as a single agent and in combination with their anti-PD-L1 mAb atezolizumab in advanced solid or metastatic tumors [NCT02794571].

16.3.3 Co-stimulatory Pathways

As a complementary strategy to reversing the inhibition impinged on the TME through the co-inhibitory pathways, mAb strategies designed to engage and activate stimulatory pathways to enhance antitumor immunity are also being evaluated in the clinic (Table 16.3). In this strategy, the antibodies are therefore selected for their ability to agonize and activate signaling pathways rather than antagonize a cellular response through ligand blocking or elimination of target expressing cells. The TNFR superfamily members OX40, 4-1BB, GITR, CD40, and CD27 are particularly attractive for such a strategy based on their functional role in mediating diverse immune cellular responses that can be leveraged to support antitumor immunity and their shared structural design amenable to agonism.

OX40 (CD134) and its corresponding ligand OX40L are inducibly expressed on immune cell subsets following activation. OX40L expression has been observed on professional APCs, while OX40 expression was observed primarily on memory T-cell subsets, in particular CD4 T cells [200] and effector CD8 T cells [201], in addition to NKT cells, NK cells, and regulatory T cells [202]. Together, the OX40/OX40L interactions form a potent co-stimulatory signaling axis that regulates antigen-specific T-cell expansion, and promotes the generation, maintenance, and survival of memory T [203, 204]. In addition, OX40/OX40L co-stimulation can both skew T-helper cells toward a T_H2 phenotype while, conversely, controlling and limiting the activity of T regulatory cells, through the production of IL-4 [205] and inhibition of TGF-β, and IL-10 cytokines, respectively [206]. With the observation that OX40/OX40L interactions play important roles on both the regulatory and effector side of T-cell biology, therapeutic interventions that target this axis are being evaluated for effectiveness in autoimmune diseases, immunization strategies, and cancer [207, 208]. Indeed, several preclinical studies have demonstrated OX40 mAb-mediated generation of tumor-eradicating immunity sufficient to lead to tumor regression either through T-regulatory suppression and/or T-effector cell agonism [209–212]. OX40 agonism has been shown to increase T-cell infiltrates into tumors while reducing the number of suppressive macrophages in tumor-bearing hosts [213]. Given the diverse effector functions and antitumor activity generated following OX40 engagement, dual agonist approaches, as in the case of OX-40 and 4-1BB mAbs [214], or agonist/antagonist (push/pull) approaches, as in the case of OX40 and PD-L1 [215] or CTLA-4 [216] mAbs, have elicited synergy. Furthermore, OX40 is known to be overexpressed on T-regulatory cells [217] and selectively expressed with PD-1 and CTLA-4 on TILs in head and neck

Table 16.3 Costimulatory antibodies in clinical development.

Target antigen	Mechanism of action	Antibody format	Name(s)	Indications	Stage[a]
CD137 (4-1BB)	Priming and activation of T cells	Human IgG4	Urelumab (BMS-663513)	Solid tumors	Phase I/II
		Humanized IgG2	PF-05082566	Lymphoma	Phase I/II
OX-40	Priming and activation of T cells	Murine IgG1	MEDI-6469	Recurrent or metastatic solid tumors	Phase I
		Human IgG2	PF-04518600	Select advanced or metastatic carcinoma	Phase I
		Humanized IgG1	GSK3174998	Advanced solid tumors +/1 pembro	Phase I
		Humanized IgG1	MEDI-5062	Advanced solid tumors	Phase I
		Humanized IgG1	MOXR0916	Metastatic/locally advanced solid tumors	Phase I
CD40	Cancer antigen presentation	Human IgG2	CP-870, 893	Solid tumors, MM, pancreatic	Phase I
		Humanized IgG1 Fc-optimized	SEA-CD40	Advanced metastatic tumors/unresectable solid malignancies	Phase I
		Humanized IgG1	APX005M	Carcinoma, neoplasms, and MM+/− pembro	Phase I
		Human IgG1	ADC-1013	Neoplasms; solid tumors	Phase I
		Fully human IgG1	Lucatumumab HCD122	Multiple myeloma	Phase II
		IgG2	RO7009789 (Roche)	Advanced metastatic tumors/unresectable solid malignancies	Phase I
		Chimeric IgG1	ChiLob 7/4	CD40+ solid tumor/refractory DLBCL	Phase I
CD27	Priming and activation of T cells	Fully human IgG1 mAb	Varlilumab (CDX-1127)	Select solid tumors or hematologic cancers, combined with atezo	Phase I/II
GITR	Priming and activation of T cells and/or depleting immunosuppressive $CD4^+CD25^+FOXP3^+$ T_{reg} cells by inducing loss of FOXP3 expression	Human IgG1	GWN323	Solid tumors	Phase I
		Human IgG1	INCAGN1876	Solid tumors	Phase I/II
		Not disclosed	MK-4166	Advanced solid tumors	Phase I
		Aglycosyl fully humanized IgG1	TRX518	Solid tumors/malignant melanoma	Phase I
		Not disclosed	BMS anti-GITR	Cancer, indications not disclosed	Phase I

cancer [218]. In several Phase I clinical trials, MedImmune has evaluated two anti-OX-40 mAbs, namely MEDI-6469, a murine IgG1 [NCT01862900] across tumor types, and MEDI-0562 [NCT02318394], a humanized IgG1 mAb in solid tumors and in combination with either tremelimumab (anti-CTLA-4 mAb) or durvalumab (anti-PD-L1) [NCT02705482]. Pfizer has also demonstrated safety and pharmacokinetic/pharmacodynamic (PK/PD) finding from their first in human study with PD-04518600, a fully human IgG2 mAb targeting OX40, indicating that it was well tolerated at the low 0.3 mg/kg repeat dose-escalation point [219]. Pfizer also is recruiting patients with neoplasms for combination study with their OX-40 mAb and their 4-1BB agonist mAb PD-05082566 [NCT02315066]. Finally, Genentech is also evaluating their anti-OX40 mAb, MOXR0916, a humanized antibody, in a Phase Ib clinical safety study in solid tumors in combination with atezolizumab (anti-PD-L1) and/or bevacizumab (anti-VEGF mAb) [NCT02410512].

CD137 (4-1BB) together with its corresponding ligand 4-1BBL forms a second co-stimulatory axis that mirrors the OX-40/OX40L interaction across an overlapping but also distinct set of immune cell populations. 4-1BB is transiently induced on CD8 T cells, but a broader expression of 4-1BB on CD4 T cells, NK cells, T-regulatory cells, in addition to DCs, mast cells, endothelium, thymocytes, progenitor cells, and tumor cells, has been observed [220–222] while 4-1BBL is expressed on forms APCs [223] and thymic epithelial cells[224]. Together with its ligand expressed on APCs, the 4-1BB/4-1BBL forms another co-stimulatory axis in parallel to OX40/OX40L axis to promote T-cell growth and differentiation, in particular with thymocyte development. In contrast to OX40, which is seen to play more dominant role with CD4 T cells, 4-1BB seems more biased toward enhancing primary antigen-driven CD8+ T-cell responses rather than inhibiting T-regulatory responses against tumors. With regard to T-regulatory cells, the role of 4-1BB stimulation is controversial, where 4-1BB signaling may inhibit T-reg differentiation or, alternatively, maintain T-reg expansion and T-cell suppressor function [222]. In contrast, the role of 4-1BB agonism in murine tumor studies is more consistent, demonstrating that anti-4-1BB mAbs can generate potent antitumor memory responses against a variety of tumor cell lines when given as a single agent [225–227]. In general, however, the tumor-eradicating immunity observed in murine models relied more on mechanisms that enhanced NFκB activation and IL-2 production via CD28 co-stimulation of tumor-specific memory CD8 T cells [227] and permitted their survival by avoided antibody induced cell death (AICD) [228] or, in the case of poorly immunogenic tumors, required IFN-γ in order to traffic to the tumor and break tolerance [229]. While agonist 4-1BB antibodies are known to augment CD8 antitumor responses, they are also capable of activating NK cells through Fc interactions. Specifically, the Fc region of the antibody can bind to Fc receptors expressed on NK cells and subsequently inducing 4-1BB expression on their surface, thereby converting NK cells into innate targets receptive to 4-1BB agonism [230]. This observation has provided the rationale for combination strategies that employ one antibody, such as cetuximab (anti-EGFR) [231], rituxamab

(anti-CD20) [232], or trastuzumab (anti-HER2) [233], in order to activate NK cells so that 4-1BB antibodies can subsequently stimulate them in addition to T cells.

As single agents, two agonist 4-1BB antibodies are under early clinical development: urelumab, a fully human IgG4 that is currently in a Phase I clinical trial for advanced colorectal and head and neck cancers [NCT02110082], and PF-05082566, a fully human IgG2 mAb [234]. It should be noted, however, that the clinical development of urelumab as single agent was initially hampered because of off-target effects, leading to marked and fatal hepatic toxicities [235–237], highlighting the need for careful dosing or further lowering the dose of anti-CD137 mAbs when given alone or in combination with additional check-point antibodies.

Given that significant experience exists with regard to managing adverse events associated with immune check-point blockade, and recent studies in mouse tumor models that have demonstrated synergy between agonist 4-1BB antibodies and the antagonistic check-point antibodies PD-1 [238], PD-L1 [239], or anti-CTLA-4 [233], there is renewed interest and further clinical development in employing 4-1BB antibodies in combination-based strategies. Indeed, while both urelumab and PF-05082566 underwent clinical trials as single agents, there are several combination approaches reported. Urelumab is being evaluated with rituximab (anti-CD20) in NHL and leukemia [NCT01775631, NCT02420938], nivolumab (anti-PD-1) in bladder cancer [NCT0284532] and metastatic malignant tumors [NCT02534506], and elotuzumab (anti-SLAMF7) in multiple myeloma versus elotuzumab+lirilumab (KIR2DL1/2L3) combination [NCT02252263], and cetuximab (anti-EGFR) in colorectal and head and neck cancer [NCT02110082]. Similarly, the anti-4-1BB antibody PF-05082566 is being evaluated in clinical combinations with PF-0451866 (anti-OX40) in neoplasms [NCT02315066], pembrolizumab (anti-PD-1) in advanced solid tumors [NCT02179918], avelumab (anti-PD-L1) in advanced cancers [NCT02554812], and rituximab (anti-CD20) [NCT01307267] in many liquid tumor types, while urelumab is being evaluated with rituximab (anti-CD20) in NHL and leukemia [NCT01775631, NCT02420938], nivolumab (anti-PD-1) in bladder cancer [NCT0284532] and metastatic malignant tumors [NCT02534506], and cetuximab (anti-EGFR) in colorectal and head and neck cancer [NCT02110082].

CD40 has attracted much attention as a cancer therapeutic target for agonistic mAbs based on its role as a mediator of antigen presentation and priming of antigen-specific cytotoxic T-lymphocyte (CTL) responses. On hematopoietic cells, CD40 is expressed primarily on APCs such as dendritic cells, B cells, and monocytes. Upon engagement with the CD40 ligand, which is expressed on T cells and platelets, CD40 receptor signaling leads to the maturation of APC function and the expression of co-stimulatory molecules, leading to an increase in the activation of antigen-specific T cells. Studies in a syngeneic mouse lymphoma model demonstrated agonism of CD40 through treatment with an anti-CD40, resulting in eradication of lymphoma accompanied by a 10-fold expansion of CD8+ T cells including anti-tumor CTLs, which protected against tumor re-challenge

without further treatment [240]. Indeed, CD40 agonistic mAbs elicit antitumor activity through potentially three mechanisms of action. The primary mechanism mimics the natural CD40/CD40L interaction by activating APCs, thereby licensing in particular DCs to induce tumor-specific immune responses [241]. Additional mechanisms, which are not necessarily mutually exclusive, include the generation of macrophage tumoricidal activity [242] and tumor death via antibody-dependent, cell-mediated cytotoxicity (ADCC)/complement-mediated cytotoxicity (CMC)/program cell death (PCD) [243], which can release tumor antigens and cross-prime CD8 T cells independent of CD4 T cell help. Clinically, the most advanced molecule is CP-870,893, a fully human IgG2 antibody, as a single agent initially targeted for solid tumors, melanoma, pancreatic carcinoma, and mesothelioma [NCT02225002] and in combination with tremelimumab (anti-CLTA-4) for Stage IV metastatic melanoma [NCT01103635]. To alleviate toxicity concerns, local rather than system administration has been proposed and is currently being investigated with the human anti-CD40 IgG1 mAb ACD-1013 [NCT02379741] [244].

CD27 (TNFRSF7) is an additional member of the TNFR superfamily being targeted in the clinic to enhance T-cell immune responses against cancer through engagement with an agonistic mAb. In contrast to 4-1BB and OX40, the expression of CD27 is constitutive on naïve and memory T cells, memory B cells, and a subset of NK cells. Its expression is further upregulated upon T-cell activation, but is then lost at the fully differentiated effector phase [245]. CD70, the ligand for CD27, is transiently expressed on matured DCs and activated T and B cells. Its expression is then further amplified upon CD28 or CD40 activation of APC serving as a key costimulator through engagement of CD27 during the T-cell priming phase by counteracting apoptosis in activated T cells during their clonal expansion [246]. The CD27/CD70 axis then continues to regulate T-cell responses during the clonal expansion of T cells and by fine-tuning CTL effector cells – in particular the memory differentiation of CD8+ T cells [247]. Within the tumor setting, transient CD27/CD70 co-stimulation can contribute to tumor control, through the induction of NK cells responses and greater CTL effector and tumor-specific memory responses, supporting the appeal for CD27 agonism through an antibody-based approach. The fully human IgG1 anti-CD27 mAb, varilumab, is currently being evaluated as a single agent in multiple solid cancer types [NCT01460134] and in combination with nivolumab (anti-PD-1) for advanced refractory solid tumors [NCT02335918] and with ipilimumab (anti-CTLA-4) for Stage IV melanoma [NCT02413827].

Glucocorticoid-induced TNFR-related (GITR) protein is an additional TNFR member constitutively expressed on T-regulatory cells and transiently expressed on CD4 and CD8 T cells [248]. In addition, GITR is also expressed on B cells, NK cells, and activated DCs [249]. The ligand for GITR is expressed on activated APCs and endothelial cells, and appears to provide co-stimulatory activity to effector T cells [250]. GITR agonists elicit tumor regression through a novel mechanism that causes T-regulatory cells, which overexpress GITR, to lose lineage stability, thereby removing the suppressive function [251]. GITR agonists are also capable

of enhancing both T-cell proliferation and effector function on both CD8 and CD4 T cells. Taken together, this suggests that GITR agonists can skew the T-effector/T-regulatory ratio by increasing CD8 T cells and decreasing T-regulatory cell numbers, leading to enhanced antitumor activity. Indeed, preclinical studies with the mouse anti-GITR mAb DTA-1 and anti-CTLA-4 mAbs have supported such mechanisms and, furthermore, demonstrated synergy in an adoptive T-cell transfer model that is sufficient to eradicate several established tumors [249, 252]. This suggests that combination strategies employing co-stimulatory agonists and check-point antagonists may generate enhanced antitumor responses [253]. At least five agonist GITR antibodies are currently under clinical evaluation (Table 16.3). TRX518, a glycosylated fully human IgG1 mAb currently being evaluated in late-stage malignant melanoma or solid tumors [NCT01239134], was reported to be safe in single doses up to 8 mg/kg and achieved T-cell saturation at doses greater than 0.5 mg/kg [254]. GWN323 is an anti-GITR mAb being tested as a single agent or in combination with PDR001 (anti-PD-1 mAb) in patients with advanced cancer or lymphomas [NCT02740270].

In conclusion, the aforementioned antibodies that target check-point and costimulatory molecules, as outlined above, seek to antagonize negative signaling axes that diminish and ultimately exhaust T-cell effector function, while potentiating positive signaling axes that augment T-cell effector function. While this view is central to the role of cytolytic effector CD8 T cells, it is worth noting that additional immune cell subsets, such as T-regulatory cells, NK cells, myeloid-derived suppressor cells (MDSCs), and macrophages, express many of these immune modulators and therefore may be targeted and exploited to elicit tumor-eradicating immunity. While such strategies are beyond the scope of this chapter, novel approaches that deplete T-regulatory cells, as well as MDSCs, in addition to approaches that convert macrophages toward generating antitumor activity, together with approaches that can both stimulate and inhibit NK cells, are actively being evaluated. While the arsenal of antibodies available to target novel immune checkpoints and their co-stimulatory counterparts, as alluded to above, is impressive, it also underscores the need to evaluate those combinations that can potentiate the greatest antitumor activity, at the same time minimizing off-targeting and adverse immune responses. Such approaches are not only being evaluated as antibody combination, but as described in the following in the context of bispecifics, may impart improved targeting, minimize the number and severity of adverse events, and offer greater antitumor efficacy than antibody combinations alone.

16.4 Bispecific Antibodies

Cancer is a multifactorial disease, with many signaling pathways implicated in pathogenesis and disease progression. While targeting a single pathway through single-antigen-dependent immunotherapy, as described previously, has been

successful, many patients receiving mAb therapy may develop drug resistance or fail to respond to treatment due to the overriding contribution of an additional aberrantly activated pathway. Hence, incorporating two therapeutic targets into single molecule in the context of a BsAb is an attractive alternative to combined pathway targeting using separate mAbs, which may obviate resistance and in certain cases may provide potential synergy not observed with simple combination of two separate antibodies. Furthermore, bispecific molecules combining the specificities for tumor antigens and antigen on the effector cells provide the opportunity to harness the effector-cell cytotoxicity toward tumor cells, as best exemplified by blinatumomab and catumaxomab, which are approved for ALL and malignant ascites. This strategy requires close cell–cell association, which is not possible with combination mAb therapies. The BsAb approach for redirecting T cells to eradicate tumor cells may be more cost effective and an off-the-shelf alternative to cell-based therapies, such as chimeric antigen receptor (CAR) T cells, which, in addition to their high cost, are often associated with manufacturing challenges, as they require *ex vivo* expansion of transduced autologous T cells. Details of structural and biophysical properties of various BsAb formats are presented in Chapter 8. In this section, we will describe bispecific antibodies that are in clinical development covering both redirected immune cell killing and dual cancer pathway targeting (Table 16.4).

16.4.1 Immune Cell Re-Targeting

16.4.1.1 Lymphoid Effectors

Redirected tumor target cell killing by immune effector cells is the most established application of bispecific antibodies in cancer immunotherapy, wherein bispecific antibodies are designed to simultaneously bind to a cytotoxic effector cell (through a receptor on cytotoxic effector cell like CD3 on T cells and CD16 on NK cells) and a target cell expressing a tumor-cell-specific antigen. These, antibody-mediated, co-association of tumor cells and target cells results in the formation of an artificial synapse between two cells, culminating in the activation of the cytotoxic pathway in effector cells and tumor cell death. CD3 is an invariant complex of proteins that is required for expression of, and signaling through, the antigen-specific T-cell receptor (TCR) on T cells. T lymphocytes also play an important role in the immune response against cancer. T cells are activated when they are presented with cognate peptide antigens by major histocompatibility (MHC) molecules, which are expressed on the surface of APCs. However, tumor-specific T-cell responses in particular are limited by immune escape mechanisms utilized by tumor cells. CD3-based BsAbs can bypass the requirements of pre-existing antigen-specific TCR or co-stimulation by co-engaging T cells and tumor cells and stimulating T cells. This process is accompanied by the formation of an artificial transient cytolytic synapse between the T cell and the targeted tumor cell, which eventually results in the subsequent activation and proliferation of T cells leading to tumor cells lysis. Recent developments in

Table 16.4 Bispecific antibodies in clinical development or approved for the treatment of cancer.

Target antigens	Mechanism of action	Format	Name	Targeted cancer type	Stage
CD123 × CD3	Redirects T-cell cytotoxicity	DART®	MGD006/S80880	AML	Phase I
A33 × CD3		DART	MGD007	Colon cancer	Phase I
B7H3 × CD3		DART	MGD009	Solid tumors	Phase I
CD19 × CD3		DART	MGD011	ALL	Phase I
P-cadherin × CD3		DART	PF-06671008	Solid tumors	Phase I
CD19 × CD3		BiTE®	Blinatumomab	NHL and B-ALL	FDA Approved
CD33 × CD3		BiTE	CD33 × CD3	AML	Phase I
CEA × CD3		BiTE	MEDI-565 (AMG-211)	Colorectal	Phase I
		IgG-based BsAb	RG7802	Advanced and/or metastatic solid tumors	Phase I
EpCAM × CD3		BiTE	Solitomab AMG110	Lung, colorectal	Phase I
PSMA × CD3		BiTE	BAY2010112 (AMG 212, Bayer; Amgen)	Prostate cancer	Phase I
		ADAPTIR™	MOR209/ES414	Prostate cancer	Phase I
EpCAM × CD3		Triomab®	Removab	Malignant ascites	EU approved
HER2 × CD3		Triomab	Ertumaxomab	Metastatic breast	Phase II
CD20 × CD3		Triomab	FBTA05 (Bi20)	B-cell malignancies	Phase I/II
CD20 × CD3		Cross-linked IgGs	REGN1979	B-cell malignancies	Phase I
GPC3 × CD3		ART-Ig	ERY974	Solid tumors	Phase I
BCMA × CD3		BiTE	AMG420 BI836880	Neoplasms	Phase I

(*continued overleaf*)

Table 16.4 (*continued*)

Target antigens	Mechanism of action	Format	Name	Targeted cancer type	Stage
CD30 × CD16	Redirects NK, macrophage, monocyte cytotoxicity	TandAb	AFM13	Hodgkin disease	Phase II
CD30 × CD16		(Fab′)2	HRS-3/A9	Hodgkin disease	Phase II
HER2 × CD64		(Fab′)2	MDX-210	Breast/ovarian	Phase I
EGFR × CD64		(Fab′)2	MDX-447	Lung, colorectal, etc.	
HER2 × HER3	Interferes with signaling receptor targeting	Biclonics®™	MCLA-128	Advanced solid tumors	Phase II
		scFv	MM-111		Phase I
Ang2 × VEGF-A		Cross-linked IgGs	RG7221 (RO5520985)	Colorectal	Phase II
EGFR × HER3		CrossMAb	RG7597 (MEDH7945A)	Colorectal, head and neck cancer stem cell	Phase II
HER3 × IGF-1R		IgG scFv	MM-141	Solid tumors	Phase I
MET × EGFR		mAb-scFv	LY3164530	Advanced solid tumors	Phase I
		DuoBody®	JNJ-61186372	Non-small-lung cancer	Phase I
DR5 × FAP		CrossMAb	RG7386	Advanced or metastatic solid tumors	Phase I
DLL4 × VEGF-A		BiMAb	OMP-305B83	Solid tumors	Phase I

the engineering of BsAbs with improved stability and manufacturability open up many new potential therapeutic applications, as evidenced by the more than 20 BsAbs in clinical development (Table 16.4). Here we review the approved bispecific molecules along with those in clinical development for therapeutic use in different cancers.

16.4.1.2 **Hematological Malignancies**

Blinatumomab (Blincyto) is based on the bispecific T-cell engager (BiTE) platform and was the first BsAb approved in 2014 by FDA. BiTE is composed of the two binding domains (variable heavy and light chain domains) of two different human IgG antibodies flexibly linked by a short peptide. Blinatumomab binds to CD3 on T cells and CD19-expressing B cells, resulting in the targeted depletion of B cells coupled with T-cell expansion. It was approved for treatment of acute B-cell lymphoblastic leukemia and is also in a Phase II trial for diffuse large B-cell lymphoma (DLBCL), and a Phase I study for NHL. Blinatumomab-mediated co-engagement of T cell and tumor cell results in the upregulation and secretion of various granzyme proteases that provide essential components for the cytolytic synapse formed between the T cells and target cell. Blinatumomab is potent in redirecting T cells to CD19+ lymphoma cells at very low concentrations of 10–100 pg/ml. Doses of 15 μg/m^2/day or higher lead to the depletion of tumor cells in humans. Blinatumomab has a short serum half-life of less than 2 h, requiring patients to receive it through continuous infusion via an implanted port system. In a Phase II trial in patients with high-burden relapsed or refractory B-cell ALL (B-ALL), treatment with blinatumomab resulted in an impressive 43% complete response rate and a median overall survival of 5 months [255]. Some patients treated with blinatumomab suffer from central nervous system toxicity and show symptoms of cytokine-release syndrome. Because of its small size, blinatumomab can be easily eliminated by the kidneys, and patients need to be administered continuously. Some patients receiving blinatumomab develop drug resistance most likely due to the loss of CD19, extramedullary relapse, and upregulation of PD-L1 on tumor cells [256]. Duvortuxizumab (MGD011), a CD19 × CD3 targeting dual-affinity re-targeting (DART) protein comprising an Fc domain to enhance half-life and support more convenient dosing, is also in clinical testing. The DART platform, developed by MacroGenics, is based upon covalently linking Fv regions of two distinct antibodies specific for different antigens in a bispecific diabody arrangement. The association of the two chains is stabilized through a covalent carboxy terminal disulfide linkage [257]. As stated earlier, MGD011 also incorporates an Fc domain to prolong the circulating half-life similar to that of conventional mAbs. In comparison, blinatumomab, which is engineered as a single-chain Fv pair, is administered by continuous IV infusion for repeated 4-week courses, owing to its short ~2 h half-life, and hence duvortuxizumab provides the potential for more convenient dosing regimen for patients. MGD011 demonstrates potent antitumor activity against localized and disseminated human B lymphomas in human PBMC-reconstituted mouse models and humanized BLT mice [258, 259]. Once-weekly intravenous infusion of MGD011 in monkeys was well tolerated and

resulted in a dose-dependent, durable decrease in circulating B cells accompanied by profound reductions of CD20+ B cells in lymphoid organs [259, 260]. A Phase 1 study is currently going on in relapsed or refractory B-cell malignancies including diffuse-large B cell lymphoma (DLBCL), follicular lymphoma (FL), mantle-cell lymphoma (MCL), chronic lymphocytic leukemia (CLL), and acute lymphoblastic leukemia (ALL).

An alternative B-cell surface antigen, CD20, is also being targeted by redirected T-cell-based bispecific molecules. REGN1979, a human IgG-based CD20 × CD3 bispecific antibody comprising a common light chain, targets CD20 expressed on normal and malignant B cells and the CD3 component of the T-cell receptor. *In vitro* studies using REGN1979 demonstrated a CD20-target cell-dependent activation, cytokine release by T cells, and efficient redirected T-cell lysis of target tumor cells. REGN1979 induced tumor regression in large, advanced Raji tumors, associated with long-lasting tumor control superior to rituximab therapy in suppressing established Raji tumors. Preclinical studies in cynomolgus monkeys showed that a single injection of REGN1979 was more potent at depleting CD20+ B cells in the lymph nodes than a high dose of rituximab [261]. A Phase I study is currently under way in patients with CD20+ B cell malignancies. FBTA05 (Bi20) is another CD20 × CD3 BsAb that connects B cells and T cells via its variable regions and also recruits FcγRI(+) accessory immune cells via its Fc region (Triomab). Bi20 mediated efficient and specific lysis of B-cell lines and of B cells with low CD20 expression levels from CLL patients [262]. In pediatric patients with B-cell malignancies, treatment with FBTA05 showed clinical response in 90% of patients, and 40% of patients show prolonged remission [263]. Another CD20 × CD3 bispecific molecule is BTCT4465A/RG7828, which is a full-length IgG1 molecule constructed using the "knobs-into-holes technology" [264]. It is currently in clinical trial in patients with NHL or CLL (NCT02500407). A third B-cell target, the B-cell maturation antigen (BCMA) exclusively expressed by plasma cells, is being targeted by AMG420 (BI836880), and a BCMA × CD3 BiTE is currently in Phase 1 study in patients with multiple myeloma (NCT02674152) [265].

Regarding the targeting of myeloid leukemia, MGD006, a CD3 × CD123 DART molecule designed to treat acute myeloid leukemia (AML) by redirecting T cells to kill leukemic cells, is being developed [266]. CD123 (IL3RA), is an antigen upregulated in several hematological malignancies and differentially expressed in AML blasts and leukemic stem cells compared to normal hematopoietic stem and progenitor cells. MGD006 simultaneously binds T lymphocytes through CD3 and cells expressing CD123, and mediates dose-dependent T-cell-redirected killing of CD123+ cell lines as well as primary AML blasts. Furthermore, MGD006 has demonstrated potent activity in tumor-bearing mice engrafted with human peripheral blood mononuclear cells or when tumors were co-inoculated with human T lymphocytes. MGD006 binds to both human and cynomolgus monkey antigens and can redirect T cells from either species to kill CD123-expressing cells. Monkeys infused with escalating doses of MGD006 on continuous or intermittent schedules over a period of 4 weeks showed depletion

of circulating CD123+ cells and persistance throughout the treatment period. In human AML xenograft model, where primary AML patient samples were transplanted into immunodeficient NSG mice, MGD006 treatment resulted in near-complete eradication of AML blasts in peripheral blood, bone marrow and spleen [267]. Currently, MGD006 is in Phase I trial in patients with relapse or refractory AML. Subsequently, two other CD123 × CD3 bispecific molecules, XmAb14045 and JNJ-63709178, have also entered clinical development for patients with CD123-expressing hematologic malignancies (NCT02730312 and NCT02715011, respectively). CD33, an alternative cell-surface target expressed on AML, is also being pursued via evaluation of AMG 330, a CD33 × CD3 BiTE. In preclinical studies, AMG 330 showed highly potent killing of CD33+ AML cell lines or primary human AML cells in the presence of healthy donor T cells or autologous T cells from AML patients at low effector to target ratio. Antitumor activity was observed in tumor-xenograft, immunodeficient mice models *in vivo* [268, 269]. Currently AMG 330 is in Phase I study in patients with relapsed refractory AML.

16.4.1.3 **Solid Tumors**

The approach of redirecting T-cell cytotoxicity to cancer cells is also in development for targeting solid tumors. Catumaxomab, an EU-approved BsAb for treatment of malignant ascites, is a rat and mouse bispecific trifunctional hybrid mAb (Triomab) that binds to EpCAM and CD3 with the Fc region that provides a third functional binding site that is able to selectively bind and activate Fcγ receptor-expressing cells [270]. It can crosslink T cells through CD3 binding, and also Fc-γ expressing effector cells such as NK cells and macrophages. Mainly all human adenocarcinoma, certain squamous cell carcinoma, retinoblastoma, and hepatocellular carcinoma express EpCAM (CD326). *In vitro* and *in vivo* preclinical studies using mouse surrogate demonstrated potent antitumor activity in the 10 pM range. In a Phase I/II clinical trial, ovarian cancer patients with malignant ascites were treated with catumaxomab (4–5 doses of 5–200 μg); 22/23 patients did not require paracentesis between the last infusion and the end of study at day 37, with a reduction of EpCAM-positive malignant cells in ascites [271]. A pivotal study involving 258 patients demonstrated improvement of puncture-free survival, leading to approval in EU [272]. A similar triomab strategy has also been employed to target HER2-expressing cancer cells. Ertumaxomab, an intact bispecific antibody targeting HER2/neu and CD3 with selective binding to activatory Fcγ Type I/III receptors (triomab), demonstrated cytotoxicity against tumor cell lines that express HER2/neu at high and low levels, in the presence of immune effector cells (PBMC) *in vitro* at low effector to target cell ratios [273]. In a Phase I study in patients with metastatic breast cancer, antitumor response was seen in 5 out of 15 evaluable patients at dose levels of 100 μg or higher. Treatment is also associated with a strong T-helper cell Type 1-associated immune response with elevated cytokines (IL-6, IL-2, TNF-α, and IFN-γ) and mild adverse events [274].

Several BiTE molecules have also been evaluated clinically for solid tumors – with molecules developed for targeting EpCAM, CEA, and PSMA.

Solitomab, an EpCAM × CD3 BiTE, showed potent activity on EpCAM-expressing cell lines *in vitro*. Tumor growth prevention was observed at nanogram doses *in vivo* in NOD/SCID mice implanted with human colon carcinoma cells mixed with unstimulated human peripheral mononuclear cells. Solitomab was also active against human ovarian metastatic tissues grafted in immunodeficient mice in the absence of human peripheral mononuclear cells [275]. Solitomab is currently being evaluated in clinical trials for lung, colorectal, and gastrointestinal cancer. AMG211, a CEA × CD3 BiTE, targets the carcinoembryonic antigen (CEA or CEACAM5), which is a tumor antigen highly expressed in CRC, and also in a substantial number of carcinomas of the lung, pancreas, stomach, ovary, uterus, and breast, and a subset of melanomas [276]. CEA × CD3 BiTE mediated T-cell-directed killing of CEA positive tumor cells within 6 h, at low effector to target ratios, which were independent of high concentrations of soluble CEA [277]. AMG 211 is currently in Phase I study in patients with advanced gastrointestinal cancer. BAY2010112, a PSMA × CD3 BiTE, is being developed for treatment of patients with prostate cancer. BAY2010112 induced target cell-dependent activation and cytokine release of T cells, and efficiently redirected T cells for lysis of target cells expressing PSMA at half the maximum BiTE concentrations between 0.1 and 4 ng/ml (1.8–72 pmol/l). In an *in vivo* NOD/SCID mice xenograft model, antitumor activity was demonstrated using PC-3-huPSMA and 22Rv1 cells at a dose level as low as 0.005 mg/kg/day [278]. A Phase I dose escalation study tested PSMA × CD3 BiTE in prostate cancer patients. MOR209/ES414 is another potent PSMA × CD3 bispecific molecule (ADAPTIR) in which the PSMA and CD3 scFv domains are linked to an Fc domain to extend the half-life [279]. It is currently in clinical trial in patients with metastatic castration-resistant prostate cancer (NCT02262910).

Three DART molecules, each incorporating an IgG1 Fc domain to enhance the circulating half-life but mutated to avoid undesired FcγR interactions, are also being applied to recruit T cells for solid tumor targeting and lysis. MGD007, a humanized, long-acting gpA33 × CD3 DART protein, was designed to redirect T cells to target gpA33-expressing colon cancer. MGD007 has enhanced pharmacokinetic properties via the incorporation of a neonatal FcR-binding Fc domain that is mutated to abolish FcγR binding and potential molecular cross-linking through such associations. The gpA33 target was selected based on its ubiquitous expression in CRC, including reactivity with putative cancer stem cell populations [280]. MGD007 displays the anticipated bispecific binding properties and mediates potent lysis of gpA33-positive – but not gpA33-negative – cancer cell lines through recruitment of T cells, with both T-cell activation and expansion observed in a gpA33-dependent manner. Currently, MGD007 is in Phase I trial in relapsed CRC (NCT02248805). P-cadherin × CD3 (PF-06671008) is an additional Fc-bearing DART molecule being clinically evaluated. P-cadherin overexpression has been reported in various tumors, including breast, gastric, endometrial, colorectal, and pancreatic cancers. P-cadherin overexpression correlates with increased tumor cell motility and invasiveness. PF-06671008, a P-cadherin × CD3 DART (an Fc domain bearing DART), was highly potent

at picomolar concentrations in CTL killing assays using P-cadherin-expressing tumor cells. Potent *in vivo* antitumor efficacy was observed in human colorectal and peripheral blood mononuclear cell (PBMC) co-mix xenograft mouse models. PF-06671008 is currently in Phase I study in patients with solid tumors expressing P-cadherin [281]. The B7H3 × CD3 DART molecule MGD009 is the third Fc-bearing DART being clinically evaluated in patients with B7H3-positive solid tumors (NCT02628535). An additional long-acting, CD3-based bispecific, ERY974, which is based on the ART-Ig platform and co-targeting Glypican 3 (GPC3), has showed potent preclinical activity against a wide variety of tumor cells *in vitro* and *in vivo* xenograft models [282] and is currently in Phase I study in patients with solid tumors (NCT02748837).

While not strictly bispecific antibodies, ImmTACs (immune-mobilizing monoclonal TCRs against cancer) are another class of bispecific proteins designed to co-engage tumor cells and T cells. However, in contrast to the BsAb approach described earlier, recognition of tumor antigens is mediated through the incorporation of a tumor-associated epitope-specific monoclonal TCR, which is engineered to possess extremely high affinity for cognate tumor antigen. After binding to tumor cells, the anti-CD3 effector arm of the ImmTAC drives the recruitment of polyclonal T cells to the tumor site, leading to a potent redirected T-cell response and tumor cell destruction [283, 284]. One of the potential advantages of ImmTACs is their ability to target intracellular antigens presented by HLA molecules on tumor cells, although tumor cells express very low numbers of the HLA peptide on the surface. IMCgp100 binds to gp100, a melanoma-associated antigen, and redirects all T cells to kill the cancer cells. In a Phase I study, IMCgp100 showed a favorable safety profile with prolonged responses observed in both uveal and cutaneous melanomas.

In addition to T cells, other immune cells with cytotoxic potential, such as NK cells, monocytes, and macrophages, can be recruited by BsAbs engaging surface receptors. CD16 and CD64 have been used for engaging other effector cells. CD16 is expressed on NK cells as well as on macrophages and granulocytes, whereas CD64 is expressed on monocytes, macrophages, and granulocytes. In the following, we give examples of bispecific molecules using CD16- or CD64-expressing effector cells.

MDX-H210 is a BsAb constructed chemically by cross-linking anti-HER-2/neu and anti-CD64, the high-affinity Fc receptor (FcγRI). Preclinical studies demonstrated it to be highly cytotoxic to HER-2/neu overexpressing cell lines, when FcγRI-positive PMN served as effector cells [285, 286]. A Phase II study in patients with HER2+ advanced prostate cancer and treated with the bispecific antibody MDX-H210 showed modest clinical response [287]. MDX-447 is a EGFR × CD64 BsAb showing enhanced tumor cytotoxicity when combined with granulocyte-colony stimulating factor (G-CSF) in preclinical studies. However, in a Phase I study in patients with advanced solid tumors, MDX-447 alone was generally well tolerated, but did not achieve objective tumor responses [288].

CD30 × CD16 (AFM13) is a bispecific, tetravalent chimeric antibody construct (TandAb) designed for the treatment of CD30-expressing malignancies. AFM13

is a CD30 × CD16A tetravalent bispecific tandem diabody (TandAb), which crosslinks NK cells with CD30+ target cells. AFM13 demonstrated superior cytotoxicity relative to Fc-enhanced IgG antibodies against CD30+ target cells, and the activation of NK cells was strictly dependent on the presence of CD30+ target cells [289]. In a Phase I dose-escalation study of 28 patients with heavily pretreated, relapsed, or refractory Hodgkin lymphoma with AFM13 at doses of 0.01 – 7 mg/kg, 3 of 26 evaluable patients achieved partial remission (11.5%) and 13 patients achieved stable disease (50%), with an overall disease control rate of 61.5%. AFM13 treatment resulted in a significant NK cell activation and a decrease of soluble CD30 in peripheral blood [290].

16.4.2 Dual Targeting of Two Receptor Pathways on Cancer Cell Using BsAbs

Tumor development and progression often depend on growth signals mediated by receptors that are upregulated in many tumor cells, as described in an earlier section of this chapter. Examples include members of the EGF receptor family, that is, EGFR, HER2, HER3, and HER4, and the IGF-1 receptor (IGF-1R), VEGF, and Ang-2, which play essential roles in regulating cell proliferation, survival, differentiation, and migration. These receptors operate through multiple downstream signaling pathways including Ras/Raf/ERK/MAPK and PI3K/AKT. Resistance to therapy against a singular receptor is often associated with pathway switching between two receptors. Several BsAbs targeting two different signaling molecules on the tumor cells are in clinical development, as shown in Table 16.4 and described below.

HER2 × HER3: Dual targeting of HER2- and HER3-expressing tumor cells was described for a bispecific molecule generated by fusing scFvs directed against HER2 and HER3 to the N- and C-termini of human serum albumin (scFv-HSA-scFv) [291]. This molecule (MM-111) combines targeting of HER2-overexpressing tumor cells with potent inhibition of ligand-induced phosphorylation of HER3, with IC_{50} values in the subnanomolar range. MM-111 is currently being tested in Phases I and II studies in patients with advanced HER2-amplified cancers (NCT01774851, NCT00911898, NCT01097460, and NCT01304784). MCLA-128 is another Her2 × Her3 BsAb that incorporates an effector function enhanced Fc-domain. Weekly dosing of MCLA-128 at 25 mg/kg in preclinical models reduced tumor burden significantly compared to trastuzumab + pertuzumab treatment [292]. In a Phase I/II study in patients with relapsed or refractory solid tumors, MCLA-128 was well tolerated and showed partial response in 1/28 patients and stable disease in 11/28 patients [293].

EGFR × HER3: MEHD7945A (duligotuzumab) is an EGFR × HER3 Cross-MAb. In cancer cells resistant to cetuximab and erlotinib, it was found that MEHD7945A, but not single target EGFR inhibitors, could inhibit tumor growth and cell-cycle progression in parallel with EGFR/HER3 signaling pathway

modulation. MEHD7945A was more effective than a combination of cetuximab and anti-HER3 antibody at inhibiting both EGFR/HER3 signaling and tumor growth [294]. MEHD7945A was well tolerated with a favorable safety profile in patients with advanced tumors treated at doses up to 30 mg/kg dosing every 2 weeks. However, in a Phase II study, in recurrent/metastatic squamous-cell carcinoma of the head and neck (RMSCCHN) patients, MEHD7945A did not improve outcomes compared to cetuximab [295].

HER3 × IGF-1R**:** MM-141 is a novel tetravalent bispecific mAb (IgG scFv) that binds IGF-1R and ErbB3 and blocks both ligand-dependent and ligand-independent IGF-1R/ErbB3/PI3K/AKT/mTOR signaling. MM-141 enhanced the biologic impact of receptor inhibition *in vivo* as a monotherapy and in combination with the mechanistic target of rapamycin (mTOR) inhibitor everolimus, gemcitabine, or docetaxel, through the blockade of IGF-IR and ErbB3 signaling and prevention of PI3K/AKT/mTOR network adaptation [296]. In a Phase I study in patients with advanced solid tumors, MM-141 was well tolerated as a monotherapy, and translational analysis of pharmacodynamic parameters suggested appropriate target engagement. A Phase II study is currently going on in biomarker-selected patients with metastatic pancreatic cancer.

MET × EGFR: EGFR and MET are RTKs, both playing a key role in cancer signaling. A number of tumor types have coexpression and activation of MET and EGFR. LY3164530 is a mAb-scFv bispecific antibody that binds to extracellular domains of MET and EGFR with high affinity, inhibits signaling via both MET and EGFR receptors, and has superior activity in overcoming HGF-mediated resistance to erlotinib, gefitinib, lapatinib, or vemurafenib as compared to the combination of individual mAbs targeting these receptors in cell-based assays. *In vivo*, LY3164530 showed potent antitumor activity that was equivalent, and in some cases superior, to the combination of obinutuzumab and cetuximab in multiple cell line-derived NSCLC and gastric xenografts. In a Phase I study in patients with advanced solid tumors, LY3164530 appeared to be safe when administered as single agent and in combination with erlotinib [297] (NCT01287546**)**. JNJ-61186372 is another BsAb targeting MET and EGFR generated by controlled Fab arm exchange method (DuoBody). In preclinical studies, JNJ-61186372 inhibited EGF and cMet downstream signaling pathways and demonstrated efficacy in multiple *in vivo* tumor models with EGFR mutations, including both cell-line- and patient-derived xenografts where single agent EGFR inhibitors were less effective [298]. Also, a preference in BsAb binding toward the higher expressed of the two receptors EGFR or c-Met was observed, which resulted in the enhanced *in vitro* potency against the less highly expressed target. This avidity-based BsAb design with differential affinities of each binding arm based on receptor expression may be useful for selectively targeting tumor cells and sparing normal cells [299]. A Phase I study in patients with advanced NSCLC is currently under way (NCT02609776).

Ang2 × VEGF-A (vanucizumab): RO5520985 is a novel bispecific human IgG1 antibody (CrossMAb), acting as a dual-targeting inhibitor of the two key angiogenic factors VEGF-A and Ang-2. RO5520985 showed potent tumor growth inhibition in a panel of orthotopic and subcutaneous syngeneic mouse tumors and patient- or cell-line-derived human tumor xenografts. In contrast to Ang-1 inhibition, anti-Ang-2-VEGF-A treatment did not aggravate the adverse effect of anti-VEGF treatment on physiologic vessels [300]. In a Phase I study in advanced solid tumors, 67% PFS rate at 8 weeks was demonstrated with an acceptable safety profile with favorable PK and PD effects [301].

DLL4 × VEGF-A (OMP-305B83): DLL4 × VEGF-A is a BiMAb that simultaneously targets DLL4 and VEGF and shows potent antitumor activities *in vivo* mouse xenograft models using a broad spectrum of tumor cell lines (Wan-Ching Yen, AACR 2014). It is currently in clinical trial in patients with previously treated solid tumors (NCT02298387).

DR5 × FAP (RG7386): Dysregulated cellular apoptosis and resistance to cell death are hallmarks of neoplastic initiation and disease progression. Activation of the extrinsic apoptotic pathway is strongly dependent on death receptor (DR) hyperclustering on the cell surface. RG7386 is a bispecific antibody (CrossMAb) that simultaneously targets fibroblast-activation protein (FAP) on cancer-associated fibroblasts in tumor stroma and DR5 on tumor cells. RG7386 trigger potent tumor cell apoptosis *in vitro* and *in vivo* in preclinical tumor models with FAP-positive stroma. RG7386 demonstrated single agent activity against FAP-expressing malignant cells, due to cross-binding of FAP and DR5 across tumor cells and in combination with irinotecan or doxorubicin in patient-derived xenograft models [302]. DR5 and FAP dual targeting could be particularly attractive as it restricts DR5 agonism to the tumor microenvironment where FAP is expressed while avoiding DR5 agonism in tissues without FAP expression.

16.5 Conclusions and Future Directions

The development of antibody-based therapeutics for cancer is in the midst of a rewarding period, exemplified by the FDA approval over the last 5 years of a battery of new treatments spanning various modalities and cancer indications. Recent approvals include mAbs targeting blockade of signaling pathways aberrantly expressed in cancer cells (e.g., next-generation anti-HER2 and anti-EGFR mAbs and first in class inhibitors of PDGFRa2), those targeting cell-surface antigens expressed on tumor cells for destruction through recruitment of immune effector cells pathways (e.g., anti-GD2, CD38, SLAMF7 mAbs), and those targeting axis supporting tumor angiogenesis (anti-VEGFR2 mAb) or immune evasion (e.g., anti-CTLA4, PD-1, and PD-L1 mAbs). The recent approval of antibody–drug conjugates targeting CD30 and HER2 (see Chapter 9) and the

bispecific antibody blinatumomab co-targeting CD19 and CD3 (as described above) has also energized efforts with those respective modalities. Collectively, these clinical successes have fueled the effort to explore both additional antibody-based modalities and targets expressed either on cancer cells themselves or in the TME – many of which are presently undergoing clinical evaluation, as described in this review, and many more in preclinical development.

Despite the significant progress achieved in the field of cancer therapy and the hope for the next wave of mAb-based targeting therapies, for many indications the improved responses through targeting of an individual pathway remain low. This has led to increasing efforts to combine mAb-based therapies to achieve more significant improvement in clinical response as obtained through a combination of trastuzumab (anti-HER2) and pertuzumab (anti-HER3) for the treatment of metastatic breast cancer and the combination of nivolumab (anti-PD-1) and ipilimumab (anti-CTLA4) for the treatment of metastatic melanoma. Moving forward, it is clear that significant effort will be placed to evaluate combination strategies across various mAb modalities and targets to increase response rates further, in addition to layering in of chemotherapeutic strategies to potentiate further efficacy. Though individual patient responses will vary, the quest to identify biomarkers predictive of response to enable patient stratification for optimal course of therapy – including combinations – will continue to play hand in hand with therapeutic development. The combination of antibody-targeting specificities in the context of therapeutic bispecific antibodies provides further opportunity to enable biological and potentially therapeutic responses not feasible with mAb combinations (e.g., recruitment of T cells through co-engagement of tumor cells and immune cells, as illustrated by blinatumomab). There lies also the potential for bispecific and multispecific antibody-based molecules to enable targeting of multiple cancer axes in a single molecule, which may lead to biological responses not observed with simple mAb combinations due to differences in co-target interaction and molecule distribution. This, in particular, could be of importance for targeting various components of the immune system to enhance tumor immunity – whether it is combined blockade of two checkpoints, combined targeting of a checkpoint and an immune stimulator, or the desire to localize immune-based intervention through co-targeting of a cancer antigen. An appeal of such bispecific targeting molecules is also the potential to simplify dosing strategies in which each component is provided at a predetermined ratio rather than optimization of each component individually, and the ability to further multiplex targeting through combination of bispecific targeting molecules.

In summary, therapeutic antibodies have provided significant benefit to cancer patients, and continued advances in antibody technology, increased understanding of the components of cancer biology, and the ability for multiplex targeting will provide additional opportunities for continued advancement in development of improved treatments with better and more durable outcomes.

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17
Protein Therapeutics in Respiratory Medicine

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17.1
Introduction

Biologic treatments with monoclonal antibodies have been developed for a wide range of conditions across the fields of medicine, with the first licensed use in 1986 of muromonab for the treatment of organ rejection following transplant. A further seven monoclonal antibodies were licensed during the 1990s. However, the first monoclonal antibody for respiratory disease was not in use until 2003, when omalizumab was licensed for the use in persistent, allergic asthma. The marginal efficacy and high cost of this agent, as well as restrictions on use based on patient weight, a requirement for allergy, and a limited range of serum immunoglobulin (Ig)E levels, limited use of this agent, and its impact has not been as great as that of biological agents in other disease areas. This is a reflection of a dearth of novel, effective treatments in the field of respiratory medicine since the introduction of inhaled corticosteroids (ICS) in the 1960s.

A rethink of the role of inflammation in airway disease has led to progress, with many targeted therapies now under development. A key change has been the identification of clinically relevant groups (or phenotypes) based on the underlying pathology. This has been possible because of the discovery and utilization of easy-to-measure, noninvasive biomarkers that can be applied even to patients with severe airway disease. These techniques have identified eosinophilic airway inflammation as a discrete, readily identifiable, and treatable pattern of disease. Importantly, traditional categorization of airway disease on the basis of demographic details and lung function abnormalities provides only a very limited perspective on the pattern of airway inflammation. A new taxonomy of airway disease based on biological mechanisms is therefore needed in order to optimize the use of biological therapies.

By far the most fruitful area to date has been in the treatment of severe asthma, with a number of new treatments recently licensed or in late-stage clinical trials.

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In this chapter, we will give an overview of asthma and the pathological processes at work, and outline the current and emerging protein therapies in use in respiratory medicine.

17.2 Asthma

Asthma is a common, chronic inflammatory condition of the airways. It affects between 5% and 10% of the UK population and over 300 million people worldwide [1]. It is characterized by episodes of shortness of breath, wheezing, and cough due to airflow limitation as a result of an increased tendency of the airway to narrow (airway hyperresponsiveness), airway mucosal inflammation, and increased airway mucus production. The inflammation in asthma involves particularly T-helper type 2 lymphocytes, eosinophils, neutrophils, and mast cells.

Variable airflow obstruction is currently the key physiological abnormality, and its demonstration represents the standard approach to the diagnosis of asthma. Airflow obstruction can be measured in a variety of ways including spirometry, which can be done at the GP's surgery or the hospital, and peak flow meters that patients can use at home and are thus particularly suited for the demonstration of variability. Some patients with asthma do not have overt variable airflow obstruction despite having underlying a suggestive clinical picture and typical inflammation of the airways. In these patients, airway hyperresponsiveness can be tested for by administering an inhaled substance such as methacholine or histamine, which, in patients with asthma but not in healthy patients, causes progressive airflow limitation. This response is usually measured using a breathing test called the forced expiratory volume in one second (FEV_1), with a decrease from baseline of more than 20% being suggestive of asthma. Airways hyperresponsiveness is thought to be the basis of many of the symptoms of asthma, such as breathlessness and wheeze. These symptoms are often triggered by exercise, changes in temperature, exposure to irritants or allergens, and viral respiratory tract infections.

Patients with asthma are at risk of asthma attacks (asthma exacerbations), where symptoms and airflow limitation worsen and become less treatment-responsive. These attacks are the most clinically important aspects of the disease, as they can result in severe symptoms requiring unscheduled medical help and sometimes hospital admission. Asthma attacks result in around 1200 deaths every year in the UK. The National Review of Asthma Deaths (NRAD), a multiagency review of all suspected asthma mortality in the UK [2], looked in detail 195 asthma deaths in 2012–2013 and concluded that the majority were the result of basic errors in asthma management and were thus readily preventable.

The Global Initiative for Asthma (GINA) has produced internationally recognized guidelines for the management of asthma (Figure 17.1). The ultimate goal is to suppress symptoms completely, maintain normal lung function, and eliminate asthma attacks. The majority of patients with asthma are well controlled

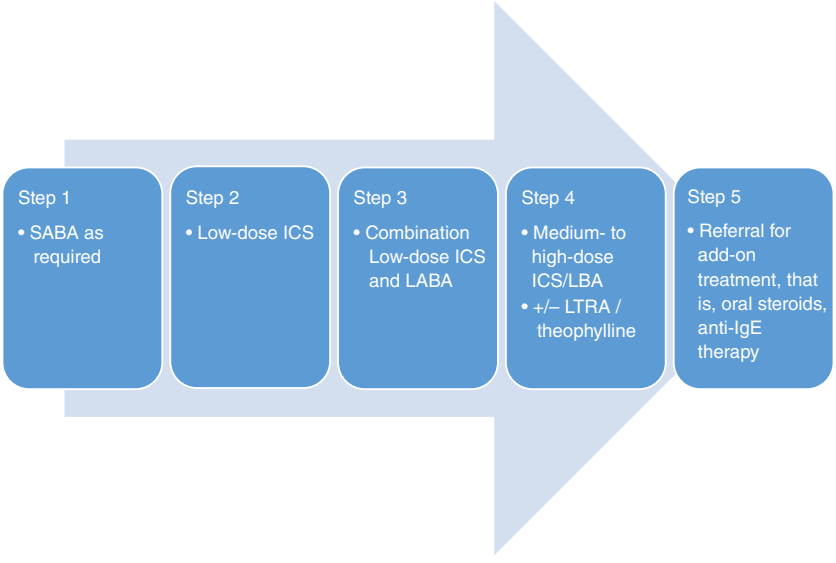

Figure 17.1 Stepwise approach to increasing asthma medications. Modified from GINA guidelines. SABA, short acting β-2 agonist; ICS, inhaled corticosteroid; LABA, long-acting β-2 agonist; LTRA, leukotriene receptor antagonist. (From Ref. [3].)

on inhaled therapy, consisting of β-2 adrenergic receptor agonists to control bronchospasm and ICS to control the underlying inflammation. The guidelines suggest a stepwise increase in asthma medications and dose to achieve control. These steps range from Step 1 – where short-acting β-2 agonists (SABA) are used as needed and the introduction of a low dose ICS can be considered – to Step 5 where patients require high-dose ICS, inhaled long-acting β2 agonist (LABA), and additional systemic treatment such as oral corticosteroids or omalizumab [3]. Long-term oral steroid treatment is associated with morbidity due to a large number of side effects, including high blood pressure, fluid retention, and osteoporosis.

Around 10% of patients have severe asthma with ongoing symptoms and/or asthma attacks despite high-dose asthma therapy at GINA Steps 4 or 5 [4]. The most recent, internationally agreed definition of severe asthma is shown in Box 17.1. Severe asthma is a complex clinical problem with a high level of morbidity and associated healthcare utilization and spending. Often, the problem is that the diagnosis is incorrect, or that asthma is uncontrolled because of a failure to master the basics of asthma management including treatment adherence, inhaler technique, and smoking cessation. Symptoms may also persist because of the presence of a comorbid condition such as obesity, rhinitis, or psychosocial issues. Because of the complexity of the condition, patients with severe asthma should be managed by an asthma specialist with access to detailed airway tests and a multidisciplinary team that includes specialist nurses, physiotherapists, and pharmacists.

Box 17.1 European Respiratory Society/American Thoracic Society definition of severe asthma 2014 [4]

"Asthma which requires treatment with guidelines suggested medications for GINA Steps 4–5 asthma (high-dose ICS and LABA or leukotriene modifier/theophylline) for the previous year or systemic CS for >50% of the previous year to prevent it from becoming "uncontrolled" or which remains "uncontrolled" despite this therapy".

Uncontrolled asthma is defined as at least one of the following:

1) Poor symptom control: ACQ consistently >1.5, ACT <20 (or "not well controlled" by NAEPP/GINA guidelines)
2) Frequent severe exacerbations: two or more bursts of systemic CS (>3 days each) in the previous year
3) Serious exacerbations: at least one hospitalization, intensive care unit (ICU) stay, or mechanical ventilation in the previous year
4) Airflow limitation: after appropriate bronchodilator withhold, $FEV_1 < 80\%$ predicted (in the face of reduced FEV_1/FVC defined as less than the lower limit of normal)

Controlled asthma that worsens on tapering of these high doses of ICS or systemic CS (or additional biologics).

*CS: corticosteroids; ACQ: Asthma Control Questionnaire; ACT: Asthma Control Test; NAEPP National Asthma Education and Prevention Program.

17.2.1 Phenotypes of Asthma

Asthma had historically been classified as allergic ("extrinsic") or non-allergic ("intrinsic") based on the age of onset and the presence of allergies.

Extrinsic asthma usually starts in the early school years and is typically associated with other allergic diseases such as rhinitis and eczema. There is often a family history of allergic disease. Patients commonly notice wheeze and other symptoms after exposure to allergens such as house dust mite, animal danders, and grass pollen. This allergic reaction occurs when inhaled allergens are recognized by antigen presenting cells (APCs) at the airway–epithelial barrier in the airways. The APCs then interact with Th0 cells and cause them to differentiate into Th2 cells, which produce the cytokines interleukin (IL) 4, IL-5, IL-9, and IL-13. IL-4 further stimulates Th0 cells to differentiate into Th2 cells and promotes B-cell (plasma cell) class switching to produce antigen-specific immunoglobulin E (IgE). This IgE binds to the high-affinity Fc-ε receptors (Fc-ƐR) on mast cells. On re-exposure to the antigen, antigen is bound by the IgE on mast cells and causes rapid degranulation of the mast cells, which results in airway inflammation due to the release

of histamine, various cytokines (e.g., IL-3, IL-4, IL-5, IL-13), chemokines (CCL3), lipid mediators (e.g., leukotriene E4, prostaglandin D2), and enzymes. IL-4 is also involved in eosinophil chemotaxis from the blood stream into the airways. A more delayed, cell-mediated allergic reaction can also occur whereby allergens are recognized by macrophages, which act as APCs that interact directly with Th2 cells to release cytokines.

Intrinsic asthma usually starts in adulthood and is more common in women. There is no clear allergic trigger identified. Intrinsic asthma tends to be more severe with a higher rate of severe exacerbations and is less responsive to usual asthma treatments. It is not clear what causes intrinsic asthma, but a post-infective immune reaction is a possible trigger.

A developing knowledge of the underlying mechanisms has shown asthma to be a heterogeneous disease involving a number of inflammatory pathways, with no pathology that is unique to "extrinsic" or "intrinsic" asthma. An alternative classification of asthma is one based on the underlying inflammatory process. This is arguably a more clinically relevant categorization because defining asthma by these inflammatory phenotypes can aid treatment decisions, with new and emerging treatments targeted at the underlying mechanistic pathways.

Inflammatory phenotypes can be broadly divided into T-helper type 1 (Th1)-mediated and T-helper type 2 (Th2)-mediated inflammation. T-helper cells differentiate from naïve $CD4^+$ T-helper (Th0) cells based on the body's reaction to various antigens, which may be bacteria, allergens, or other substances recognized by the immune system. The Th1 pathway results in neutrophil-related airway inflammation, whereas the Th2 pathway causes eosinophil-related inflammation (Figure 17.2).

Patients with Th2-high asthma are at a much higher risk of frequent and severe asthma attacks than those with Th2-low disease. It has, however, previously been difficult to identify those at highest risk. Recognition and measurement of biological markers specific to the underlying inflammatory process has allowed phenotyping patients according to their underlying pathology. This enables the identification of those at high risk and ensuring that their treatments are appropriate and that the patients are aware of the importance of taking them.

17.2.2 Biomarkers

A biomarker is a clinical, biological, or chemical measurement that is indicative of an underlying biological process. Eosinophils found in the blood and airways are biomarkers of Th2-high disease and can help in phenotyping patients and guiding treatments.

The peripheral blood can be quickly assessed in the laboratory to measure the blood white cell count and its differential make-up. A raised blood eosinophil count is associated with eosinophilic airways inflammation; however, it is not associated with neutrophilic airways inflammation.

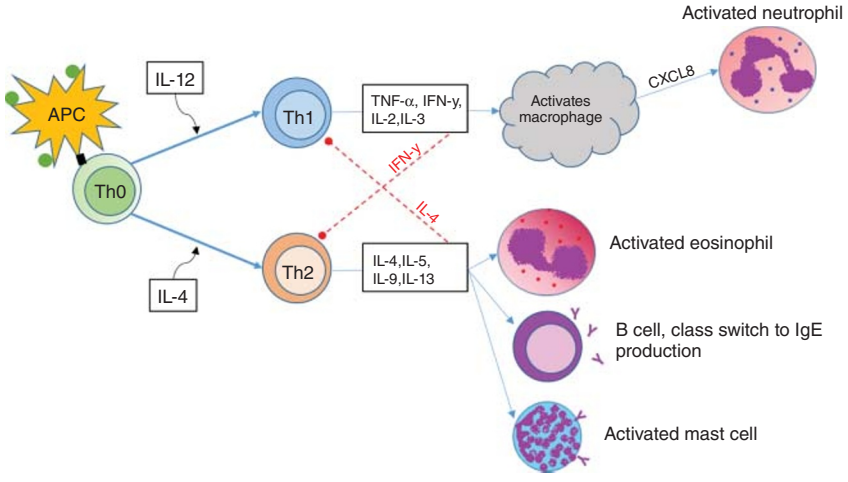

Figure 17.2 Pathways leading to Th1 (neutrophilic) and Th2 (eosinophilic) inflammation. Naïve Th0 cells differentiate following interaction with antigen -APCs and cytokine influence. The Th1 and Th2 pathways inhibit each other via IFN-γ and IL-4, respectively. APC, antigen presenting cell; Th, T helper; IL, interleukin; IFN-γ, interferon gamma.

Microscopic evaluation of the sputum gives a direct representation of the active processes within the airway. Sputum can be obtained either by collection of spontaneously produced samples or by inducing production of a sample. Sputum induction is performed by inhalation of a nebulized hypertonic saline solution to promote the production of airway secretions, which can then be coughed up and collected. Induced sputum collection can take up to an hour and can cause wheeze. Because of this, it needs to be performed by trained staff and the sputum analyzed by experienced technicians. These considerations have meant that induced sputum has previously mainly been used as a research tool, although it is increasingly being incorporated into clinical decision making in specialist centers.

Another useful biomarker of Th2-related inflammation is the fractional exhaled nitric oxide (FeNO), which is present in raised concentration in exhaled breath in patients with eosinophilic airway inflammation as a result of IL-13-mediated induction of nitric oxide synthase in the airway epithelium. FeNO is a noninvasive test and can be measured by a simple breath test in the GP's surgery or in the hospital clinic. Normal values of FeNO are between 5 and 25 parts per billion (ppb), with values over 50 ppb highly suggestive of eosinophilic inflammation.

Periostin is a promising biomarker for Th2 inflammation. It is a protein that is made by a number of cells including fibroblasts and bronchial epithelial cells. Periostin is a downstream product of IL-4 and IL-13 activity. It can be measured in the blood, although this test is mainly used for clinical trial purposes and is not available for routine clinical use. A summary of Th2-related biomarkers is shown in Table 17.1.

Table 17.1 Biomarkers of eosinophilic inflammation used in RCTs with monoclonal antibodies to preselect patients in adult asthma.

Biomarker	Association with treatment response	Invasiveness	Comments
FeNO	Corticosteroids, anti-IL-13, anti-IL-4 and13, anti-IgE	Noninvasive	Easy, quick, not specific, cheap, generally available
Serum IgE	Not associated	Minimal	Although recommended to measure, there is no clear association between IgE as a biomarker and treatment responses or clinical outcome
Serum periostin	Anti-IL-13[a)]	Minimal	Effect shown with anti-IL-13; high costs and limited availability currently
Blood eosinophil count	Anti-IL-5[a)], anti-IL4/13	Minimal	Generally available, high clinical impact, predicts anti-IL-5 response. Less clear predictor in anti-IL4/13 treatment
Sputum eosinophil count	Corticosteroids, anti-IL-5	Moderate	Specialist centers, tissue-specific, time-consuming

a) Proven clinical efficacy in combination with this treatment.
FeNO, fractional exhaled nitric oxide; IgE, immunoglobulin E; IL, interleukin.

17.2.3 The Th1 Pathway

The Th1 inflammatory process is signaled by IL-12 and IL-2, which promote the release of interferon gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α), among other mediators. This causes activation of macrophages, which leads to phagocyte-related inflammation. Sputum evaluation in this population shows a neutrophil-predominant cell type.

Th1-driven asthma is less responsive to steroid treatment, both inhaled and oral. This neutrophilic phenotype tends to be associated with a high symptom burden but a lower risk of life-threatening asthma attacks. Neutrophilic inflammation can also be caused by a Th17-mediated response through the effects of IL-17 and TNF-α produced by these cells.

Brodalumab is a fully human monoclonal antibody that blocks the IL-17A receptor. In a clinical trial of patients with moderate to severe asthma, it did not meet its primary end point of improving patient symptom scores. There was, however, an improvement in a preselected subgroup of patients who showed a large response to inhaled bronchodilators [5]. Golimumab is a fully human monoclonal antibody that targets TNF-α. It also suggested as an improvement in

patients with a large bronchodilator response, but the trial was halted because of an increase seen in the rate of cancers in the treatment group [6].

Neutrophils express the CXCR2 receptor, which recognizes a group of chemokines (designated CXC). CXCR2 is a key regulator of neutrophil chemotaxis and adhesion, and is thought to play an important role in recruiting neutrophils in to the airways. A trial of a novel small-molecule antagonist of CXCR2 in patients with severe asthma showed that it did reduce airway neutrophils by 36% but did not have a significant effect on lung function or asthma symptom scores [7]. A recent trial of another CXCR2 antagonist in chronic obstructive pulmonary disease (COPD), a predominantly neutrophilic condition, showed that sputum neutrophils were reduced and that this was associated with a clinical improvement in lung function in current smokers but not in ex-smokers [8].

These disappointing results targeting neutrophilic inflammation in asthma suggest that we do not have a full understanding of all the mechanisms at work in Th2-low inflammation and that the presence (and targeted reduction) of neutrophils in themselves do not correlate with patient symptoms and lung function.

Macrolide antibiotics, such as azithromycin, may be a useful treatment option in this group of patients with neutrophil-predominant inflammation. As well as antimicrobial agents, macrolide antibiotics have an anti-inflammatory effect via a number of mechanisms that are not fully understood. In a randomized controlled trial (RCT) of daily low-dose azithromycin in patients with severe COPD and persistent exacerbations, azithromycin was found to reduce the risk of exacerbation compared to placebo [9]. The role of macrolide therapy in patients with asthma is less clear. A number of studies have been conducted with conflicting results. These studies have largely been looking at a wide asthma population. A predefined subgroup analysis of an RCT investigating azithromycin in severe asthma showed a significant reduction in exacerbations in patients who predominantly have neutrophils in their sputum. Further trials are planned to investigate this group of patients [10].

17.2.4 The Th2 Pathway

Th2-mediated asthma is characterized by eosinophilic inflammation within the airways. Eosinophils are potent inflammatory white blood cells and are found in increased numbers in the sputum, bronchial biopsies, and peripheral blood samples of patients with this form of asthma. Th2 inflammation can be caused by the classical immediate (Type 1) hypersensitivity and delayed (Type 4) hypersensitivity mechanisms.

IL-5 is the key cytokine in the development of eosinophilic inflammation. It promotes the recruitment and maturation of eosinophils into the blood stream from the bone marrow and increases eosinophil survival. IL-5 is also an important chemo-attractant, causing migration of eosinophils from the blood into the affected tissues. IL-9 is a mast cell growth factor and also promotes further IL-4 release. IL-4, along with IL-13, promotes class switching of B cells to IgE

production. In addition, it causes increased epithelial permeability and increased mast cell survival. IL-9 stimulates the production of further Th2 cells, stimulates B cells to produce more IgE, and promotes the proliferation of mast cells. IL-13 has activity similar to that of IL-4. It has a number of other effects, including increasing mucus production, increasing mast cell proliferation and survival, increasing the production of extracellular matrix proteins, and increasing the contractility of the airway smooth muscle.

Less than 50% of patients with severe eosinophilic asthma have a raised serum IgE and a demonstrable allergy shown by skin prick or blood testing. This raises the possibility of an alternative non-allergic pathway leading to eosinophilic infiltration and inflammation in the airways. This is currently being explored, with interest around innate lymphoid cells type 2 (ILC-2), which can produce large amounts if IL-5 and IL-13 but hardly any IL-4. ILC-2 cells are not triggered by the Type 1 hypersensitivity pathway, but may be triggered by non-allergic interactions at the epithelial cell barrier with the inhaled air. It is proposed that, in response to stimulus, epithelial cells produce IL-25 and IL-33, which can act directly on ILC-2 cells and cause them to release the cytokines that cause eosinophilic inflammation [11]. This mechanism may also be potentiated by the allergic mast-cell-recruiting route via the release of prostaglandin D2 (PGD2) acting on the chemokine receptor homologous molecule expressed on Th2 lymphocytes (CRTH2), which is found on eosinophils, Th2 cells, and ILC-2 cells [12] (Figure 17.3). It has been shown that CRTH2-expressing cells are elevated in patients with Th2-high asthma [13].

Early trials of an oral, small molecule antagonist of CRTH2 have shown it to improve lung function in patients with eosinophilic asthma [14], making this receptor an attractive target for further research.

Th2 cytokines have been the focus of research to create targeted therapies in the treatment of asthma and have shown promising results. We will discuss the current and emerging therapies targeting these pathways.

17.3 Th2-Targeted Therapies

17.3.1 Immunoglobulin E

Omalizumab is a recombinant humanized immunoglobulin G (IgG) monoclonal antibody against human IgE. It is delivered as a subcutaneous injection dosed according to body weight and total serum IgE every 2–4 weeks. It binds to IgE and so lowers free IgE levels in the blood and downregulates the high-affinity IgE receptor on mast cells. It was approved in 2003 for use in moderate to severe, persistent, allergic asthma. To be eligible for treatment, patients need to have poor asthma control despite high-dose ICS treatment, a raised serum IgE level, and a positive skin prick test, or in vitro reactivity to a perennial aeroallergen as measured by a specific IgE blood test.

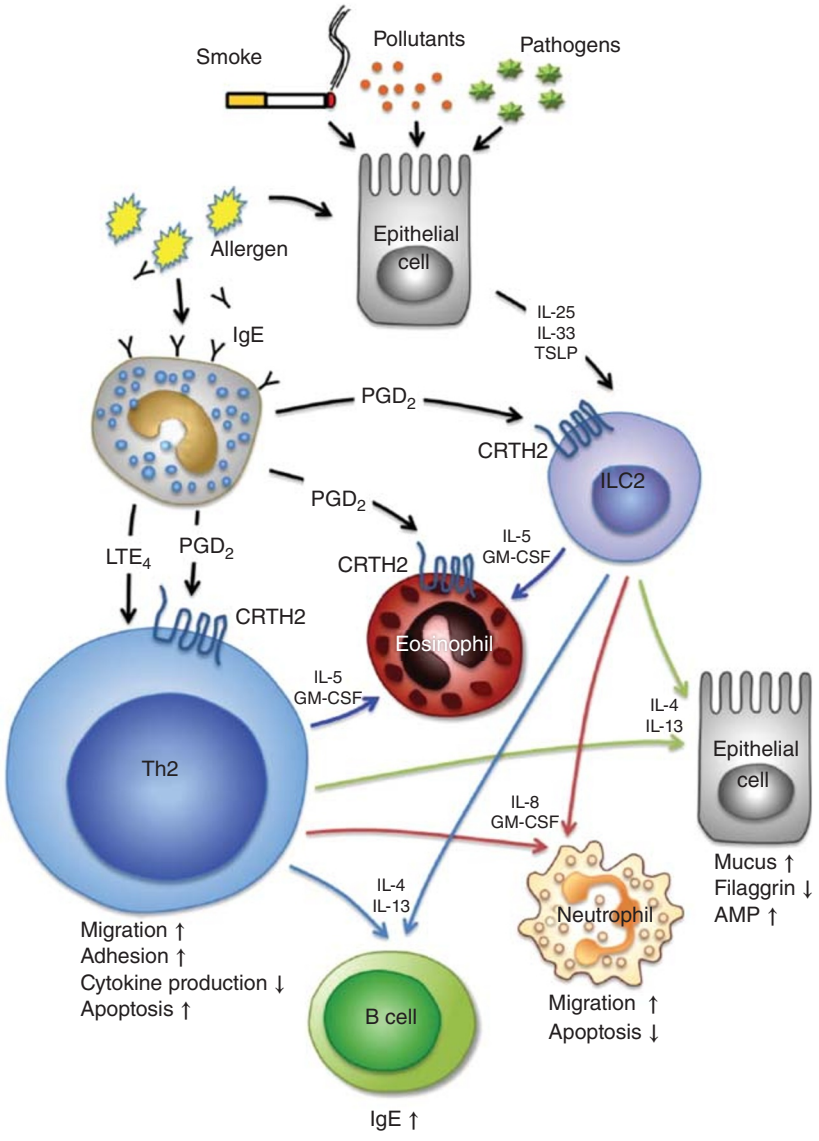

Figure 17.3 Proposed parallel mechanisms of eosinophilic inflammation due to interactions of antigens and irritants at the air/epithelial layer interface. Antigens and irritants can induce eosinophilic inflammation via the IgE-mediated hypersensitivity pathway and also by IL-25- and IL-33-mediated activation of ILC-2 cells. IgE, immunoglobulin E; PGD2, prostaglandin D2; IL, interleukin; Th2, T-helper type 2; ILC2, innate lymphoid cell type 2; LTE4, leukotriene E4; CRTH2, chemokine receptor homologous molecule expressed on Th2 lymphocytes.

A Cochrane systematic review from 2014 reviewed 25 placebo-controlled RCTs of omalizumab in asthma with a total of 6382 participants [15]. This showed that, when compared to placebo, omalizumab reduced the absolute risk of asthma attacks by 10%, from 26% to 16%, over the course of 16–60 weeks. Anti-IgE treatment reduced hospitalization due to exacerbations, with an absolute risk reduction of 3% with placebo to 0.5% with omalizumab. Compared to placebo, treatment reduced the need for high-dose ICS treatment in some patients, but no significant difference was noted in the number of subjects who were able to withdraw oral steroid. There was some evidence that omalizumab treatment improved asthma-related symptom scores, with a significant improvement in 7 out of the 11 studies in the review that reported asthma symptom outcomes. Overall, there was very little change in lung function measurements when compared to placebo.

Interestingly, a favorable response to omalizumab treatment can be predicted by the level of eosinophilic inflammation, as shown by the peripheral blood eosinophil count, FENO, and serum periostin, rather than the total serum IgE, as might be expected [16]. The lack of a direct link between the total serum IgE level and a response to anti-IgE therapy is curious, as is evidence that therapy is helpful in patients with non-allergic severe eosinophilic asthma [17]. The mechanisms of action of omalizumab are not fully understood. One theory that may explain why omalizumab is effective in non-allergic patients is that it improves the patients' immune response to viruses and so reduces exacerbations due to viral infection [18]. Dendritic cells are a key part of the immune system in recognizing and mounting a response to viral infections. Dendritic cells, like mast cells, express the high-affinity Fc-ƐR IgE receptor. It is thought that IgE cross-linking this receptor may reduce the efficacy of dendritic cells in viral infections and that, by reducing the amount of IgE binding with omalizumab, they may become more effective at fighting viruses.

Omalizumab is also licensed for use in dermatology for chronic idiopathic urticaria that has not responded to first-line treatment.

17.3.2 Interleukin 5

IL-5 is essential for the recruitment, survival, and migration of eosinophils. It mediates its effect via the IL-5 receptor found on eosinophils, basophils, and some mast cells [19]. Neutralizing the action of IL-5, either by targeting IL-5 itself or the IL-5 receptor, is therefore an attractive target for treatment.

17.4 Mepolizumab

Mepolizumab is a recombinant humanized IgG monoclonal antibody against IL-5. One of the earlier trials of mepolizumab showed that it dramatically reduced

the blood and sputum eosinophil count in patients with mild allergic asthma but did not improve airway responsiveness or the airway response to inhaled allergen [20]. A subsequent large RCT looking at the safety and efficacy of mepolizumab in 362 patients with moderate asthma showed, again, that blood eosinophil counts were significantly lowered by the drug. However, the clinical end points of lung function and quality of life were not significantly improved [21]. This disappointing result led some people to question whether eosinophilic inflammation played as important a role in asthma as initially thought, as greatly reduced numbers of eosinophils did not translate to improved symptoms. Another explanation for the observed lack of clinical efficacy was that the drug was being used in patients who did not have active eosinophilic inflammation, or that the trials were looking at the wrong outcomes.

Patients with active eosinophilic disease are at risk of exacerbations; however, this increased risk does not correlate with increased symptoms or decreased lung function. This means that it is not possible to assess the risk on clinical grounds alone. It follows that reducing the amount of eosinophilic inflammation may not improve symptoms, but may decrease the exacerbation rate. This change in thinking led to the design and development of trials to investigate patients with eosinophilic disease who would be likely to benefit from IL-5-targeted therapy.

An RCT was conducted from 2006 to 2008 and reported in 2009 to evaluate the effect of monthly intravenous dosing of mepolizumab or placebo on exacerbation rates over 1 year. It studied patients who had proven, severe eosinophilic asthma. Eosinophilia was defined as a sputum eosinophil count of >3%, with the normal sputum eosinophil count being <2%. This trial studied 61 patients who took their regular medications in addition to the trial medications. Mepolizumab showed a 43% relative risk reduction of severe exacerbations and a modest improvement in symptom scores [22]. Treatment with mepolizumab showed a reduction in the sputum and blood eosinophil counts as seen in previous trials.

Another RCT of mepolizumab was reported in 2009. This trial compared monthly intravenous mepolizumab to placebo over 6 months in 20 patients with severe asthma who were on oral steroid treatment and had a phased withdrawal of oral steroids during the trial period. To be eligible, patients had to demonstrate persistent eosinophilia by way of sputum eosinophils despite high-intensity treatment. This trial showed that mepolizumab reduced blood and sputum eosinophils, and allowed steroids to be reduced significantly compared to placebo. Improvements were also seen in FEV_1. The patients taking mepolizumab treatment were able to reduce their oral steroid dose by a mean of 83.8% compared to 47.7% for those taking placebo [23]. This magnitude of steroid reduction was much larger than seen in the only other monoclonal antibody available for asthma at the time, omalizumab. The Steroid Reduction with Mepolizumab Study (SIRIUS) trial looked specifically at reduction in need for oral steroid treatment [24]. It found that patients treated with mepolizumab were able to reduce their oral steroid dose by a median of 50%, compared to no reduction for those taking placebo. The mepolizumab-treated group in this trial, despite lowering their

steroids, had a lower rate of exacerbations and an improved symptom score compared to the placebo group.

A much larger RCT, the Dose Ranging Efficacy And safety with Mepolizumab in severe asthma (DREAM) trial, was designed to define the dose of mepolizumab and identify the optimum biomarker. The DREAM study was a multicenter, placebo-controlled trial that evaluated three doses of mepolizumab (75, 250, and 750 mg) delivered by monthly intravenous injection. Six hundred and twenty-one patients with severe eosinophilic asthma with a history of exacerbations were randomized with over 150 per treatment arm. To be eligible for the trial, patients needed to demonstrate evidence of eosinophilic airway inflammation as reflected by one or more of the following: sputum eosinophils >3%, blood eosinophils $>0.3 \times 10^9$/l, a FENO >50 ppb, and/or a prompt deterioration in asthma following a 25% reduction in CS treatment. This large study showed a large reduction in the number of severe exacerbations, with ~50% reduction at all doses compared to placebo (Figure 17.4) [25]. Significant reductions in the blood and sputum eosinophil count were seen, and while a dose–response relationship was seen for the latter, no such effect was seen for clinical outcomes and blood eosinophil reductions. Moreover, the study showed that the blood eosinophil count was more predictive of a response to mepolizumab treatment than any other measure, suggesting that the treatment target is circulating eosinophils. The identification of the blood eosinophil level as a predictor of response to mepolizumab treatment is important, as it is easily measurable across all hospitals, unlike induced sputum.

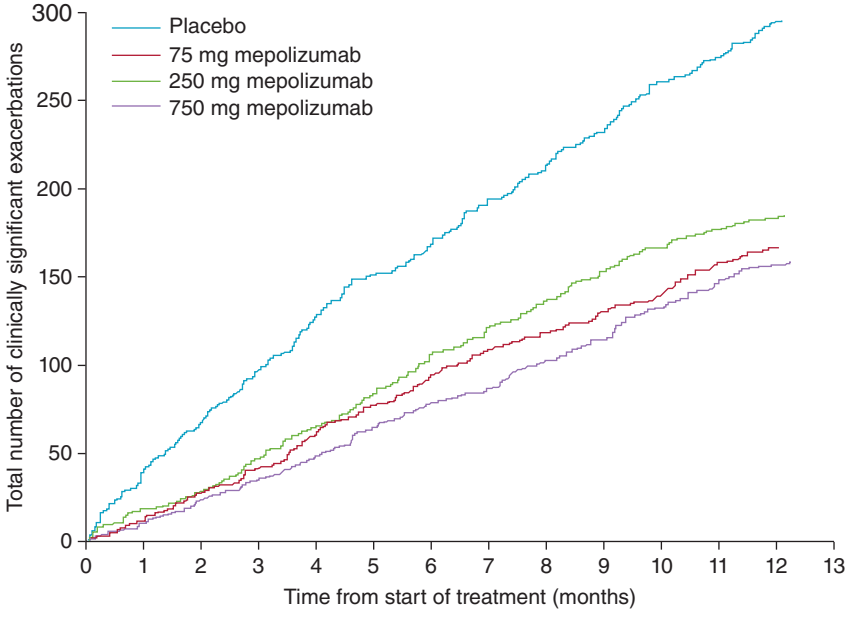

Figure 17.4 Cumulative number of exacerbations in each treatment group – DREAM study, 2012 [25]. Significantly reduced exacerbation rate at all doses of mepolizumab.

The finding that lower doses of mepolizumab were just as effective as higher doses meant that subcutaneous administration was feasible as lower volumes of drug were required. The Mepolizumab as Adjunctive Therapy in Patients with Severe Asthma (MENSA) trial used the key characteristic of the blood eosinophil count to identify eligible patients. Subjects needed to have a blood eosinophil count of $>0.15 \times 10^9$/l at study entry or of $>0.3 \times 10^9$/l in the past 12 months. This study used the lower dose of 75 mg intravenously and compared it with a subcutaneous dose of 100 mg and placebo. This trial confirmed the results of previous trials, with a marked reduction in severe exacerbation rates and a clear relationship between benefit and baseline blood eosinophil count. Unlike in other studies, there was clear evidence of improvement in lung function and symptom scores, potentially reflecting better identification of a treatment-responsive population. Subgroup analysis showed a very large beneficial effect in the 30% of patients, with a blood eosinophil level of $>0.5 \times 10^9$/l. No difference was seen between subcutaneous and intravenous dosing [26]. The efficacy of subcutaneous dosing means that administering the drug is much simpler, as it does not require the placement of an intravenous needle but can be given as a small injection under the skin.

In combination, these trials have shown that mepolizumab is very effective at reducing the risk of severe asthma exacerbations when used in patients with active eosinophilic asthma as shown by the sputum or blood eosinophils. It also allows the reduction in oral steroid dose with no worsening of asthma control. There were no concerns about the safety of the drug, and it was well tolerated. The subcutaneous monthly dosing allows a practical administration schedule. These findings have led to mepolizumab being granted its license for use in severe asthma in 2015.

17.5 Other Anti-IL-5-Targeted Treatments

Reslizumab is another humanized IgG antibody against IL-5 that has recently completed Phase III clinical trials in patients with poorly controlled, moderate to severe eosinophilic asthma. It is delivered via intravenous injection. It showed a similar effect to mepolizumab with a marked reduction in exacerbations [27], although patients did need to have a higher blood eosinophil count to enter the trail when compared to the mepolizumab trials.

Benralizumab is a humanized IgG antibody against the α subunit of the IL-5 receptor. Benralizumab inhibits the proliferation of IL-5-dependant cell lines by binding to this receptor. The drug has also shown increased eosinophil cell death via antibody-dependant, cell-mediated toxicity *in vitro* [28]. These mechanisms result in a rapid eosinopenia (low eosinophil count) following dosing with the drug. Benralizumab is currently undergoing Phase III clinical trials in severe asthma, having shown efficacy in reducing exacerbations in Phase IIb trials over the course of treatment for 1 year [29].

It was noted that eosinopenia occurred rapidly after the first dose of benralizumab and that this effect was sustained for 8–12 weeks [30]. A trial was conducted to evaluate whether a single dose of benralizumab given, alongside usual treatment, at the time of an acute asthma exacerbation reduced the occurrence of further exacerbations. The trial showed that a single dose of benralizumab reduced the rate of exacerbations over the next 3 months by 49% [31]. This finding raises the possibility of using benralizumab as an add-on or alternative to usual treatment with oral steroids for exacerbation. It could be argued that using benralizumab in place of oral steroids may be logical, as the main effect of oral steroids in an exacerbation is reducing eosinophilic inflammation. Further trials would need to be conducted to answer this question.

A summary of anti-IL5 trials programs and their efficacy are shown in Tables 17.2 and 17.3, respectively.

Table 17.2 Anti-IL-5 programs.

		Phase III		Phase II
		Mepolizumab (anti-IL-5)	**Reslizumab (anti-IL-5)**	**Benralizumab (anti-IL-5R)**
Biomarker (cut-off)		Blood EOS ≥ 150/μl at initiation or 300/μl in previous 12 months	Blood EOS (≥400/μl)	Weighted for eosinophilic based on proprietary index or FeNO ≥40 ppb
Baseline biomarker mean		290 ± 1050	590 (100–2300)	530–560
Background therapy		≥880 μg FP/day + another controller	≥440 μg FP/day ± another controller	High/medium-dose ICS + another controller
Baseline patient demographics	Exacerbations required for inclusion (mean observed)	>2 (3.8 ± 2.7)	No requirement for FEV_1 study (55% ≥1 in past year)	2–6 in last year
	FEV_1 pre-BD (ml)	1730 ± 660 (FEV_1 <80% predicted in adults)	2190	Not reported
	Asthma symptoms (ACQ5/6)	2.26 ± 1.27	2.6	3.6–3.8
	Asthma QOL (AQLQ)	Not reported	4.2	3.6–3.8
	Maintenance OCS use % (mean dose, mg)	27% 12.6 (2–50)	Not reported	4–11%
	Age	≥12 years	≥12 years	≥18 years

ACQ , asthma control questionnaire; EOS, eosinophils; FP, fluticasone propionate; ICS, inhaled corticosteroid; QOL, quality of life; TBD, to be determined.

Table 17.3 Anti-IL-5 efficacy and safety.

		Phase III		Phase II
		Mepolizumab (anti-IL-5)	**Reslizumab (anti-IL-5)**	**Benralizumab (anti-IL-5R)**
Efficacy from most recent trial readout	Exacerbation reduction relative to PBO	54% (SC) and 47% (IV) 80% at ≥500 blood EOS (SC)	50–60% (top-line data) at 400 cut-off	57% for 20 mg dose in ≥300 cells/μl group
	FEV_1 improvement (% FEV_1)	98 ml (6%) SC	160 ml (7%) and 270 ml (12%)[†]	0.23 l for ≥300 cells/μl group
	Asthma symptoms (ACQ5/6)	0.44 0.75 in ≥500 PBO-corrected	0.36 PBO-corrected	0.44 PBO-corrected (>300 cut-off)
	Asthma QOL Oral steroid sparing (min 5 mg/day prednisone)	Unknown 50% median reduction in baseline OCS dose	Unknown No OCS sparing study	Not reported Unknown planned for P3
	Expected frequency and dosage	q4w 100 mg	q4w 3 mg/kg	3 × q4w then q8w (30 mg for P3)
	ROA	SC lyophilized powder	IV	SC prefilled syringe
Safety		One potential anaphylaxis case	One case of anaphylaxis related to drug	Balanced

ACQ, asthma control questionnaire; EOS, eosinophils; OCS, oral corticosteroid; PBO, placebo; QOL, quality of life; ROA, route of administration.

17.5.1 Interleukin 4

Given the early involvement of IL-4 in the Th2 pathway, both in response to allergy and in stimulating further Th2 cell production, blocking the effects of IL-4 and therefore its downstream effects is another attractive proposition for treating Th2-high asthma.

Early small clinical trials showed that the soluble recombinant human IL-4 receptor (sIL-4R), altrakincept, had a positive effect in moderate asthma. It is designed to work as a decoy receptor and bind free IL-4. It is delivered via an inhaled nebulized solution. Improvements were seen in lung function, airway inflammation as measured by FeNO, and a reduction in the amount of inhaled medication required [32]. Subsequent trials, however, did not confirm this effect [33].

A monoclonal humanized murine antibody against IL-4, pascolizumab, diminished Th2 cytokine release *in vitro* in human cell lines. A large Phase IIa trial with

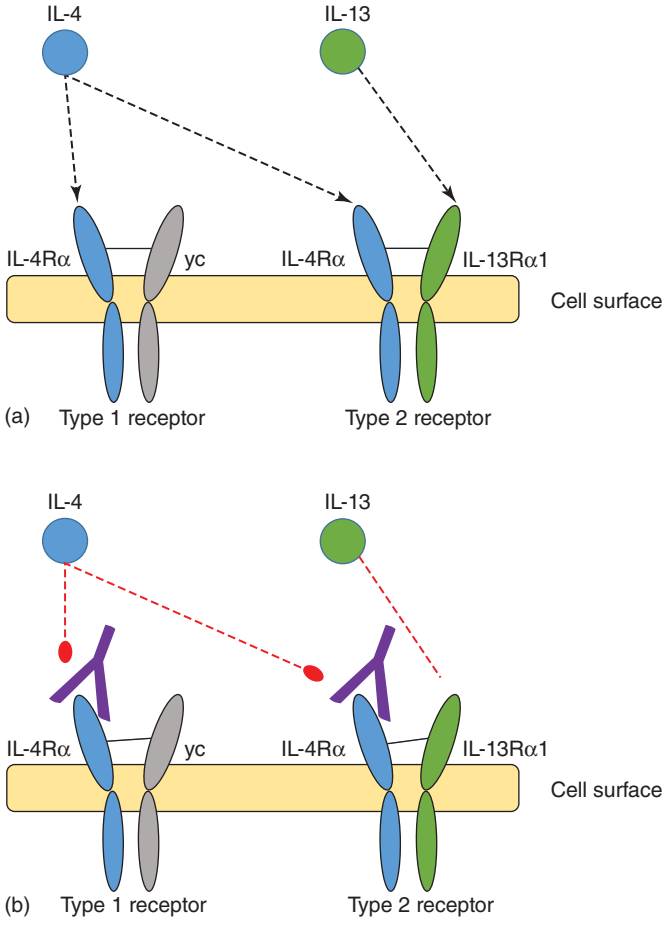

Figure 17.5 (a) IL-4Rα Type 1 and Type 2 receptors. Type 1 comprises the IL-4Rα and common cytokine receptor γ-chain (γc). Type 2 receptor is made from IL-4Rα subunit and IL-13Rα1. IL-4 binds to Type 1 and Type 2 receptors, while IL-13 binds to only the Type 2 receptor. (b) Dupilumab binds to the IL-4Rα subunit of both Type 1 and Type 2 receptors, stopping the binding of IL-4 and IL-13. IL, interleukin.

pascolizumab was, however, stopped early because of a lack of clinical response. It has more recently been shown that sIL-4R can alter the effect of IL-13. The mechanism of this may be by stabilizing the IL-13 receptor due to its proximity and relationship to the IL-4 receptor (Figure 17.5a). This change in the IL-13 receptor can stabilize the IL-13 and IL-13R complex and actually increase the effects of IL-13. This increase in IL-13 activity may explain the lack of efficacy of pascolizumab [34]. There is also redundancy in the Th2 pathway, possibly allowing IL-13 to continue Th2 inflammation despite diminished IL-4.

Dupilumab is a fully human monoclonal antibody that targets the α subunit of the IL-4 receptor (IL-4Rα). There are two types of the receptor with the IL-4Rα

subunit, Type 1 and Type 2 (Figure 17.5a). Type 2 receptor is comprised of IL-4Rα and IL-13Rα1. By blocking IL-4Rα, dupilumab inhibits binding of IL-4 and IL-13 to these receptors [35] (Figure 17.5b).

A placebo-controlled Phase IIa RCT of dupilumab has been conducted in patients with moderate to severe asthma with evidence of eosinophilia, as demonstrated by a sputum eosinophil count of >3% or a blood eosinophil level of 0.3×10^9/l or higher. The trial started with all patients taking a combination of ICS and LABA with the addition of subcutaneous dupilumab or placebo injections weekly. After 4 weeks of treatment, the LABA component was stopped, and the ICS dose was tapered over the following 4 weeks, with patients continuing on either dupilumab or placebo therapy. This trial showed an 87% reduction in exacerbations on dupilumab compared to placebo. FEV_1 improved as a percentage of the predicted value with dupilumab. Nocturnal symptoms and asthma symptom scores also improved with dupilumab treatment to a greater extent than had been seen with any other monoclonal antibody asthma therapy. This may be because of its effect of blocking two relevant cytokines. FeNO and serum IgE reduced markedly on dupilumab, but the peripheral blood eosinophil count increased on treatment [36]. This suggests that it has an effect on eosinophil movement into the airway but not on the production of eosinophils, which is not unexpected as the latter is an IL-5-dependant process. Whether the treatment-associated increase in blood eosinophils is clinically important is unclear. A recent dose-ranging Phase IIb trial of dupilumab has shown efficacy in increasing lung function and decreasing exacerbations in subjects with moderate to severe asthma [37]. An analysis by blood eosinophil level showed efficacy in subjects with blood eosinophils both greater than 0.3×10^9/l and less than 0.3×10^9/l, with the largest effect seen in the former. Large Phase III trials are currently under way in patients with asthma.

Dupilumab has also completed clinical trials in atopic dermatitis, another condition with an IgE-related eosinophil response, and has shown a marked improvement in clinical features and a reduction in serum IgE [38].

17.5.2 Interleukin 13

Lebrikizumab is a humanized monoclonal antibody against IL-13. An RCT studying lebrikizumab in patients with moderate to severe asthma showed only a modest improvement in lung function and some reduction in FeNO [39]. Subgroup analysis revealed that the improvements in lung function, exacerbation rate and airway inflammation were more marked in patients who had high levels of FENO and periostin, both biomarkers of IL-13 activity.

Two replicate design RCTs were conducted to further evaluate lebrikizumab in patients with asthma that was uncontrolled despite high-dose inhaled medication. Four hundred and sixty-three patients were randomized to one of three doses of subcutaneous lebrikizumab or placebo. Randomization was stratified by the baseline serum periostin level, exacerbation history, and current medications.

In patients with a periostin level $\geq$50 ng ml^{-1}, lebrikizumab reduced exacerbations by 60%, compared to a 5% reduction in patients with a periostin level <50 ng ml^{-1}. Improvements in FEV_1 of 9.2% and 2.6% were seen in the periostin-high and periostin-low groups, respectively. Treatment with lebrikizumab caused a larger drop in the serum periostin, blood eosinophil count, and FeNO in the periostin-high group than in the periostin-low group. No significant change in symptoms was seen [40].

Another anti-IL-13 drug, tralokinumab, has also been evaluated in patients with severe, uncontrolled asthma with a history of exacerbations. In this study, patients were randomized to either tralokinumab or placebo treatment, twice weekly or four times weekly. This study evaluated potential biomarkers of response and measured periostin as well as dipeptidyl peptidase-4 (DPP-4). DPP-4 is a gene that is highly induced by IL-13 in normal and asthmatic airways. The median periostin for all patients within the trial was ~22 ng ml^{-1}. Overall, no difference was seen in exacerbations, lung function, or asthma symptoms between placebo and the active medication, or between the dosing schedules. Subgroup analysis showed that patients who had high levels of DPP-4 or high levels of periostin did show improvements in lung function with two weekly dosing compared to four weekly dosing or placebo. These improvements were not seen in DPP-4-low or periostin-low groups [41].

Phase III trials for both these anti-IL-13 drugs are currently under way, prospectively targeting subjects with biomarkers of high IL-13 activity. LAVOLTA I and LAVOLTA II, two identical Phase III trials of lebrikizumab in subjects with severe asthma, have just concluded. The results are currently unpublished, but a press release from the pharmaceutical company reports that one study met its primary end point, showing that lebrikizumab significantly reduced exacerbations, but the second study did not meet this primary end point. The full analysis is to be published in due course.

Collectively, these Th2-targeted therapy trials have shown a response to treatment that is related to the peripheral blood eosinophil count. It is clear that this simple biomarker is key to stratifying patients into responders and nonresponders. What is currently less clear is the optimum blood eosinophil level cut-off to make this stratification. It has been shown that exacerbation risk has a direct relationship with the blood eosinophil level, with higher values conveying a higher risk. Recent post hoc analysis of large trials of ICS/LABA combinations have also shown an increasing risk of exacerbations with increasing blood eosinophil percentage [42]. In this analysis, exacerbation risk was reduced by inhaled corticosteroid/LABA combination in subjects with raised blood eosinophil percentage (>2% of the absolute white cell count), but not in those with a lower blood eosinophil percentage. Ongoing analysis of these trials, stratified by the blood eosinophil count, is required to determine whether there is an optimum cut-off for all Th2 medications, or whether the cut-offs used will need to be treatment-specific. These trials should also assess the possibility whether the relationship between clinical effects of cytokine blockade and biomarker differs. For example, anti-IL-13 and anti-IL-4 and 13 reduce FeNO markedly (Table 17.4), and FeNO might be a better biomarker

Table 17.4 Effects of different cytokine blockers on clinical measures and biomarkers.

	Effect on clinical measures				Effect on biomarkers			
	FEV_1	Symptoms	Exac	PC_{20}	Bleos	Speos	FeNO	IgE
Oral steroids	+	+	++	++	↓↓	↓↓	↓↓	↓
Anti-IL-5	0/+	0/+	++	0	↓↓	↓↓	←→	←→
Anti-IL-13	+	+	++ (?)	0 (?)	↑	↓	↓↓	↓
Anti-IL-4/13	+	+	++ (?)	0 (?)	↑	↓	↓↓	↓↓
Anti-IgE	+	+	+	0	←→	↓	↓↓	↓

FEV_1, forced expiratory volume in 1 s; Exac, exacerbations; PC20, test of airway hyperresponsiveness; Bleos, blood eosinophils; Speos, sputum eosinophils; FeNO, fractional exhaled nitric oxide; IgE, immunoglobulin E; (?) early evidence, further confirmation required.

of response to these agents whereas it is not responsive to anti-IL-5 and is not a good biomarker of response to mepolizumab in the DREAM study (Table 17.4). The future might be to sub-stratify type-2-high asthma based on biomarker profiles.

17.5.3 Interleukin 9

IL-9 enhances the proliferation and activity of a number of cell types involved in Th2 response when studied *in vitro* [43]. This led to the development of an anti-IL-9 monoclonal antibody, MEDI-528, which has been studied in patients with uncontrolled moderate to severe asthma. Although well tolerated, it was not associated with an improvement in lung function, exacerbation rates, or patient symptom scores [44].

17.6 Other Respiratory Uses of Monoclonal Antibodies

Although the majority of targeted protein therapies in respiratory medicine so far have been asthma-related, there are a number of other potential uses.

There is an unmet need for effective therapies in patients with inflammation of the airways but do not have Th2-mediated disease. Further understanding and research of the underlying mechanisms in these patients may yield new targets, or identify which subgroups of patients may benefit from existing and previously trialed therapies.

COPD is a primarily smoking-related inflammatory disease of the small airways, which can have both Th1- and Th2-type inflammation. A Phase IIa trial of the anti-IL-5R benralizumab in patients with COPD who have had previous exacerbations has shown that it may have a beneficial effect on lung function in a subgroup of

patients who have a raised blood eosinophil count [45]. Further trials are under way to investigate this subgroup of patients.

Patients with idiopathic pulmonary fibrosis (IPF), a progressive inflammatory lung condition, have been found to overexpress IL-13 in the lung, which is thought to lead to excess fibroblast proliferation and resultant damage [46]. Lebrikizumab, which has been shown to have an effect in serve asthma, is being investigated in patients with IPF in a large Phase IIb trial.

Churg–Strauss syndrome is an eosinophilic condition that causes inflammation of small to medium-sized blood vessels and can affect the lungs, skin, and other organs. It is diagnosed by biopsy of the affected area, which shows microscopic inflammation of the blood vessels. It is associated with a markedly raised blood eosinophil count. Churg–Strauss is a condition that is very responsive to treatment with steroids. Anti-IL-5 treatment with mepolizumab is being trialed in patients with this condition, as it has been found to deplete blood eosinophil levels in patients with asthma.

Monoclonal antibody therapies are used in the treatment of lung cancer, which are covered in another chapter.

17.7 Summary

The development of these new targeted medicines is promising and offers treatment alternatives to patients with severe Th2-high asthma who currently remain uncontrolled and suffer the side effects of long-term oral steroid treatment.

It is clear that the presence of eosinophils, either in the blood or in the sputum, is a key biomarker for identifying Th2-high asthma. The various monoclonal antibodies available have differing target molecules that are involved in the development of Th2 inflammation. Their effects on clinical and biological markers are shown in Table 17.4. A priority is to now identify which patients are most likely to benefit from which therapy.

An important consideration in appropriately identifying patients is the cost, with treatment with omalizumab costing upwards of £20 000 per patient per year depending on the dosage and frequency. The cost of the individual emerging monoclonal antibody therapies is not yet known, but will be substantial when compared to current standard inhaled treatments and oral steroids.

Two of the discussed emerging monoclonal antibody therapies are likely to become available for clinical use during 2016. The anti-IL-5 drug mepolizumab is very effective in reducing severe exacerbations, and this effect seems to be more pronounced in patients with higher blood eosinophil counts. Mepolizumab has modest effects on lung function and asthma symptom scores. The ideal patient population for this drug would be those who have high blood eosinophil counts and a history of severe exacerbations but are not troubled by frequent symptoms. Dupilumab, which blocks IL-4 and IL-13, seems to have a greater improvement

in symptoms than mepolizumab with a similar reduction in exacerbations and also has an effect on allergic dermatitis and nasal polyps – both conditions that frequently coexist with asthma because of their similar underlying pathologies. The presence of troublesome symptoms or these coexistent diseases may make dupilumab a more attractive option for this group of patients.

The measurement and interpretation of biomarkers is essential not only for the identification of patients with Th2-high disease but also for tailoring specific treatments. It is likely that, due to the highly heterogeneous nature of airways disease, there will be a number of clinically relevant patient groups within the Th2-high population, for example, predominantly IL-5-high disease and predominantly IL-13-high disease. In the near future, we may well be categorizing patients by the relevant underlying pathological mechanisms rather than the resultant inflammation, and targeting treatment accordingly.

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18
Antibodies for the Prevention, Treatment, and Preemption of Infectious Diseases

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18.1
Prophylaxis and Precision Medicine

The advent of the "age of antibiotics" in the 1940s had a major impact on extending lives but it also brought with it a false sense of security and led to an overenthusiastic use of antibiotics, sometimes as a substitute for good sanitation and hygiene. We now know that resistance to these important agents can and does arise and this may have dire consequences for patients. Finding new ways to prevent and treat infections caused by resistant organisms will be a major challenge in the twenty-first century. Identifying "at risk" patients, for example, those colonized with *Staphylococcus aureus*, and taking preemptive measures such as decolonization or perhaps developing a protective vaccine may well obviate the use of the antibiotics that have made the practice of medicine in the late twentieth and early twenty-first centuries possible. A new term of art has been coined "Precision Medicine" and we now have available (although not routinely employed) the ability to identify an infecting pathogen within 2 h, if not less. This should allow for the use of narrow-spectrum agents which, in and of themselves cannot obviate either toxicity or the development of resistance, but may well prevent cross-resistance to current antibiotics as well as ameliorate, if not prevent, horizontal gene transfer. In addition to the continued hunt for effective vaccines, currently there are multiple monoclonal antibodies in clinical development to prevent and/or treat infections caused by *Pseudomonas aeruginosa, S. aureus, Clostridium difficile* and others. To date, these monoclonal antibodies, mainly of human origin or design, have had an enviable safety record as may well be predicted, and these may represent the future of antibacterial treatments.

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18.2
Antibacterial Immune Therapy – A Nineteenth Century Breakthrough

Emil von Behring pioneered the use of sera derived from inoculated animals as a treatment for infections, with diphtheria being the first bacterial infection so treated. For this groundbreaking work, von Behring won the first Nobel Prize for Physiology or Medicine in 1901, and then 7 years later his former associate Paul Ehrlich (sharing the prize with Ilya Metchnikof) also won the award for his contributions to immunotherapeutics for infection [1]. The importance and utility of serum therapy was dramatically demonstrated in 1925 when Nome, Alaska, suffered an outbreak of diphtheria and a "Great Race of Mercy" was undertaken to deliver, by a relay of dog sled teams, anti-diphtheria antiserum from the railhead, at Nenana near Fairbanks, 674 miles west of Nome. In just over 5 days, the final leg of the relay, with musher Gunnar Kaasen and lead dog Balto, delivered the antiserum to Dr. Curtis Welch without a single broken ampule through some of the worst winter weather Alaska had seen in two decades. While there were reportedly only seven deaths, it is not clear how many of the Inuit (native Alaskans) actually perished in the epidemic but it was clear that the antiserum played a large role in bringing the epidemic to an end and underscored the importance of drug delivery (then and now).

18.2.1
Why Has It Taken So Long for Novel Immunotherapeutics?

In 1995, Casadevall and Scharff called for novel antibody-based therapies for infectious diseases in what many, even then, referred as a "back-to-the-future" approach [2]. Dr Casadevall himself has been directly involved in the discovery and development of such agents and continues to be one of its chief proponents. It was a mere 13 years from the publication of Fleming's discovery of a Penicillium mold that could lyse *S. aureus* to the first successful therapeutic use of penicillin; more importantly this began a period of natural product research that brought us many new, broad-spectrum antibacterial agents from the aminoglycosides (kanamycin, streptomycin) to the tetracyclines to the macrolides (erythromycin) to the rifamycins all within the 15 years since Anne Sheafe Miller was cured of her Group A streptococcus infection by penicillin treatment in April of 1942. In the 21 years since Casadevall and Scharff proposed the use of novel immunotherapeutics for infectious disease not a single monoclonal antibody has been used clinically in the infectious disease space. However, today's picture is far brighter, perhaps necessitated by the continued and inexorable increase of microbial drug resistance as well as improved methodologies in both target identification and antibody discovery, not to mention the continued futility in the discovery and development of the next generation of broad-spectrum antibiotics [3]. There are several novel agents currently in clinical development, and many more in preclinical development. Moreover, we are starting to see a hint of efficacy for those agents in addition to published positive results in animal models of infection.

18.2.2 Monoclonal Antibodies for the Prevention and Treatment of Viral Infections

To date, the first, and still the only, monoclonal antibody used for the treatment of an infectious disease is palivizumab for the prevention of respiratory syncytial virus (RSV) infection in high-risk infants [4], who are primarily premature infants of less than 29 weeks gestational age. Because of the seasonality, usually during the colder months and over a 5-month period, of RSV infection, palivizumab is mostly used during the RSV "season" and dosed on a monthly basis. Currently, two "next-generation" mAbs, also targeting RSV, are in late-stage development. These are REGN2222 and MEDI8897, both of which may allow for less frequent dosing, perhaps once or twice during the RSV season, and therefore may expand upon the utility of the mAb approach to prophylactic treatment of a wider population including term infants [5, 6].

Other respiratory viruses that are potential targets for monoclonal antibody prevention or treatment include influenza (both A and B strains). Because influenza A causes about 70% of the influenza seen annually with year-to-year variation, and generally can cause a more severe disease, including mortality seen mostly with H3 serotypes, mAbs targeting Influenza A strains, including and especially pandemic strains, have been prioritized and several are currently in clinical development (all for treatment of diagnosed influenza) [7]. To date, clinically efficacy has not been established. Some of the clinical testing, using "challenge" studies have produced equivocal results. In these studies, attenuated strains of influenza infect otherwise healthy volunteers with disease progression and viral load followed. The issues with these studies are that what is observed is mainly upper (rather than lower) respiratory disease, that is, more like a common cold than influenza and that the infection is virtually self-limiting. Therefore, it is difficult to see a therapeutic effect even with currently approved anti-influenza drugs. The "real" patient population will be those people hospitalized with diagnosed influenza A. Reduction in hospital stay would be a key outcome as would be prevention of secondary bacterial pneumonias, which is an important cause of influenza-related mortality. As indicated, there are currently several mAbs in development for the treatment and possible prevention of influenza (both A and B). In many instances, these fully human mAbs were discovered either from postconvalescent patients or vaccinated subjects, by harvesting B cells and identifying potent antibodies [8]. Most interestingly, the antibodies appear to have fairly broad coverage across serotypes maintaining a high degree of potency. Finally the open question is whether these monoclonal antibodies will be superior to the current marketed neuraminidase inhibitors zanamivir and oseltamivir, superiority in animal models of influenza infection notwithstanding.

Monoclonal antibodies currently in clinical development include: RG7745 (Genentech), VIS410 (Visterra), CT-P27 (Celltrion) all in Phase 2; MEDI8852 (MedImmune) and MHAB5553A (Genetech) both in Phase 1 (Table 18.1).

Table 18.1 mAbs in development.

Organization	Target organism	Name or number	Status as of Q2 2016
Merck	*C. difficile*	Bezlotoxumab	3 (complete)
MedImmune	*S. aureus*	MEDI4893	2b
Aridis	*P. aeruginosa*	AR-101	2a (complete)
Aridis	*S. aureus*	AR-301	2a
MedImmune	*P. aeruginosa*	MEDI3902	2b
XBiotech	*S. aureus*	514G3	1
Arsanis	*S. aureus*	ASN100	1
Genentech	Influenza A	MHAA4549A/RG7745	2b
Genentech	Influenza B	MHAB5553A	1a
Visterra	Influenza A	VS410	2a
Celltrion	Influenza A	CT-P27	2a
MedImmune	Influenza A	MEDI8852	2a
Regeneron	RSV	REGN2222	3
MedImmune	RSV	MEDI8897	2a

18.2.3
S. aureus: No Longer the Hospital Scourge?

Recent data published by the Center for Disease Control and Prevention (CDC) and the Veterans Administration suggests that there has been a precipitous decline in rates of hospital infections due to methicillin-resistant staphylococcus, for example, approximately a 50% drop between 2006 and 2011 [9]. However, this drop in the United States is not consistent with the observation that linezolid (the antibiotic used for methicillin-resistant gram-positive bacterial infections) sales have continued to increase both in the United States and worldwide and, even more dramatically, another methicillin-resistant *Staphylococcus aureus* (MRSA) drug, daptomycin, has seen sales in the United States go from $189 million in 2006 to $977 million in 2014 [10]. It is hard to rationalize a 50% drop in infection rates with a 517% increase in sales for a drug whose primary utility is for MRSA infection. There are several ways to look at this apparent paradox but a likely explanation is either an aggressive prophylaxis in high-risk patients or aggressive empiric therapy where an infection is suspected but as yet undiagnosed. In either event, this would represent a significant change in how anti-staphylococcal drugs are being used and suggests an important role in the use of specific agents aimed at preventing fulminant staphylococcal infections.

18.2.4
Monoclonal Antibodies to Prevent, Treat or Preempt Staphylococcal Infections

There are currently at least three mAbs in clinical development that target *S. aureus* alpha toxin. AR-301 (Aridis) and MEDI4893 (MedImmune/AstraZeneca)

have both been shown to neutralize alpha toxin (formerly known as alpha hemolysin), a virulence factor that has multiple activities and is key in establishing lung and skin infections [11]. In addition to these two mAbs, Arsanis, a Vienna-based biotechnology company, has initiated a Phase 1 study of ASN100 a combination of two mAbs that, in addition to alpha toxin, also neutralize five other secreted toxins via binding to a related structural motif [12].

SAR279356 (F598) (Sanofi/Alopexx) is a mAb directed toward a surface carbohydrate alternatively called PNAG (poly *N*-acetyl glucosamine) or PIA (polysaccharide intercellular adhesion). Since PNAG is also found on the surface of coagulase negative staphylococci (e.g., *Staphylococcus epidermidis*), this mAb may have broader utility as *S. epidermidis* is an important pathogen in high-risk neonates. It is currently listed as in Phase 2.

It is not currently clear what the optimum use will be for these mAbs: whether it be treatment in combination with antibiotics (i.e., "adjunctive therapy"), prevention of staphylococcal diseases in high-risk patients, for example, dialysis patients, or "preemption" by identifying patients, perhaps mechanically ventilated patients in intensive care units, who are colonized but asymptomatic. The latter would also require a rapid, point-of-care diagnostic. Encouragingly, the preclinical data for all of these mAbs suggests that all of the above may be possible.

Less has been disclosed on XBiotech's Phase1/2 mAb "514G3" derived from a postconvalescent patients and apparently targeting staphylococcal protein A, which, by binding the Fc portion of IgG antibodies, helps *S. aureus* evade adaptive immune response.

18.2.5 *P. aeruginosa*: The Bacterial Cockroach

P. aeruginosa is not usually associated with infections in otherwise healthy individuals, rather those with compromised immune systems, other underlying disease (e.g., cystic fibrosis), trauma and/or burns patients, patients in intensive care units, and especially those receiving mechanical ventilation who are prime targets for this ubiquitous bacterium. Because *P. aeruginosa* is so adaptable to its environment, it is intrinsically resistant to many antibiotics and, probably owing to its large (6.3 Mb) genome, it can often become resistant to virtually any novel agent in a matter of days to weeks. To state that novel approaches to the prevention and treatment of pseudomonal infections are urgently needed is certainly an understatement.

KB001-A (Kalabios) is a pegylated Fab targeting PcrV, a protein that is best described as the tip of the *P. aeruginosa* Type 3 secretion system [13]. As a Fab, KB001-A can block the secretion of toxins through the "injectisome," thereby reducing the virulence of the bacterium but, lacking an Fc (and therefore effector function), KB001-A would not lead to a more rapid clearance of the bacterium and is therefore more suitable as a prophylactic rather than a therapeutic. Currently, KB001-A appears to be on hold as it may have to have its dose adjusted upward.

Medi3902 (MedImmune/AstraZeneca), also targeting PcrV, is a bispecific mAb which simultaneously targets Psl (a manose-rch surface polysaccharide) [14]. This mAb has been shown to retain effector function as well as synergize with antibiotics (even vs drug-resistant strains). This mAb has recently started clinical development.

"Aerucin," an IgG1 mAb that reportedly binds to alginate, has completed Phase 1 studies by Aridis, which also is developing an IgM mAb specific for *P. aeruginosa* serotype 011. Both are being developed for hospital-acquired pneumonias [15].

18.2.6
Immune Evasion: A Bridge Too Far?

Just because serum therapy has worked for several diverse pathogens does not necessarily mean that this technology is broadly applicable to all bacterial or viral pathogens. It is telling that in Casadevall and Scharff's scholarly paper [2], none of the serum therapeutics listed were for either staphylococcal or pseudomonal infections and also telling is that most of the bacterial and all of the viral infections for which serum therapies were developed are diseases that today are preventable by vaccination, whereas repeated attempts to develop vaccines to prevent staphylococcal or pseudomonal infections lay somewhere between bitter disappointments and abject failures. Of the 300–400 virulence factors encoded on the *S. aureus* genome, the large majority of them are likely to have a role in immune evasion. Work by Schneewind and colleagues at the University of Chicago has been targeting perhaps the keystone of staphylococcal immune evasion, Protein A, with positive preclinical results [16].

18.2.7
Monoclonal Antibodies for *C. difficile* Infection: "A Winning Bet or a Crap Shoot"

A disease that arises as a consequence of a therapy is called "iatrogenic" and *C. difficile*-associated diarrhea is just such a disease. It is most often associated with elderly patients in the healthcare system who are receiving antibacterial treatments. It is likely that perturbations in the patient's gut microflora allow for the germination of *C. difficile* spores and this bacterium then expresses two related toxins that give rise to pseudomembranous colitis, a potentially life-threatening condition.

A project begun by MassBio and Medarex and subsequently licensed by Merck has resulted in a pair of monoclonal antibodies which neutralize the A and B toxins of *C. difficile* (TcdA, TcdB) and has been shown clinically to prevent relapse [9]. After completion of Phase 2 studies, Merck has disclosed that only the mAb neutralizing TcdB ("Bezlotoxumab") demonstrated clinically significant activity in the prevention of relapse. This clinical finding is somewhat inconsistent with preclinical animal models and may possibly reflect a lack of potency for the TcdA-neutralizing monoclonal that was used in the studies [17].

For a recent review of biologic drugs in development, see Ref. [18]."

18.2.8
Are Two Antibodies Enough; Is Six Too Many?

In addition to the *C. difficile* combination tested by Merck, other combination approaches are being studied both clinically and preclinically. Aside from the Arsanis staphylococcal combination discussed above, Symphogen, a Danish Biotech, discovers and develops recombinant antibody mixtures for therapeutic use and lists Sym009 as "an undisclosed infectious disease target funded by Genentech" on their website.

18.2.9
Prophylaxis or Treatment? Beware of False Dichotomies

A point raised above is the question about how these agents should be used: as a potential treatment post infection or in a. prophylactic setting. The view here is that either approach is viable but pricing would be quite different depending on the use (treatment being more expensive). The current thinking is that prophylaxis would be a "lower bar" for the demonstration of clinical utility but would likely require larger clinical trials than for therapeutic use.

18.3
Other Potential Anti-infective mAbs

In addition to palivizumab for RSV (discussed above), there is a second approved monoclonal antibody in the infectious disease space and that is an anti-anthrax mAb called *raxibacumab* (GlaxoSmithKline by way of Human Genome Sciences/Cambridge Antibody Technology). Raxibacumab targets the protective antigen of *Bacillus anthracis* and has only been shown to have *in vivo* efficacy in animal models of anthrax [19]; it was approved under the so-called animal rule (for select agents) and has been stockpiled by the US government. A similar approach is currently ongoing for identifying and developing mAbs that are active against Ebola virus, an example of which is a cocktail of three chimeric mAbs called ZMapp [19] which is produced in tobacco plants, probably an impractical production method in the longer term. However, the general strategy of harvesting B cells from postconvalescent patients is currently being employed to find more potent mAbs that demonstrate efficacy in primate models [20].

18.3.1
Safety: Human Enough for You?

The irony of the replacement of serum therapy by antibiotics was that the antibiotics were perceived to be safer owing to the early days of serum therapy when "serum sickness [21]" was not uncommon and most likely due to an immune

reaction to foreign, mainly equine, protein but probably not to the antibodies in the serum as the structural similarity between human and equine antibodies is quite high. It should be noted that, today, several chronically administered monoclonal antibodies are chimeras and, even with the development of antidrug antibodies, rarely does this cause serious adverse events. Therefore, the foreign proteins that led to serum sickness were probably not the antibodies in the serum. Indeed, even during the time of Paul Ehrlich safer and safer preparations were being manufactured and by the mid-1930s serum sickness was rare. Today's ultrapure preparations of monoclonal antibodies, which are "fully human" in nature are likely to be safer still. Unlike small molecule drugs, there is little likelihood of drug–drug interactions with other therapeutics agents and, as such, they would also be suitable for "adjunctive therapy" in combination with other drugs, even broad-spectrum antibiotics.

18.3.2 Another Precinct is Heard from: Immunomodulatory Agents for the Treatment of Chronic Infections

A conundrum of chronic infections is why our immune system does not rise up and kill the infected cells rather than allowing for these "zombie cells" to harbor bacteria (e.g., *Mycobacterium tuberculosis*) or viruses (e.g., HBV, HIV) where the pathogen can hide, or worse replicate. Recent work by Michael Starnbach at Harvard has demonstrated that the obligate intracellular bacterial pathogen *Chlamydia trachomatis* unregulates the host cell's PD-1 pathway [22]. PD-1 ("programmed death") is a receptor for an immune checkpoint inhibitor, a fail-safe system to prevent autoimmune disease, and as such effectively blocks an immune counterattack upon the affected cells. In oncology, immune modulation is emerging as a very productive area of research where the potential for actual cures is emerging. It is possible, if not likely, that these agents may well be active against the chronic infections discussed above.

18.3.3 Are We There Yet? Easy-to-Use, Fast-Turnaround, Point-of-Care Diagnostics

As bacterial and viral-specific antibodies and other, similarly targeted anti-infectious agents enter clinical practice, they are going to necessitate, in parallel, the development of companion diagnostics that are easy to use, with a modest unit cost per diagnosis, and a sampling to result time of under 2 h. A platform using polymerase chain reaction (PCR) coupled with molecular beacons, such as the GeneXpert® system developed by Cepheid appears to be capable of delivering with ease and on time, with an admirable degree of flexibility, while the question of cost remains an open one [23]. Other technologies, such as MALDI-TOF mass spectroscopy, are promising but reducing them to practice will require a significant amount of additional research and development.

18.3.4

Are Biologic Drugs Going To Be Too Expensive to Treat Infections?

It is an increasingly recognized fact that broad-spectrum antibiotics, which, by virtue of that broad spectrum, facilitated the aggressive and empiric use of these drugs. But there is another important fact, also becoming realized, that antibiotics are grossly underpriced in relation to their effectiveness. Even those still protected by patents and/or data exclusivity most often cost less than $1000 for a course of therapy which in most instances results in a complete cure of the infection. In contrast, oncology drugs, that extend patients' lives for as little as 2 months can cost upward of $100 000. Ironically, oncology patients also often require antibacterial therapy by virtue of the immunosuppressive nature of many oncology therapies. That being stated, in today's resource and/or financially constrained environment, virtually no drug will gain wide acceptance, much less routine use, if it does not have a pharmacoeconomic advantage over standard of care.

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19
Rescue Therapies

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19.1
Introduction

There are clinical situations in which an immediate reversal of the action of a medical treatment is required. This applies to intoxication, such as accidental overdose, or when serious side-effects occur. Sometimes, a medical treatment can also interfere with appropriate management of a clinical emergency situation, requiring the action of the medicine to be turned off. In such situations, administration of an antidote or reversal agent can be life-saving. Conversely, animals such as snakes or scorpions, as well as microorganisms, can be the source of potentially life-threatening intoxications. Specific antivenoms and antitoxins can rescue the lives of people who have been exposed to such poisons. The present chapter aims to provide a selection of examples of rescue therapies with therapeutic proteins in clinical use.

19.1.1
Clinical Development Peculiarities

The clinical development program of rescue medications often does not follow the typical design of "full-development" programs for new medicines. Probably the most striking difference is that only data from patient case studies/patient case series trials instead of traditional confirmatory Phase III studies may be available. Placebo-controlled trials are frequently not conducted. This can be due to the rarity of some emergencies for which these rescue medications are indicated. Logistical challenges in identifying appropriate patients as well as ethical considerations, such as denying patients with life-threatening conditions the rescue medicines that proved highly effective in preclinical settings, account for additional complexity.

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Authorities to some extent appear to appreciate the value of these medications as well as the associated challenges. For example, new reversal agents for direct oral anticoagulants, including the specific dabigatran reversal agent idarucizumab (see the following), were granted breakthrough designation by the FDA and accelerated approval processes have been granted by various regulatory agencies. The design of clinical development programs for these agents was discussed in a dialog including academia, industry, and members of regulatory authorities. The participants proposed that initial regulatory review and conditional approval for these new medicines could primarily be based on healthy volunteer data, supported by only a limited data package obtained in patients [1]. Such regulatory framesetting also enables rapid clinical development processes. Remarkably, the first clinical study for idarucizumab was initiated in the second half of 2012 and regulatory approval by the US FDA and the European EMA was granted approximately 3 years later [2, 3].

19.2 Antidotes/Reversal Agents

19.2.1 Introduction

Antidotes are traditionally used to reduce or even abrogate the toxic effects of poisons, toxins, or other substances that have a dangerous effect on an organism. Antidotes belong to a variety of substance classes and have distinct modes of action, including tight binding of the toxic substance, chemical modification to a less toxic substance, displacing the toxic substance from its target in the body, or enhancing its elimination from the body. A considerable fraction of antidotes are small molecules and as such not in the focus of this book, which focuses on proteins. Clinical applications of proteins designed to reverse the action of a certain agent include emergencies due to overexposure to a drug with accompanying toxicities, as observed with the narrow-therapeutic index compound digoxin or the anticancer treatment methotrexate. On the other hand, even the intended action of a drug can cause challenges in critical clinical situations. Anticoagulant drugs interfere with normal blood coagulation and are used in millions of people worldwide to prevent thromboembolic events. In rare emergency situations, immediate restoration of normal hemostasis can be achieved with respective reversal agents.

Tables 19.1 and 19.2 provide examples of protein-based reversal agents by mode of action.

19.2.2 Anti-digoxin Fab

19.2.2.1 Background

Digoxin is a cardiac glycoside derived from *Digitalis lanata* [12]. It is "indicated for the treatment of mild to moderate heart failure and for the control of resting

Table 19.1 Antidotes/reversal agents acting through high-affinity binding.

Reversal agent; company	Target	Type and origin	Phase
Digibind®; GlaxoSmithKline DigiFab®; BTG DigiDot®; Roche	Digoxin	Polyclonal, ovine anti-digoxin Fab	Launched [4]
Praxbind® [5]; Boehringer Ingelheim	Dabigatran	Humanized anti-dabigatran Fab	Launched [6]
Andexanet alfa; Portola	Rivaroxaban, apixaban, edoxaban	Recombinant modified human Factor Xa decoy protein	Submitted for regulatory approval [7]
IXT-m200; InterveXion	Methamphetamine	Chimeric anti-methamphetamine monoclonal antibody	Phase I [8]

Table 19.2 Antidotes/reversal agents acting as degrading enzymes.

Reversal agent; company	Target	Type and origin	Phase
Voraxaze® [9]; BTG	Methotrexate	Recombinant carboxypeptidase G2	Launched [9]
Protexia®; PharmAthene	Nerve gas	Recombinant human butyrylcholinesterase	Phase I [10]
TV-1380; Teva	Cocaine	Recombinant monomeric albuminated butyrylcholinesterase	Phase II [11]

ventricular rate in patients with chronic atrial fibrillation" [4]. Digoxin has a narrow therapeutic index; "toxicity can occur at levels of digoxin only slightly exceeding therapeutic levels" [4]; and mortality in severe digoxin poisoning is high. Emergency management of acute cardiac glycoside poisoning comprises decontamination with activated charcoal, cardiac pacing, electrical cardioversion as well as the administration of antidotes, including anti-digoxin Fab [13]. As the oldest protein-based antidote still used in clinical practice, anti-digoxin Fab is presented first in this chapter.

19.2.2.2 Mode of Action

Anti-digoxin Fab acts through tight binding of digoxin. Its binding affinity to digoxin is higher than the affinity of digoxin for the Na^+-K^+ ATPase, its target receptor [14]. The resulting stable Fab–digoxin complex thereby sequesters free serum digoxin in plasma [15] and thus reduces its interaction with other structures in the body.

Initially, anti-digoxin whole antibodies were generated from sheep [16] and used to demonstrate dampening of digoxin toxicity in animals [17]. However, whole

antibodies have some challenging properties with respect to their use as rescue medicine for digoxin. Owing to their tight binding to digoxin and long half-life in plasma, they interfere with digoxin elimination from the body. Eventually, this may result in the release of large quantities of digoxin back into circulation when the antibody–digoxin complex is degraded [15]. Additionally, whole antibodies convey the potential for (unwanted, in this case) Fc-mediated immunologic actions (for details refer to Chapter 3). These challenges were overcome by the generation of anti-digoxin Fab preparations, achieved via papain digestion of the whole antibodies and subsequent separation and purification of the Fab fraction [4].

19.2.2.3 Studies in Volunteers

A comparison of the pharmacokinetics of two anti-digoxin Fab products (DigiTab vs Digibind) was conducted in the presence of 1 mg digoxin [18]. The central compartment volume of distribution of both preparations was similar and indicated limited distribution outside the blood compartment; the terminal half-life ranged between 15 and 23 h. Both products showed similar binding and neutralization of digoxin, as evidenced by a drop of unbound digoxin concentrations to below the quantification limit immediately after the end of the 5 min infusion. Differences with respect to systemic clearance of the anti-digoxin Fab and, consequently, digoxin were considered to be likely of no clinical relevance for efficacy [18].

19.2.2.4 Dose Considerations

Each Fab molecule binds one molecule digoxin and thus each unit (e.g., vial) of anti-digoxin Fab has a defined digoxin binding capacity. Thus, the total amount of digoxin in the body is important for dose calculation. The digoxin body load can be calculated in different ways: if the ingested amount of digoxin is known, the amount in the body can be calculated by multiplying this ingested amount with a correction factor of 0.8, accounting for digoxin's bioavailability of approximately 80%. Alternatively, the serum concentration of digoxin can be multiplied with its volume of distribution and the patient's body weight. In both cases, multiplying the digoxin amount in the body with $[MW_{Fab}/MW_{digoxin}]$ then provides the equimolar amount of anti-digoxin Fab [19]. Lower than equimolar doses have been used on the basis of pharmacokinetic considerations [20]. Most digoxin is not in the central compartment (volume of distribution ~6 l/kg [19]) and therefore not immediately available for binding. A smaller dose of Fab is therefore sufficient to rapidly neutralize all digoxin in the central compartment. Excess Fab may be partly wasted (eliminated in urine) because it takes time for peripheral digoxin to diffuse back into the central compartment [21]. Additional doses can be administered after 1 h in case of inadequate response or recurrence; or earlier if there is a clinical deterioration [13].

19.2.2.5 Cost Considerations

Fab preparations are relatively expensive treatments; the use of these preparations is therefore typically limited to clinical emergency situations in which other

therapies are likely to fail [22]. Anti-digoxin Fab is indicated in patients with life-threatening tachy-bradyarrhythmias, hyperkalemia (>6 mmol/l) or hemodynamic instability with digoxin concentrations indicating that digoxin may be a contributing cause. Conversely, anti-digoxin Fab is *not* indicated in asymptomatic patients [21]. Cost-effectiveness considerations have to be made for less severe cases, in which the shortened stay in hospital has to be balanced with the costs for the anti-digoxin Fab treatment [23]. For example, an *in silico*-based decision analysis model found that anti-digoxin Fab was associated with an incremental cost of $54 compared with standard therapy, but reduced length of hospital stay by 1.5 days. It was concluded that anti-digoxin Fab treatment could be a cost-saving alternative to standard therapy in many clinical scenarios, such as patients with high serum digoxin concentrations and renal dysfunction [23].

19.2.2.6 **Studies in Patients**

The clinical evidence for the efficacy of anti-digoxin Fab is limited to observational data. Several case series have reported benefits from anti-digoxin Fab for acute and chronic digoxin toxicity [24–34], yet the effect in chronic poisoning has recently been questioned [35]. This is also reflected in the observed differences in response rates to anti-digoxin Fab, which ranged from ~50% to 90% and tended to be higher in studies with more acute and more severe digoxin poisoning. However, owing to variability in baseline toxicity, different rates of use in chronic versus acute toxicity as well as differences in comorbidities, differences in response rates across studies are difficult to interpret [21]. Of note, anti-digoxin Fab has also been effective in managing other cardiac glycoside poisonings such as oleander [36, 37], toad venom [38], or Chinese herbal medicines [39].

19.2.2.7 **Safety**

Infusion of anti-digoxin Fab is generally well tolerated [26, 36]. Adverse events attributable to the Fab are rare and generally not serious, comprising exacerbation of heart failure, increased ventricular response in atrial fibrillation and hypokalemia. Conversely, allergic reactions are seldom observed [21].

19.2.3 **Idarucizumab and Andexanet Alfa: Reversal Agents for Oral Anticoagulants**

19.2.3.1 **Background**

Anticoagulant drugs interfere with the normal blood coagulation, thereby prolonging the time for clot formation. These drugs are important treatment options for patients at risk for thrombotic events, such as patients with atrial fibrillation [40]. A side effect inherent to the mode of action of all anticoagulants is the risk for bleeding, which in rare cases can be life-threatening. Clinical care for patients was advanced by the development of a novel group of direct oral anticoagulants (DOACs), comprising the direct thrombin inhibitor, dabigatran, as well as the Factor Xa inhibitors, rivaroxaban, apixaban, and edoxaban. DOACs have shown similar or better efficacy and safety compared to Vitamin K

antagonists [41–44], and their more predictable pharmacokinetic as well as pharmacodynamic properties obviate the need for regular monitoring of plasma concentrations. Nevertheless, bleeding, although rare, is still an important and potentially life-threatening condition associated with these compounds and reversal agents provide an additional treatment option in emergency situations. Beyond that, the fear of bleeding is also a factor influencing treatment decisions. For example, it was estimated that up to 50% of patients with atrial fibrillation, that is, patients in need of anticoagulant treatment for the prevention of strokes, are not receiving appropriate treatment [1]. Therefore, specific reversal agents could additionally increase the confidence in the safety of anticoagulant drugs and contribute to a better overall management of patients at risk for thromboembolic events.

19.2.3.2 **Idarucizumab**

Mode of Action Idarucizumab is a fully humanized, monoclonal antibody fragment (Fab), derived from an IgG1 isotype molecule [45]. It binds dabigatran and its active metabolites in a 1:1 molar ratio with high affinity, >300-fold higher compared to the affinity of dabigatran to thrombin. The resulting complex is very stable; and within the complex, dabigatran is prevented from its action of inhibiting thrombin activity. Advantages of the Fab approach include the highly specific binding of dabigatran, with no known off-target binding and consequently no undesired effects such as activation of the coagulation cascade [45]. The intravenous administration allows for an immediate onset of action; and the short half-life (in the range of hours compared to days for full monoclonal antibodies) enables rapid restoration of anticoagulation 24 h after treatment, when clinically indicated [46].

Using recombinant DNA technology, the Fab is expressed in a mammalian cell line [45]. The direct expression as a Fab avoids the need for cleavage of a whole antibody, which is relevant as papain cleavage has been linked to increased risk for immunogenic reactions.

Studies in Volunteers Doses from 20 mg to 8 g idarucizumab were administered as 5 min or 1 h infusion to volunteers in a total of three Phase I studies. The pharmacokinetics (PK) of idarucizumab was characterized by rapid achievement of maximum plasma concentrations followed by rapid, mainly renal, elimination. Four hours after administration, plasma concentrations had already dropped by more than 90% from peak with an initial half-life of ~45 min. Idarucizumab had limited extravascular distribution [47].

To achieve clinically relevant concentrations of dabigatran, healthy volunteers were pretreated with 220 mg dabigatran etexilate twice daily to steady state [48]. The anticoagulant effect of dabigatran, as well as its reversal by idarucizumab, was then explored measuring the prolongation and subsequent normalization of plasma clotting times, following dabigatran and idarucizumab administration, respectively. Infusion of idarucizumab at total doses of 1, 2, 4, and 7.5 g resulted

in immediate and complete reversal of the anticoagulant effect of dabigatran, as consistently observed with a panel of clotting-time assays. Doses ≥2 g idarucizumab, reflecting an amount of idarucizumab at least 1 : 1 molar to the total amount of dabigatran in the body, resulted in sustained normalization of clotting times over the entire observation period of 72 h [47]. The clotting times correlated well with the concentrations of unbound (active) dabigatran. These were reduced to undetectable levels upon idarucizumab treatment and remained for doses ≥2 g at very low concentrations.

Studies in Patients A prospective, case-series study in dabigatran-treated patients with life-threatening bleeding or patients who needed emergency surgery/urgent intervention was initiated in more than 300 participating study sites [49, 50]. The dose of idarucizumab was set to 5 g to achieve complete reversal of dabigatran anticoagulation even in patients with very high dabigatran exposure. Considering the total dabigatran body load in most patients, 5 g is considered to be an "overwhelming" dose. In an interim analysis based on 90 patients with mean age of 76.7 years, complete normalization of clotting times was achieved in 88–98% of the patients, depending on the clotting assay taken into consideration. Normal intraoperative hemostasis was reported in 33 of 36 patients who underwent an emergency procedure [50].

Safety Administration of doses from 20 mg to 8 g was safe and well tolerated in healthy volunteers as well as in the elderly and volunteers with mild or moderate renal impairment [47, 48, 51]. There was no relationship observed between idarucizumab dose and frequency of adverse events; no severe or serious adverse events, no dose-related events, and no discontinuations due to adverse events were reported [47, 48, 51]; there were also no safety concerns among the 90 participants in the patient study [50].

Absence of Anticoagulant or Prothrombotic Properties One clinically relevant concern in the bleeding management of anticoagulated patients is the risk for a procoagulant (and thus prothrombotic) state induced by the rescue medication [52]. Idarucizumab does not have any procoagulant effects, as consistently demonstrated *in vitro* [45] and in clinical investigations [47, 49]. However, patients are treated with anticoagulants because they have an underlying prothrombotic risk, and reversing the actions of the anticoagulant can unmask the underlying risk of the patient.

Immunogenicity Immunogenicity was evaluated in plasma samples from 283 volunteers, including 224 treated with idarucizumab. Preexisting antibodies with cross-reactivity to idarucizumab were detected in 36 of the 283 individuals (13%); these had no impact on the pharmacokinetics or the reversal effect of idarucizumab. Of the 224 individuals treated with idarucizumab, 9 individuals (4%) had a low-titer, treatment-emergent, possibly persistent anti-idarucizumab antibody response [5].

Bleeding Cessation Owing to the lack of a control group, the efficacy of idarucizumab on bleeding cessation cannot be assessed in the clinical setting. However, preclinical animal models have been used to study this effect, including a porcine trauma model [53]. In the model, animals were pretreated with supratherapeutic doses of dabigatran, resulting in a significant increase in trauma-induced blood loss and a 100% mortality rate. Idarucizumab administration 15 min post trauma was associated with a significant reduction in blood loss, and bleeding stopped within 15 min after administration. Mortality was reduced by 83% with the administration of 30 mg/kg idarucizumab; all animals survived after doses of 60 and 120 mg/kg [53].

19.2.3.3 Andexanet Alfa

Mode of Action Andexanet alfa is a recombinant modified human Factor Xa decoy protein. It is catalytically inactive; however, it retains the active site binding pocket to allow for the binding of small-molecule Factor Xa inhibitors [54], including rivaroxaban, apixaban, and edoxaban. It thereby sequesters the Factor Xa inhibitors in a 1 : 1 molar ratio within the vascular space, restores the activity of endogenous Factor Xa, and reduces the level of anticoagulant activity. On the basis of reported interactions of Factor X and Xa, it is concluded that andexanet alfa might also interact with circulating antithrombin III, tissue factor pathway inhibitor, and factor V, protein S, or protein Z inhibitor [54]. It reduces the effective concentration of the antithrombin III–heparin complex, and thus also reverses the anticoagulant effect of antithrombin III-dependent Factor Xa inhibitors, such as enoxaparin and fondaparinux [54].

Studies in Volunteers The anticoagulant action of Factor Xa inhibitors and its reversal can be monitored by measuring Factor Xa activity as well as the concentration of unbound Factor Xa. In small cohorts of healthy volunteers, andexanet alfa administration resulted in dose-dependent, rapid reversal of the anticoagulant effects of apixaban, rivaroxaban, edoxaban or enoxaparin [55–57].

Two parallel trials tested the efficacy and safety of andexanet alfa in older volunteers (mean age 57.9 years, 39% women) for reversing the anticoagulant effects of the Factor Xa inhibitors apixaban ($N = 48$; $N = 17$ for placebo) and rivaroxaban ($N = 53$; $N = 27$ for placebo) [58]. Both studies were conducted in two consecutive parts. In part one, a bolus was administered alone and in part two a bolus administration was followed by a 120 min continuous infusion. Participants were pretreated to steady state with apixaban or rivaroxaban at the respective, highest-approved dose levels. The dose of andexanet alfa was then adjusted for each drug, that is, in part one a 400 or 800 mg bolus was administered for apixaban- and rivaroxaban-treated volunteers, respectively; which was, in part two, followed by 480 or 960 mg as 120 min infusion, respectively. Bolus administration achieved a statistically significant ($P < 0.001$) maximum reduction of anti-Factor Xa activity by 94% and 92% for apixaban and rivaroxaban, respectively, compared to 21% and

18% for the respective placebo groups. This correlated with a significant reduction of the unbound concentrations of apixaban and rivaroxaban. Factor Xa activity then returned over a time frame of approximately 2 h to the levels observed in participants receiving placebo treatment, which is in line with the earlier reported pharmacodynamic half-life of approximately 1 h [55–57]. In part two of the studies, the reduction of Factor Xa activity of >90% was prolonged for both anticoagulant drugs for the infusion duration [58].

Studies in Patients A Phase IIIb/IV study is currently evaluating the efficacy and safety of andexanet alfa in patients with Factor Xa inhibitor-associated acute major bleeding [59].

Safety, Endogenous Thrombin Generation, and Immunogenicity There were no serious or severe adverse events and no thrombotic events reported in the volunteer studies, including elderly volunteers [55–58]. In the absence of clinical thrombotic events, transient increases in prothrombin fragments 1 and 2 as well as D-dimer were observed, which are sensitive but also variable markers of a prothrombotic state [60, 61]. These elevations were not associated with high levels of thrombin generation and returned to normal range within 24–72 h [58]; the clinical relevance of these findings for patients remains to be determined. Antibodies against Factor Xa or Factor X have not been observed in the studies in volunteers. The absence of such antibodies is very important, as Factor X and Xa are endogenous proteins. In the studies in elderly volunteers, non-neutralizing antibodies against andexanet alfa were detected in 17 of 101 participants treated with andexanet alfa and 1 of 44 treated with placebo.

19.2.4 Glucarpidase

19.2.4.1 Background

Methotrexate is a folic acid analog capable of inhibiting dihydrofolate reductase, a key enzyme in *de novo* purine biosynthesis. It has been used for decades to treat a variety of cancers in pediatrics as well as adults [62]. Despite best efforts in prevention, methotrexate can induce acute kidney injury resulting in an oncological emergency. Among the consequences of acute renal failure is the delayed elimination of methotrexate from the body. Serious cases of methotrexate accumulation in the body can result in severe methotrexate-related toxicities, including paralysis, cranial nerve palsies, seizures, coma, demyelinating encephalopathy, and death [63].

19.2.4.2 Mode of Action

Glucarpidase is a recombinant form of carboxypeptidase G2, a bacterial enzyme capable of rapidly hydrolyzing the terminal glutamate residue from methotrexate [64]. The resulting inactive metabolite, 2,4-diamino-N^{10}-methylpteroic acid or "DAMPA," is then eliminated by non-renal pathways. Glucarpidase is a

homodimeric protein with a molecular weight of 83 kDa and is produced in genetically modified *Escherichia coli* [9]. Although having been available in the United States and Europe since 1993 under compassionate use [62], glucarpidase was approved only in 2012 for the treatment of toxic plasma methotrexate concentrations in patients with delayed methotrexate clearance due to impaired renal function [9].

19.2.4.3 Studies in Volunteers

A pharmacokinetic study comprised 12 adult volunteers without cancer, 4 of them having severely impaired renal function [65]. Following a 5 min infusion of 50 U glucarpidase per kg body weight, that is, the standard clinical dose, the mean plasma half-life was 9 and 10 h for healthy and renally impaired volunteers, respectively, suggesting that renal impairment has limited effect on the elimination of glucarpidase. The volume of distribution was 3.6 l, indicating that glucarpidase distribution is mostly restricted to the plasma volume.

19.2.4.4 Studies in Patients

Glucarpidase demonstrated to be highly efficient in reducing methotrexate levels in a number of compassionate-use case reports and clinical trials (summarized by Green [63]). A pooled analysis of efficacy data from four multicenter, single-arm, compassionate-use clinical trials, comprising a total of 476 patients [62], found that in 169 patients with available concentration measurements, plasma methotrexate concentration demonstrated consistent 99% median reduction. The percentage reduction was dependent on the pre-glucarpidase methotrexate concentration; the reduction, however, was >85% in nearly all patients. Pre-glucarpidase methotrexate concentration was dependent on cancer type and methotrexate dose. "Sixty-four percent of patients with renal impairment greater than or equal to Common Terminology Criteria for Adverse Events grade 2 recovered to grade 0 or 1 at a median of 12.5 days after glucarpidase administration" [62]. Glucarpidase only hydrolyzes methotrexate in the vascular space, and intracellular methotrexate is only accessible after release into the vascular space. Rebound, defined as a post-glucarpidase increase of methotrexate >2 times the nadir methotrexate concentration, occurred in 14–23% of patients [62]. This suggests that not all intracellular methotrexate is inactivated and additional intracellular rescue may be indicated, such as leucovorin rescue [66]. Leucovorin is a mixture of the diastereoisomers of the 5-formyl derivative of tetrahydrofolic acid and can counteract the therapeutic and toxic effects of methotrexate [67]. It should not be administered within 2 h of glucarpidase administration, as it is an alternative substrate for glucarpidase and may compete with methotrexate.

19.2.4.5 Safety

In a broad range of clinical studies and case reports, glucarpidase was generally safe and well tolerated. Side effects were rare and included paresthesia, flushing, nausea/vomiting, hypotension, and headache [9, 63].

19.2.4.6 Immunogenicity

In clinical trials, 121 patients who received one ($n = 99$), two ($n = 21$) or three ($N = 1$) doses of glucarpidase were evaluated for anti-glucarpidase antibodies. Of these 121 patients, 25 (21%) had detectable anti-glucarpidase antibodies following Voraxaze® administration (19 received single dose and 6 two doses). Neutralizing antibodies were observed in 11 of the 25 patients [9].

19.2.5 Selected Reversal-Agent Approaches in Clinical Testing

19.2.5.1 Butyrylcholinesterase (Protexia; TV-1380)

Butyrylcholinesterase (BChE) belongs to the family of serine hydrolases, found in several tissues in the body. Its activity is irreversibly inhibited by organophosphorus compounds, for example, the nerve agent sarin and the pesticide metabolite chlorpyrifos oxon. Broomfield *et al.* [68] demonstrated that monkeys pretreated with BChE were protected from the toxic effects of nerve gas. This is achieved through a covalent modification, whereby the active site of BChE reacts with the organophosphorus ester, resulting in simultaneous inactivation of BChE and the organophosphorus poison [69]. The observation raised obvious interest for military application as a protective agent for nerve gas poisoning. Consequently, Protexia®, a recombinant human butyrylcholinesterase, has been tested in a Phase I trial [10] for the development as a "pre- and post-exposure therapy for casualties on the battlefield or civilian victims of nerve agent attacks" [70].

Future indications for butyrylcholinesterases are not limited to military applications. For example, TV-1380, a recombinant, monomeric, albuminated butyrylcholinesterase, has been shown to rapidly eliminate cocaine in the plasma, thus preventing the entry of cocaine into the brain and heart. Early clinical testing showed that TV-1380 was well tolerated and safe with a predictable PK profile. The authors concluded that TV-1380 might offer a safe once-weekly pharmacological treatment for treating cocaine dependence [11].

19.2.5.2 Anti-methamphetamine Antibodies

Similarly to treating cocaine dependence, protein therapeutics have been implicated in the treatment of methamphetamine abuse. IXT-m200 (also known as Ch-mAb7F9), a chimeric anti-methamphetamine monoclonal antibody, was designed to bind methamphetamine with high affinity and specificity. In a first Phase I clinical trial, Ch-mAb7F9 has demonstrated to be safe in healthy volunteers [71]. Anti-methamphetamine antibody medications are a promising pharmacological approach for treating methamphetamine use and addiction.

19.3 Antivenoms and Antitoxins

A venom (*venenum* (lat.) = poison) is a poisonous secretion by animals such as snakes, spiders, and scorpions which is typically injected into prey or aggressors

by biting or stinging [72]. Venoms usually comprise many different protein components of variable structure and toxicity.

A toxin (*toxicum* (lat.) = poison; *toxikonpharmakon* (greek) = poison on arrow) is a poison of plant or animal origin, especially one produced by – or derived from – microorganisms [72] such as *Clostridium botulinum, Clostridium tetani*, or *Bacillus anthracis.*

Antivenoms and antitoxins are used therapeutically in individuals exposed to such poisons. A common principle to both is that toxic agents are neutralized by passive immunization, that is, transfer of antibodies or antibody fragments. In the following, the terms are, therefore, used synonymous and interchangeably with the term "antisera". These antisera are often generated by immunization of large animals, such as horses or sheep. Plasma from these animals is then concentrated and purified. They may also be derived from postexposure donations from individuals that have undergone active immunization, so-called convalescent sera, as described for botulism or ebola infection [73, 74], or they may be generated by recombinant technologies, such as monoclonal antibodies against botulism or anthrax [75].

Antisera represent immunoglobulin (-fragment) preparations derived from heterologous plasma, still containing the host protein to some degree. Consequently, their administration is frequently associated with severe adverse effects such as anaphylaxis or serum sickness with an incidence of up to 40% [76]. Therefore, administration of antisera as antivenoms or antitoxins is generally limited to life-threatening conditions induced by acute poisoning, for example, through venomous snakes, spiders, or scorpions or through bacteria-derived toxins, such as in diphtheria, tetanus, or botulism.

19.3.1 Background and History

The first infectious disease that was successfully treated by passive immunization was diphtheria. It is caused by toxins derived from the gram-positive bacterium *Corynebacterium diphtheriae.* Mortality ranges between 5% and 10% overall and up to 20% in children under 5 years of age. In the second half of the nineteenth century in Germany, more than 50 000 children per year died from the disease. At that time, there was no known treatment other than palliative tracheotomy to prevent suffocation from the obstructed upper airways. Emil von Behring and Shibasaburo Kitasato discovered that it was possible to neutralize bacterial toxins *in vivo* with cell-free plasma from previously immunized animals [77]. In 1891, Emil von Behring successfully treated the first child with diphtheria antiserum. In 1901, he received the first Nobel Prize in Medicine and Physiology for the invention and development of diphtheria antitoxin therapy [78]. In the same era, Phisalix and Bertand demonstrated the antitoxic activity of the blood of animals immunized with the venom of the European viper (Viperaaspis) [79], while Calmette optimized immunization protocols to obtain protective antisera for the treatment of cobra bites [80]. During World War I, the same concept of

passive immune transfer was used for the prophylaxis and treatment of tetanus in soldiers and in farm animals.

When Fleming discovered penicillin in 1928, the importance of serum therapy decreased substantially as cheaper antibiotics became available for the treatment of infections. Today, use of antisera is generally restricted to treatment of acute poisoning from venomous animals, such as snake or spider bites and scorpion stings or they are used as convalescent sera against other incurable diseases such as ebola. However, in the future it may face a revival as additional treatment option for antibiotic-resistant infections [81] or possibly as countermeasure for biowarfare agents [82].

19.3.2 Epidemiology of Envenoming

Globally, about 5 million people per year are estimated to be bitten by snakes, causing about 125 000 fatalities and an additional 400 000 who are permanently disabled or disfigured [83, 84]. Scorpions account for more than 1 million cases of envenomation per year worldwide [85].

Envenomation or envenoming is rated by WHO as a neglected public health issue [86]. It represents an area of very high medical need as it affects mostly a poor agricultural population with insufficient access to medical care. Climate conditions in affected regions complicate storage of antibody preparations so that they are not densely distributed. Lack of commercial attractiveness for industry adds further to the undersupply.

19.3.2.1 Effects of Immunoglobulin Design on Antiserum Pharmacokinetics

The choice between complete IgG, $F(ab')_2$, or Fab as neutralizing agents in antivenoms has been discussed controversially [87]. Ideally, the antivenom should possess a similar pharmacokinetic profile as the respective poison, so that the two molecules are present in the same compartments and are able to interact and form complexes. Additionally, it should be present in the circulation long enough to bind toxin that is redistributing from tissues into plasma, after the circulating toxin has been complexed and eliminated.

Generally, the molecular mass of these molecules determines their pharmacokinetic profiles. Animal venoms contain a large variety of toxins, most of them proteins and peptides, with varying structures and a wide spectrum of molecular weights. Some scorpions and snake venoms contain potent neurotoxins, predominantly peptides, with low molecular mass, around 3–4 kDa. Some snake and insect venoms contain toxic phospholipases A of about 14 kDa. Latrotoxin, the venom of the black widow spider, is a 130 kDa high-molecular-weight neurotoxin. If possible, this should be considered in the selection of the respective antiserum. Fab and $F(ab')_2$ molecules are smaller and have a larger volume of distribution than IgG [88]. Fab distributes more rapidly into tissues than $F(ab')_2$ or IgG.

Fab molecules have a single antigen-binding site and lack the hinge region, thus they are incapable of cross-linking. Consequently, they are less prone to induce

Table 19.3 Molecular weights and estimated pharmacokinetic properties of antibodies and fragments.

Molecule type	Molecular weight (kDa)	Plasma elimination half-life ($t_{1/2}$; hours)	Volume of distribution (V_d; l/kg)
IgG	150	45–116	0.1
$F(ab')_2$	100	18–96	0.2
Fab	50	4.4–28	0.1–0.3

Source: Adapted from Lavonas (2012) [87].

aggregates or histamine release from mast cells. However, owing to their size, they distribute mainly in the plasma and are eliminated rapidly by the kidneys. The half-life of Fab molecules may even be shorter than that of the toxins [87]. $F(ab')_2$ molecules are divalent and are able to cross-link antigen or form conglomerates. An overview of the molecular weights and corresponding estimated pharmacokinetic properties of antibodies and their fragments is provided in Table 19.3.

Furthermore, the volume of distribution of IgG and $F(ab')_2$ is significantly larger in envenomed animals than in controls [88]. Thus it seems that envenomation itself promotes vascular permeability and distribution into tissues, also for larger molecules.

The preferred route of administration for antisera is intravenously, as it implies immediate and complete bioavailability. However, in the field, this may not always be feasible and intramuscular injection may be the only option. Following intramuscular injection, absorption of $F(ab')_2$ and IgG is slower and their elimination half-life is longer compared with Fab. Bioavailability of $F(ab')_2$ by this route is 36–42%. Further differences in kinetics are attributable to species differences: The terminal half-life of equine or ovine antibodies in humans is shorter than that of human antibodies.

19.3.3 Generation of Antivenoms and Antitoxins

In Behring's times, serum therapy for a vast target population required enormous volumes of antisera. Therefore, large-scale production was performed by immunization of large animals such as sheep and horses from which great volumes of serum could be withdrawn repeatedly. Even today, horses and sheep remain the donor species of choice for the same reasons. After isolation of the bacterial toxin or collection of venom, for example, by "milking" a snake, donor animals are immunized by repeated injections of the purified antigen or antigen mixtures with or without adjuvant. The immunized animals are bled and plasma or serum is separated from the blood cells. Immunoglobulins are concentrated and purified by precipitation and/or fractionation. A fragmentation step, for example, papain or pepsin cleavage, may be included to generate Fab or $F(ab')_2$ fragments and to remove the Fc parts of the IgG molecules.

Manufacturing of IgG preparations is relatively simple and inexpensive as there is no loss of yield due to a cleavage process. However, they are more frequently associated with hypersensitivity reactions. The pepsin digestion for cleavage into $F(ab')_2$ molecules is usually conducted under acidic conditions and can increase the amounts of aggregates. Additional virus inactivation/removal steps by sterile filtration and/or pasteurization are mandatory. The final product is filled aseptically, occasionally lyophilized, tested and released [89, 90].

19.3.4 Specificity

"Scorpion venoms are highly complex mixtures of peptides, enzymes, mucoproteins, free amino acids, nucleotides, lipids, amines, heterocyclic components, inorganic salts and probably other unknown substances" [87]. Similarly, toxins or venoms from other animals used for immunization are often a mixture of various antigens and only some of them represent the actual poison. Thus, only a small fraction of the induced antibodies in the antiserum will be directed specifically against the toxin [91]. In consequence, the potency is highly variable between donors as well as antigens. Besides difficulties in quality control, this may also infer variability in efficacy. Some antisera are monovalent, that is, prepared by immunization with venom of a single species, while others are generated with a mixture of toxins from a variety of species, strains, or subtypes [88]. In the clinical situation, often the exact envenoming species cannot be identified. Therefore, polyvalent sera can provide efficacy benefit by a wide spectrum of specificities.

Targets for human- or animal-derived antisera that represent a high medical need are, for example, rabies, ebola, tetanus, diphtheria, botulinum, anthrax, and animal venoms (snake, scorpion, spider). Examples for antitoxin or antivenom preparations that are commercially available or in clinical development are listed in Table 19.4.

19.3.4.1 Anti-anthrax Approaches

Owing to the complex pathogenesis and the time course of the infection, treatment of anthrax requires additional considerations which are outlined below. Anthrax is a lethal infectious disease caused by the spore-forming bacterium *B. anthracis.* The spores are very resilient to extreme environmental conditions. Once they enter a mammalian host, they germinate into rapidly dividing vegetative cells which produce a variety of anthrax toxins. Lethality of the anthrax infection is mainly attributed to the capsule and protein virulence factors, that is, protective antigen (PA), lethal factor [34], and edema factor (EF) [92]. PA can be detected in the blood approximately 24–48 h after exposure to *B. anthracis* spores [93]. Immediate postexposure vaccination with recombinant PA together with antibiotic coverage was fully protective in rabbits without bacteremia [94]. Without the recombinant vaccine, only 56% survived and 50% developed bacteremia.

Table 19.4 Examples for antitoxins/antisera.

Antitoxin/antivenom; company	Target	Type and origin	Development phase
Polyvalent snake antivenin; Biological E	Snake venom: cobra, common krait, Russels viper, and saw scaled viper	Equine plasma of hyperimmunized horses; polyclonal equine antibodies	Launched
EchiTAbG; MicroPharm	Carpet viper	Fab fragments of sheep polyclonal antibodies	Launched
ViperaTAb; Protherics	Viperaberus	Fab fragments of sheep polyclonal antibodies	Launched
CroFab; Protherics	Pit viper	Fab fragments of sheep polyclonal antibodies	Launched
Anascorp; InstitutoBioclon Sa De CV	Centruroides scorpion	Equine antibody fragment F(ab')2	Launched
Anavip; InstitutoBioclon Sa De CV and Rare Disease Therapeutics	Pit viper	Equine antibody fragment F(ab')2	Approved
NP-018; Cangene Corp.	Botulinum toxin (A, B, C, D, E, F, G)	Heptavalent mixture of equine-derived antibody fragments	Launched
Anthrasil; Emergent	Anthrax	Purified polyclonal human IgG from healthy. immunized donors	Approved
Raxibacumab, GlaxoSmithKline	Protective antigen (PA) component of anthrax toxin	mAb	Approved
Anthim (obiltoxaximab); Elusys Therapeutics Inc.	Anthrax	mAb	Approved
Analatro; InstitutoBioclon Sa De CV and Rare Disease Therapeutics	Black widow spider	Equine antibody fragment F(ab')2	Phase 3
Xoma-3AB; Xoma	Botulinum toxin A1, 2, 3	IgG1 mAb, trivalent	Phase 1

Source: Thomson Cortellis, PharmaProject.

Efficient postexposure treatment needs to be initiated as early as possible to be able to counteract the rapid progression of the infection. Repeated administrations over weeks are required for full protection. Antibiotics are unable to neutralize circulating toxins. If therapy is not initiated early enough, patients may succumb to toxin-induced pathology, even if all bacteria have been killed [92].

Antibiotics can only be effective against the vegetative form of the bacteria, so that ungerminated spores may be retained and act as a "depot" [95]. These

may lead to recurring infections after cessation of the antibiotic treatment. Consequently, the combination of antibiotic treatment with active or passive immunization can enhance the efficiency of postexposure prophylaxis and treatment. On the basis of the significantly longer half-life of antibodies compared with antibiotics, the duration of efficacy may be extended considerably [92]. As PA neutralization blocks the disease progression, neutralizing anti-PA antibodies are viewed as appropriate life-saving therapy after confirmed infection [96]. The complex pathogenesis mechanisms involve the capsule as well as several virulence factors. Thus, passive immunization using a polyvalent "cocktail" of neutralizing antibodies against several virulence factors and the capsule may provide additional benefit [92].

19.3.5 Safety and Tolerability

Recommendations for management of envenomation and especially for the dose of antiserum are so far only supported by scarce clinical data and case reports [97]. Most data are empirical and their benefit has not been proven in well-controlled clinical studies. Polyclonal, often heterologous, antivenoms/antisera bear an inherent clinical risk of hypersensitivity, serum sickness, shock, transmission of infectious agents, or antigenicity. Thus, in selecting the treatment for such potentially fatal or debilitating poisoning conditions, the expected benefits need to be weighed against the risks on an individual basis. Although most stings cause mainly local effects, severe envenomation may be lethal, especially in children. The only specific treatment is the administration of antiserum which should additionally be combined with symptomatic and supportive treatment [98]. Owing to the high incidence of adverse events, they need to be managed clinically in addition and complicate the underlying condition of intoxication.

Severe systemic anaphylaxis may occur in as many as 40% of the cases [76]. These may be related to Type I hypersensitivity but they are also observed in naïve patients receiving antiserum for the first time. Such acute reactions may additionally involve complement activation and effects of aggregates and Fc. The associated generalized shock can be prevented or mitigated by concurrent administration of corticosteroids, antihistamines, fluids, and adrenaline. Fever can also occur and is a result of pyrogen contamination, mostly bacterial lipopolysaccharides, during manufacture [76].

Serum sickness represents a delayed-type hypersensitivity reaction. It can be observed 1–2 weeks after administration of an antiserum. The pathophysiology involves circulating immune complexes and complement activation. Circulating immune complexes may lead to kidney damage by clogging the renal filter, and immunoglobulin aggregates may activate the complement.

As with other immunoglobulins, tolerability may theoretically be improved by modification (papain or pepsin cleavage) of complete immunoglobulins to $F(ab)_2$, Fab [87] or other antigen-specific antibody fragments such as scFv,

dimers, tandems, or nanobodies [99]. These lack the Fc part, thereby avoiding Fc-receptor-mediated adverse effects.

Safety and tolerability can be further improved by further purification steps, for example, removal of other host serum proteins by precipitation, purification, chromatography, or removal of pathogens by pasteurization [90, 100].

Further technological improvement is possible with the availability of recombinant technologies and engineering of neutralizing monoclonal antibodies or fragments with improved homogeneity, specific activity, and possibly safety [91]. The commercial attractiveness to pharmaceutical companies is, however, limited owing to the small market in contrast to high development and production costs involved. A further approach to circumvent the poor tolerability of animal-derived antisera is the use of human immunoglobulins generated from individuals who have overcome a certain (infectious) disease and developed antibodies by active immunization. So-called convalescent plasma was used with considerable value in the treatment of infectious diseases such as lassa or ebola [74].

Further antiserum properties and constituents that may be associated with poor tolerability comprise (heterologous) protein aggregates, presence of residual donor proteins or LPS, and poor stability of the plasma-derived proteins. Depending on the donor species, species-specific glycosylation of the antibodies may additionally compromise safety and efficacy of the antiserum [91].

19.4 Conclusion

Rescue medications are primarily used as countermeasure in acute emergencies and life-threatening conditions. While the pharmacologic activity of selected drugs can be safely reversed with highly specific, state-of-the-art recombinant proteins, treatment of envenoming often follows the same principles as that followed over a century ago. Availability of antisera is directed by the endemic occurrence of venomous agents. In principle, technologies are available to construct antisera with improved safety profiles; however, manufacture of antisera is often guided by compromises between safety, efficacy, specificity, and costs. Reversal agents are increasingly available widespread, matching the distribution of the drug they were designed for. These agents not only serve as rescue medication in case of emergencies; their availability can also increase the confidence in the safety of the underlying drug and thus be beneficial for overall patient care.

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20
Biosimilars

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20.1
Introduction

Biosimilars (or follow-on biologics (FOB)) are officially approved subsequent versions of innovator biologic products made by a different sponsor following patent expiry on the innovator products. They have demonstrated similarity to the innovator biologic products in terms of quality, safety, and efficacy. The development of biosimilars provides an opportunity to lower the cost of expensive biologic drugs and increase patients' access to life-saving biologic medicines. However, unlike the generic small-molecule drugs, the development of biosimilars is much more complicated because of the fundamental differences between chemical drugs and biologic products. Therefore, the standard methodology for the assessment of bioequivalence is not appropriate for the assessment of biosimilars. The European Medicine Agency (EMA) has taken the lead in the regulatory approval framework for biosimilar products, and the US Food and Drug Agency (FDA) was authorized to approve biosimilars by the Biologics Price Competition and Innovation (BPCI) Act passed by the US congress on March 23, 2010. In this chapter, we will introduce the concept and definition of biosimilars, discuss the rationale and significance of developing biosimilar drugs, review the current landscape of biosimilar regulation and discuss the future trends and challenges of biosimilar development.

20.2
Concept and Definition of Biosimilars

20.2.1
Generic Small Molecule Drugs Compared with Biosimilars

In the United States, when an innovator drug is going off patent, pharmaceutical companies may file an abbreviated new drug application (ANDA) for approval

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of the generic copies of the innovator drug product. In 1984, the US FDA was authorized to approve generic drug products under the Drug Price Competition and Patent Term Restoration Act, also known as the "Hatch-Waxman Act" [1]. According to FDA's definition, the generic drug products should be comparable to the reference drug product in strength, dosage form, route of administration, quality, performance characteristics, and intended use.

For the approval of generic (small molecule) drug products, the FDA requires that evidence of average bioavailability be provided through the conduct of bioavailability and bioequivalence studies. The assessment of bioequivalence as a surrogate for evaluation of drug safety and efficacy is based on the *fundamental bioequivalence assumption* that if two drug products are shown to be bioequivalent in average bioavailability, it is assumed that they will reach the same therapeutic effect and hence can be used interchangeably [2]. Under this fundamental assumption, regulatory requirements, study design, criteria, and statistical methods for the assessment of bioequivalence have been well established.

However, unlike generic drugs (small molecule drugs) with well-defined composition and structure, biosimilar products (biologics) are much bigger in size and much more complex in structure (Table 20.1). The molecular weights of small molecule drugs are typically below 1 kDa. In contrast, the molecular weights of biologic products can range anywhere between several kilodalton (e.g., insulin) to thousands of kilodalton (e.g., virus particles). Small molecule drugs have clear and well-defined structures and modifications, and are stable and easy to characterize. Thus, identical copies can be made. However, biologic drugs are much more complex. For example, recombinant-protein drugs fold into three-dimensional structures; the activity and biological functions are highly dependent on their unique spatial conformation, and slight variations in the manufacturing processes may affect the spatial conformation and the efficacy of the biologic drugs [3].

The manufacturing processes of small molecule drugs and biologics are very different. Small molecule drugs are produced by chemical synthesis, while biologics are produced in cell cultures or living organisms, and the structures and modifications of biologics are highly dependent on the exact manufacturing

Table 20.1 Characteristics of small molecule drugs compared to biologics.

	Small molecule drugs	**Biologic drugs**
Size	Small Low molecular weight	Large High molecular weight
Structure	Simple Well-defined modifications	Complex and heterogeneous Complex modifications
Manufacturing	Produced by chemical synthesis Simple and predictable	Produced in cell culture or living organisms Complex and difficult to control
Identical copies?	Yes	No

process. Manufacturing processes for biologic drugs are highly complex, including obtaining and expressing of target genes, optimization and fermentation of genetically engineered cells, clarification and purification of the biologic products, formulation and testing, and aseptic filling and packaging. Each of these procedures contains multiple steps and requires strictly controlled conditions such as pH, temperature, optical density and so forth in order to guarantee the efficacy and safety of the biologic products [3]. In fact, these manufacturing details are typically proprietary, and therefore it is difficult to duplicate the manufacturing processes, and differences in any step of the manufacturing processes may result in variations of clinical relevant parameters, such as the three-dimensional structure of the protein, the quantities of acid–base variants, and post-translational modifications. Thus, it is very difficult to completely characterize biologic drugs and it is almost impossible to make an identical copy. Therefore, there is general consensus that the standard methodology for the assessment of bioequivalence is not appropriate for the assessment of biosimilars.

20.2.2
Definition and Interpretation of Biosimilars

Since a biosimilar is not the identical copy of the innovator biologic drug, it is important to understand the definition of a biosimilar and how to interpret it. The definition and nomenclature of biosimilars are different among the various regulatory agencies across the world (Table 20.2). For example, similar biologic

Table 20.2 Definitions of biosimilar products worldwide.

Term	By	Definition
SBP	WHO	A biotherapeutic product similar to an already licensed reference biotherapeutic product in terms of quality, safety, and efficacy
Biosimilar	EMA	A biological medicine that is similar to another biological medicine that has already been authorized for use
FOB	US FDA	A product highly similar to the reference product without clinically meaningful differences in safety, purity, and potency
SEB	Canada	A biologic drug that enters the market subsequent to a version previously authorized in Canada with demonstrated similarity to a reference biologic drug
Biosimilar	Korea	Biological products which demonstrated its equivalence to an already approved reference product with regard to quality, safety, and efficacy [4]
Similar biologics	India	Similar biologics contain well-characterized proteins as their active substance. The demonstration of similarity depends upon detailed and comprehensive product characterization and preclinical and clinical studies carried out in comparison with a reference biologic [5]

drug products are generally referred to as similar biotherapeutic products (SBPs) by WHO [6]. WHO defines SBP as a biotherapeutic product, which is similar in terms of quality, safety, and efficacy to an already licensed reference biotherapeutic product [6]. EMA defines biosimilar medicine as a biological medicine that is similar to another biological medicine that has already been authorized for use [7]. In the United States, the BPCI Act passed by the US congress on March 23, 2010, indicates that an FOB product is a product that is highly similar to the reference product, notwithstanding minor differences in clinically inactive components, and for which there are no clinically meaningful differences in terms of safety, purity, and potency from the reference product [8].

On the basis of these definitions, there are three determinants in the definition of the biosimilar: (i) it should be a biologic product; (ii) the reference product should be an already licensed biologic product; (iii) the demonstration of high similarity in safety, quality, and efficacy is necessary [9]. Besides, it is well recognized that the similarity should be demonstrated using a set of comprehensive comparability exercises at the quality and non-clinical and clinical level. Products not authorized by this comparability regulatory pathway cannot be called biosimilars.

For small molecule generics, it is assumed that they will reach the same therapeutic effect as the reference drug and hence can be used interchangeably. However, there is a clear distinction between biosimilarity and interchangeability. According to the definitions given in BPCI, biosimilarity does not imply interchangeability, which is much more stringent [3]. As indicated in Subsection (b)(3) amended to the PHS Act Subsection 351(k)(3), the term interchangeability, with reference to a biological product means that the biological product may be substituted for the reference product without the intervention of the healthcare provider who prescribed the reference product [3]. A biological product is considered to be interchangeable with the reference product if (i) the biological product is biosimilar with the reference product; and (ii) it can be expected to produce the same clinical result in any given patient [3]. Besides, for a biological product that is administered more than once to an individual, the risk in terms of safety or diminished efficacy of alternating or switching between use of the biosimilar product and the reference product is not greater than the risk of using the reference product without such alternation or switch.

20.3 Rationale and Significance of Biosimilars

20.3.1 The Potential for Cost Reduction

With fast and advanced development of modern biological technology especially recombinant DNA technology, biologic drug products have played more and more important roles in treating many life-threatening and chronic diseases, such as

Table 20.3 Top 10 best-selling drugs of 2012.

Name	Company	Indication	mAb?	2012 sales (billion)
Humira	AbbVie	Auto-immune	Yes	$9.265
Remicade	J&J, Merck	Auto-immune	Yes	$8.215
Enbrel	Amgen, Pfizer	Auto-immune	Yes	$7.963
Advair	GSK	Asthma, COPD	No	$7.904
Rituxan	Roche	Non-Hodgkin's lymphoma, CLL, RA	Yes	$7.285
Lantus	Sanofi	Diabetes	No	$6.648
Herceptin	Roche	HER2-positive breast cancer	Yes	$6.397
Crestor	AstraZeneca	Reduction of cholesterol	No	$6.253
Avastin	Roche	Colon cancer, NSCLC	Yes	$6.260
Cymbalta	Eli Lily	Depressive disorder	No	$4.994

cancer, kidney failure, and arthritis. As a result, biologic drugs have comprised a growing segment in the pharmaceutical industry. The global biologics market totaled more than $200 billion in 2013 and is expected to grow to $387 billion by the end of 2019 at a compound annual growth rate (CAGR) of 10.6% [10]. However, the high unit cost of biologics has resulted in patients' concerns about sustainable access to potentially life-saving therapies. Recently, the expiration of patents for a number of blockbuster biologics has ushered in an era of the subsequent production of biosimilar products, which might contribute to increased access to biologic products at an affordable price.

The monoclonal antibody (mAb) is arguably the most important type of biologic drugs, and it is known to be very expensive. mAb is derived from a single clone of immune cells and recognizes a unique antigenic determinant. It is the most promising and rapidly growing category of targeted agents. Among the top 10 best-selling drugs in 2012, six were mAb drugs with each annual sales value exceeding US$6 billion (Table 20.3) [11]. The global sales value of mAb drugs exceeded US$50 billion in 2012. As of 2013, there were 46 mAbs in market and more than 1400 mAbs in clinical trials in the United States (Figure 20.1) [12]. The reason for the rise of mAb therapeutics is because mAbs are in general

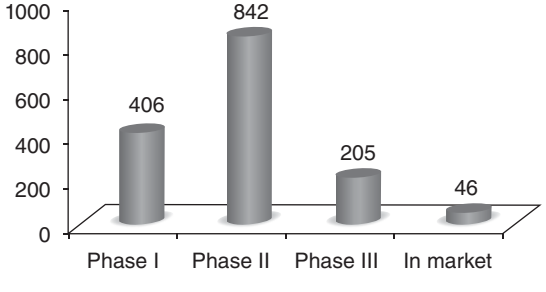

Figure 20.1 Number of mAbs clinical studies in the United States.

safer, and more specific and effective than traditional chemical drugs. However, biologic therapies are expensive to an extent that can be unaffordable to patients in middle- and low-income countries. For example, the mAb drug Enbrel used to treat rheumatoid arthritis and other auto-immune diseases costs$10 000 for a course of treatment. Notably, the median family annual income in China is only $10 000. Even for patients in developed countries, the high cost of biologic drugs creates an extremely high financial burden to patients, their family, and the whole healthcare system.

20.3.2 The Scale of the Opportunity to Reduce Cost

The expiration of patents and other intellectual property rights for originator biologics over the next decade opens up opportunities for biosimilars to enter the market and increase industry competition. Twelve biological products with global sales of more than US$67 billion will be exposed to biosimilar competition by 2020 [13]. Some of the major patents expiries for top-selling biologics are shown in Figure 20.2 including Avastin, Herceptin, Rituxan, and so on.

According to a Rand study from 2014, it is estimated that biosimilars could reduce spending on biologics by $44 billion over the next decade by US consumers and the government [14]. The impact of biosimilar drugs on global biopharmaceutical spending will be even more significant. Furthermore, the concept of biosimilar by itself and the possibility of biosimilar entering the market create pressures for originator manufacturer to lower the price. For example, when the mAb drug Herceptin, used to treat a subset of breast cancer, first became available in India, it was priced at around $2050 per dose, making it unaffordable to all except the very wealthy (Figure 20.3). In March 2012, soon after the

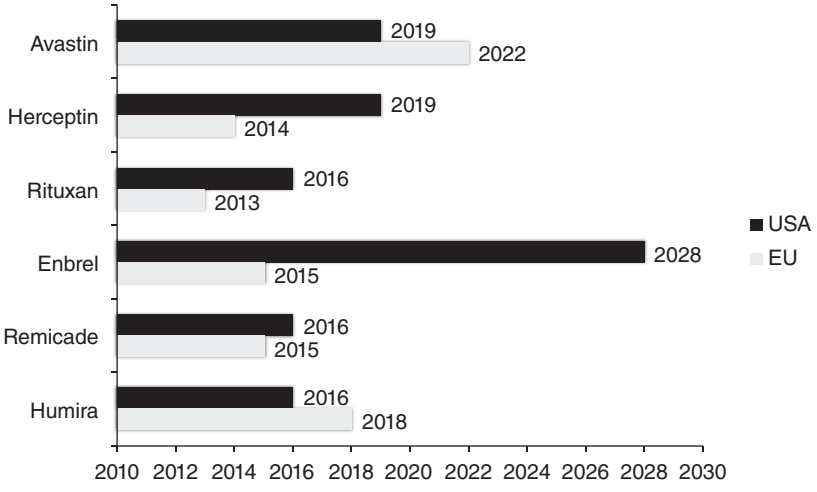

Figure 20.2 Major patents expiry for top-selling mAbs.

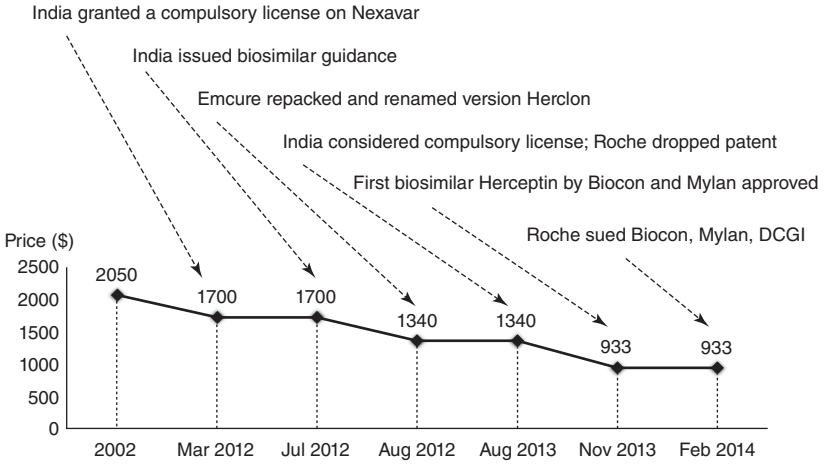

Figure 20.3 Herceptin price change in India.

decision of the Indian Patent Controller to grant a compulsory license on Nexavar (another biologic drug), Roche announced a cut in the price of Herceptin to $1700 per dose. In July 2012, India issued the regulatory guidance of biosimilars. One month later, Roche partnered with Emcure to offer a repacked and renamed version of Herceptin priced at $1340 per dose. A year later, the Indian government was considering issuing a compulsory license on Herceptin. Roche decided to discontinue the patent of Herceptin in India. In November 2013, three months after Roche discontinued the patent, the first biosimilar Herceptin made by Biocon and Mylan was approved and it further reduced the price to $933 per dose. India has achieved a 50% price reduction of Herceptin due to the pressure from government and potential competition.

This case demonstrates the potential impact biosimilars can generate on reducing the cost of biologic drugs. As the interest in biosimilars increases, multiple regulatory agencies have established regulatory pathways to regulate biosimilar approval, which will be discussed in detail in the next section.

20.4
Current Approvals and Trends

20.4.1
Biosimilar Approvals

In 2006, the EMA approved the first biosimilar product Omnitrope, which contains the active substance somatropin [7]. Since then, EMA has taken the lead in biosimilar regulation and it has authorized 20 biosimilar products (Table 20.4) [7].

Table 20.4 EMA approved biosimilar products.

Name	Active substance	Therapeutic area	Date approved	Type
Benepali	Etanercept	Auto-immune	2016	Anti-TNF
Accofil	Filgrastim	Neutropenia	2014	G-CSF
Abasaglar	Insulin glargine	Diabetes mellitus	2014	HGH
Bemfola	Follitropin alfa	Anovulation	2014	HGH
Grastofil	Filgrastim	Neutropenia	2013	G-CSF
Ovaleap	Follitropin alfa	Anovulation	2013	HGH
Remsima	Infliximab	Auto-immune	2013	Anti-TNF
Inflectra	Infliximab	Auto-immune	2013	Anti-TNF
Nivestim	Filgrastim	Cancer; hematopoietic stem cell transplantation; neutropenia	2009	G-CSF
Filgrastim Hexal	Filgrastim	Same as above		G-CSF
Zarzio	Filgrastim	Same as above	2009	G-CSF
Biograstim	Filgrastim	Same as above	2008	G-CSF
Ratiograstim	Filgrastim	Same as above	2008	G-CSF
Tevagrastim	Filgrastim	Same as above	2008	G-CSF
Retacrit	Epoetin zeta	Anemia; blood transfusion; autologous; cancer: kidney failure chronic	2007	EPO
Silapo	Epoetin zeta	Same as above	2007	EPO
Abseamed	Epoetin alfa	Anemia; cancer; kidney failure chronic	2007	EPO
Epoetin Alfa Hexal	Epoetin alfa	Same as above	2007	EPO
Binocrit	Epoetin alfa	Anemia; kidney failure chronic	2007	EPO
Omnitrope	Somatropin	Dwarfism; pituitary Prader-Willi syndrome; turner syndrome	2006	EPO

According to the data from IMS, since the introduction of biosimilar EPO products in Europe, there has been a price reduction of EPO in a lot of accessible markets in Europe. For example, the price reduction in Croatia is more than 80%; the price reduction in Germany is about 50%, and that in France is about 30% [15].

Biosimilars currently marketed in Europe fall into the following four categories:

1) Erythropoietin (EPO), a glycoprotein hormone that controls erythropoietin, or red blood cell production.
2) Human growth hormone (HGH), a peptide hormone that stimulates growth, cell reproduction and regeneration.
3) Granulocyte colony-stimulating factor (G-CSF), a glycoprotein that stimulates the bone marrow to product granulocytes and stem cells.
4) Anti-tumor necrosis factor (TNF), either dimeric fusion protein or chimeric mAb.

In contrast to Europe, US FDA is falling behind in regulating biosimilars. The US congress passed the BPCI Act in 2010, which started the official chapter of biosimilars in the United States. So far, there is only one biosimilar drug approved by FDA in 2015, Zarxio, made by Sandoz [1]. Its reference drug is Neupogen (filgrastim) sold by Amgen. But there has been a significant increase in biosimilar development over the past 5 years in the United States. According to data from clinicaltrial.gov, as of May 2014, there were 14 unique biosimilars in development: 12 in Phase III and 2 in Phase I or I/II only [12].

In developing countries, India, which has dominated the generic drugs industry for decades, has launched the most biosimilars on their domestic market and demonstrated the greatest acceptance of biosimilars. Over 50 biosimilar products have been approved for marketing in India.

20.4.2 Regulatory Pathways for Biosimilars

For the assessment of biosimilars, regulatory requirements from different agencies, such as EMA, US FDA, and WHO, are similar and yet slightly different. They share four primary principles:

1) The standard methods for the assessment of generic drug products are not appropriate for the assessment of biosimilarity.
2) The development of biosimilars requires a stepwise approach starting with characterization of quality attributes of the product followed by non-clinical and clinical evaluations. Manufacturers should submit a full quality dossier that includes a complete characterization of the product, demonstration of consistent and robust manufacture of their product, and comparability exercise between the biosimilar product and the reference product in the quality part, which together serve as the basis for the possible reduction in data requirement in the non-clinical and clinical development.
3) The regulation of biosimilars adopts the case-by-case approach. The amount of non-clinical and clinical data considered necessary is dependent on the class of the product and the results of the comparability studies in quality.
4) Pharmacovigilance is stressed. In addition to the quality, non-clinical, and clinical data, applicants need to present an ongoing risk management and pharmacovigilance plan.

In addition to these shared principles, FDA introduced another two principles in the draft biosimilar guidance published in 2012 [16, 17]:

1) When evaluating the sponsor's demonstration of biosimilarity, FDA intends to consider the *totality of the evidence* provided by the sponsor. A sponsor may be able to demonstrate biosimilarity even though there are minor differences, provided that the sponsor submits sufficient data demonstrating that the differences are not clinically meaningful.
2) FDA will ordinarily provide feedback on a case-by-case basis on the components of a development program for a proposed product. Sponsors intending

to develop biosimilar products should meet with FDA at such time as the sponsor can provide a proposed plan for its development program, manufacturing process information and preliminary comparative analytical data with the reference product, and this early discussion with FDA will facilitate biosimilar development.

In the following sections, we will use WHO's guidance as an example to introduce the specific regulatory requirements for assessing biosimilar products (Figure 20.4) [6].

20.4.2.1 **Quality**

As mentioned in an earlier section, the comprehensive comparison showing similarity of quality between a biosimilar product and a reference product is a prerequisite for applying the clinical safety and efficacy profile of reference to a biosimilar, and thus a full quality dossier for both drug substance and drug product is always required. To evaluate comparability, WHO recommends the manufacturer to conduct a comprehensive physicochemical and biological characterization of the biosimilar in head-to-head comparisons with reference. The following aspects of product quality and heterogeneity should be assessed.

Manufacturing Process The manufacturing process should meet the same standards as required by the national regulatory agency (NRA) for originator products, and implement Good Manufacturing Practices, modern quality control and assurance procedures, in-process controls, and process validation. The biosimilar manufacturer should assemble all available knowledge of the reference with regard to the type of host cell, formulation and container closure system, and submit a complete description and data package delineating the whole manufacturing process including obtaining and expression of target genes, the optimization and fermentation of gene engineering cells, the clarification and purification of the products, the formulation and testing, aseptic filling, and packaging.

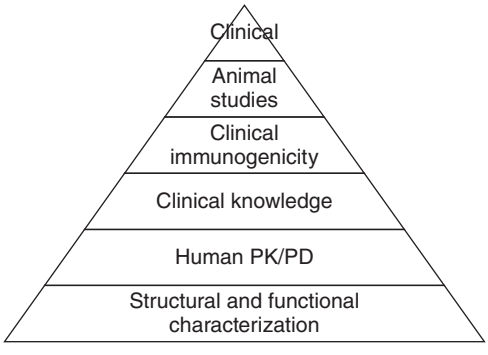

Figure 20.4 Dossier required for biosimilar approval.

Characterization Thorough characterization and comparability exercise are required, and details should be provided on primary and higher-order structure, post-translational modifications, biological activity, process- and product-related impurities, the relevant immunochemical properties, and results from accelerated degradation studies and studies under various stress conditions.

20.4.2.2 Non-clinical and Clinical Studies

After demonstrating the similarity between a biosimilar product and a reference product in quality, the proving of safety and efficacy of a biosimilar usually requires further non-clinical and clinical data. Non-clinical evaluations should be undertaken both *in vitro* (e.g., receptor-binding studies, cell-proliferation, cytotoxicity assays) and *in vivo* (e.g., biological/pharmacodynamic activity, repeat dose toxicity study, toxicokinetic measurements, anti-product antibody titers, cross reactivity with homologous endogenous proteins, product neutralizing capacity).

In terms of clinical evaluation, the comparability exercise should begin with pharmacokinetic (PK) and pharmacodynamic (PD) studies followed by the pivotal clinical trials. PK studies should be designed to enable detection of potential differences between SBP and RBP. Single-dose, cross-over PK studies in homogenous population are recommended by WHO. The manufacturer should justify the choice of single-dose studies, steady-state studies, or repeated determination of PK parameters, and the study population. Due to the lack of established acceptance criteria for the demonstration of similar PK between a biosimilar product and a reference product, the traditional 80–125% equivalence range is often used. Besides, PD studies and confirmatory PK/PD studies may be appropriate if there are clinically relevant PD markers. In addition, similar efficacy of a biosimilar product and a reference product has to be demonstrated in randomized and well-controlled clinical trials, which should preferably be double-blind or at least observer-blind. In principle, equivalence designs (requiring lower and upper comparability margins) are clearly preferred for the comparison of the efficacy and safety of a biosimilar with a reference. Non-inferiority designs (requiring only one margin) may be considered if appropriately justified. WHO also suggests that the pre-licensing safety data and the immunogenicity data should be obtained from the comparative efficacy trials.

In addition to non-clinical and clinical data, applicants also need to present an ongoing risk management and pharmacovigilance plan, since data from pre-authorized clinical studies are usually too limited to identify all potential side effects of the biosimilars. The safety specification should describe important identified or potential safety issues for the reference, and any that are specific for the biosimilar.

There are slight differences between EMA, FDA, and WHO's guidance and a complete comparison between them can be found in Table 20.5.

Table 20.5 Guidance comparison between EMA, FDA, and WHO.

	EMA	FDA	WHO
Scope	Well-characterized Vaccines/allergen: case by case Blood/plasma-derived: excluded Gene/cell-therapy: consider in the future	Therapeutic protein product	Well-established biotherapeutic products Vaccines, plasma-derived products: excluded
Reference product	Complete dossier Authorized in EU	Complete dossier Submitted under section 351 of PHS Act Exception: submitted under the FD&C Act through March 23, 2020	Complete dossier Authorized in jurisdiction with well-established regulation Same dosage form and route of administration
Structure and biology activity	Same primary and higher order structure May contain mix post-translational modifications Similar biological activities	Same primary and high order structure, post-translational modifications Similar potency	Similar as EMA
Purity	Product-related and process-related impurities Analyze samples stored under stress condition	Similar as EMA	Similar as EMA
Non-clinical	*In vitro:* receptor binding or cell based *In vivo:* PD activity At least one repeat dose toxicity study Other routine toxicological studies not required	Warranted: animal toxicity studies Helpful: animal PK and PD; animal immunogenicity Other routine toxicological studies not required	Similar as EMA
Clinical	Date expected: Both PK and PD Clinical immunogenicity Clinical safety and efficacy	Similar as EMA	Similar as EMA

20.5 Challenges and Future Trends

With the patent expiry of blockbuster biologic drugs and the establishment of biosimilar regulatory pathways in Europe, United States, and other major markets, the biosimilar industry has expanded quickly over the past few years. This presents

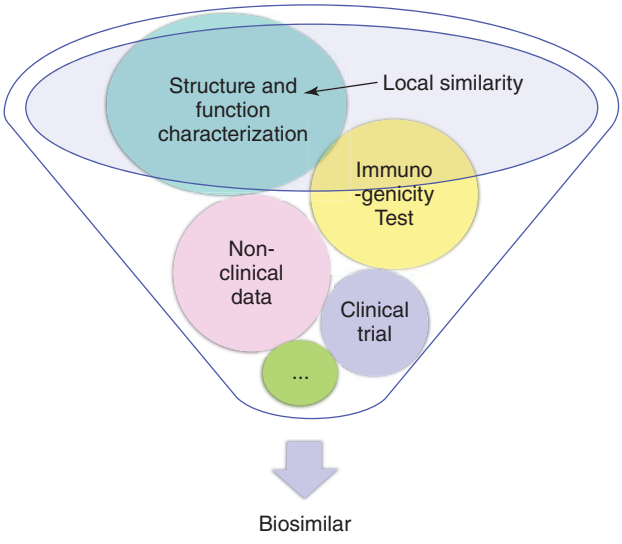

Figure 20.5 How similar is "highly similar"?

a promising opportunity to lower healthcare cost and increase access to biotherapeutics, but unlike generics, biosimilars face several significant challenges.

First, even though current criteria for the assessment of bioequivalence are useful for determining whether a biosimilar product is similar to a reference product, it does not provide additional information regarding the degree of similarity. As indicated in the BPCI Act, a biosimilar product is defined as a product that is highly similar to the reference product. However, how similar is considered "highly similar" has not been defined quantitatively. In fact, it will be extremely difficult to quantify biosimilarity on a whole. As mentioned in the previous sections, biosimilar development has to take a stepwise approach and the regulatory agencies need to consider the *totality of evidence* including structure and function characterization, immunogenicity tests, non-clinical data and clinical trials (Figure 20.5). Within each category, the similarity between the biosimilar product and the reference product can be evaluated. We can consider this similarity as local similarity. However, how we leverage different levels of local similarity to achieve global similarity becomes a much more complicated and challenging question. So far, no regulatory agency has established a comprehensive system to solve this question and biosimilar development is still evaluated on a case-by-case basis. But all regulatory agencies agree that clinical data should be the final criteria and the most important factor when evaluating biosimilarity, which means that unlike generic development, biosimilar development requires clinical trial data. All of these factors greatly increase the cost and uncertainty of biosimilar development.

Second, biosimilar development faces significant resistance from innovator companies. With the introduction of biosimilars, large biopharmaceutical companies with high-selling biologic products stand to lose significant market share

for their branded innovator products. In response, biopharmaceutical companies have been focusing on research and development to strengthen innovator product portfolios and improvements to drug to maintain market buy-in. For example, biopharmaceutical companies are focusing on ways to expand and improve drug formulations, expression systems, dosing, and delivery methods, to support the view of superiority of branded innovator drugs over their biosimilar counterparts. In addition, this strategy also provides the potential to extend patent protection. For example, Amgen's rheumatoid arthritis drug Enbrel, whose patents originally were set to expire in 2012, was granted a patent extension for another 16 years beyond the original expiration date by the US Patent and Trademark Office [18]. Therefore, in order for biosimilars to achieve significant market penetration, biosimilar developers need to have both R&D and manufacturing capacities, and commercial and legal expertise and strategies to face competitor's response.

Regardless of these challenges, the fact is that governments want cheaper drugs for their populations, biotechnology is an area of fast advance, and new competitors will keep entering into the market. Biosimilars have the potential to achieve a big impact on the healthcare industry and provide affordable medicines to meet the ever-growing needs of patients. Although progress in delivering biosimilars to the market has been highly dependent on the varied regulatory approaches around the world, the number of approvals are increasing year on year and this will be an area of significant growth over the coming decade.

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Part VI
Future Horizons

21
Future Horizons and New Target Class Opportunities

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21.1
Introduction

In this chapter, we focus on two areas. We first discuss the target spaces that are generally not amenable to biologic drugs. These are the central nervous system (CNS) and intracellular targets. Biologic agents like antibodies are very specific and highly potent, but these large molecules cannot penetrate the blood–brain barrier (BBB) to reach targets in the brain, nor can they penetrate cell membranes to access intracellular targets. If one can develop technologies to overcome these two challenges, this would significantly enhance the potential for developing new biologic medicines. Second, we provide insights on emerging classes of biotherapeutics that have the possibility to substantially shape both the industry and healthcare system. These include bi- or multispecific biologics, antibody–drug conjugates, *in vivo* expressed biologics and oral biologics. The corresponding technologies and their applications are discussed.

21.2
Targeting the Central Nervous System

21.2.1
The Opportunity

The brain is an important and complex organ and as a result requires a precisely regulated microenvironment in which to function correctly. It is separated from the main circulatory system by the presence of the BBB, which exists to protect the brain from the harsh biochemistry and molecules deleterious to the functioning of the delicate and sensitive cells of the CNS. Despite this protection, the CNS is

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subject to malfunction, and there are many diseases and disorders that originate in the CNS. Many of these diseases, such as Alzheimer's, remain untreatable or, at best, have therapies that provide only temporary palliative relief of symptoms.

As detailed in earlier chapters the development of antibody-based therapeutics has progressed rapidly over the past few decades, achieving clinical success for the treatment of many diseases. Unfortunately, development of antibody-based therapies for conditions of the CNS has not kept pace with small molecule drugs. This is mainly due to the poor BBB permeability of large molecule therapeutics. While all biologic drugs penetrate the brain to a small degree (typically less than 0.1% of the injected dose of IgG reaches the brain after peripheral administration) [1, 2] it is difficult to achieve sufficient concentrations of antibodies in the brain to produce a therapeutic response.

From a drug delivery point of view therefore, the BBB has been an insurmountable hurdle to the delivery of biologics for the treatment of disorders of the CNS. However, the BBB is not a passive barrier and in order to provide the brain with its required nutrients the BBB is a site of exchange between the blood and the cells of the CNS. With an estimated surface area of 100 cm^2/g of brain tissue in mammals [3] a huge opportunity exists to access the brain with therapies targeted to cross the BBB and treat CNS diseases.

21.2.2 The Challenge

Starting from the capillary lumen, the barrier consists of a layer of non-fenestrated capillary endothelium lining a continuous basal lamina. Pericytes, cells of mesodermal origin, are found in close association with the basal lamina and in contact with the underlying endothelial cells [4]. In addition, astrocytic end-foot processes cover large swathes of basal lamina forming an almost-completely sheathed neurovascular structure [5]. It is thought, mostly from studies carried out *in vitro,* that both astrocytes and pericytes contribute to the development of the phenotype of the BBB and to the polarization and differentiation of brain endothelial cells, thus strengthening the barrier function [6, 7].

The endothelial cells that make up the BBB are highly specialized with intercellular adhesions and tight junctions and low levels of non-specific pinocytosis. Tight junctions are composed of complex interactions between transmembrane proteins on the membranes of neighboring cells. Important tight junction proteins include occludin and members of the claudin and junctional adhesion molecule (JAM) families [8]. On the inner face of the cell membrane cytoplasmic plaques composed of peripheral membrane proteins and adaptor proteins are connected to the transmembrane proteins allowing interaction with signaling networks that modulate tight junction formation and endothelial cell polarity [9]. Tight junctions are particularly tight between cerebral endothelial cells and are mainly responsible for the physical aspect of the BBB barrier function, impeding paracellular diffusion across the BBB [8]. This leads to high electrical resistance with *in vivo* measurements of about 1500 $\Omega\,cm^2$ recorded in the pial blood vessels of 28–33-day-old

rats [10]. In addition, the tight junctions contribute to the polarized nature of the endothelial cells of the BBB allowing them to act as a transport barrier. In polarized brain endothelial cells, transport protein expression can be restricted to the luminal or abluminal surface of the endothelial layer, controlling the flow of nutrients and other solutes into or out of the brain [11, 12].

The specialization of the BBB endothelial cells and their close interaction and regulation by surrounding cells result in a complex set of conditions that are difficult to replicate fully *in vitro.* Brain endothelial cells can be isolated from microvessels of brains from a number of different species including mouse, rat, pig, bovine, and human [13–19]. It was demonstrated that brain endothelial cells could be cultured as a monolayer on plastic or on tissue culture inserts, which resulted in a degree of differentiation and polarization. Measuring the transendothelial electrical resistance (TEER) and passage of small molecules across a tissue culture insert-based model allows determination of the permeability of the cell barrier [20]. As the complexity of the BBB was revealed, the requirements for more sophisticated *in vitro* models increased and double and then triple co-culture models have been developed in which brain endothelial cells are cultured alongside astrocytes and pericytes, allowing these cells to play their part in inducing the barrier phenotype and resulting in much improved *in vitro* barrier tightness [6, 20–24]. However, primary cells quickly lose their endothelial tight-barrier properties when maintained in culture. Additionally, these models are time-consuming and expensive to set up and maintain at the scale required for drug discovery purposes. There are a number of brain endothelial cells lines available [25, 26]; however, immortalization results in a lack of differentiation and the permeability of barriers formed with cell lines is much higher than barriers formed with primary cells. Within the last few years, one group of researchers have been using human stem cells to try and mimic the development of the BBB *in vitro* and determine whether this method will result in the formation of a model that has barrier tightness and other characteristics that are more comparable with the *in vivo* BBB [27, 28].

21.2.3 Nature's Solution

While the BBB has a reputation of being a restrictive barrier preventing access to the CNS. In reality it is a highly discriminative check point, providing tight control over what can and cannot enter and leave the CNS. A true barrier function would isolate the CNS from the rest of the body and prevent the passage of essential nutrients, hormones, signaling molecules into the CNS, and prevent the escape of potentially harmful waste products. The BBB therefore contains a variety of receptors and transporters that selectively take up the molecules the CNS requires for correct function and survival. In order for a functional brain environment to be maintained, mechanisms of transport across the BBB exist for necessary molecules. Depending on the molecule, transport can be via passive diffusion, either through cells or junctions, carrier-mediated transport, or

transcytosis. Passive diffusion can occur for small lipophilic molecules and there is a general correlation between lipophilicity of a molecule and its accumulation in the brain over time. However, other factors including the polar surface area and the number of hydrogen bonds can also influence a molecule's ability to cross the BBB passively. In addition, increasing the lipophilicity of a molecule can actually increase the potential for active efflux by one of the ABC transporters expressed by the endothelial cells, and therefore this is not always an effective method for increasing brain penetrance [29, 30].

Solute carriers (SLCs) are expressed on the cell membrane of the BBB endothelial cells allowing for the active transport of solutes that cannot pass into the brain passively. Members of the huge superfamily of SLCs can be localized to the luminal, abluminal or both faces of the membrane to control influx or efflux of a range of nutrients and neurotransmitters required by the brain [31]. These transporter proteins include the ATP-binding cassette (ABC) efflux transporters. Three subfamilies (B, C, and G) of the large ABC transporter family are particularly important in tissues that require a barrier function [32]. In the endothelial cells of the BBB, the ABC efflux pumps contribute to the neuroprotective function of the BBB by making sure that unwanted and potentially neurotoxic compounds such as xenobiotics, metabolic waste products and, unfortunately, most lipid-soluble drugs are extruded into the blood [30, 32, 33].

For larger macromolecules, receptor-mediated transcytosis (RMT) or adsorptive-mediated transcytosis (AMT) mechanisms exist for transport across the BBB. In RMT, a specific cell surface receptor is engaged by a macromolecular ligand causing internalization of the ligand–receptor complex. AMT relies on a charge-dependent interaction between a positively charged macromolecule and the negatively charged cell surface. Common to both mechanisms is the subsequent transport of the macromolecule across the endothelial cell, avoiding the lysosome, and release at the abluminal surface [30]. Several endogenous cell surface receptors have been identified as transport receptors for macromolecules at the BBB, including the transferrin receptor (TfR), melanotransferrin, insulin receptor, and LDL-receptor-related protein 1 [34–37]. Examples of substrates for AMT include most cell penetrating peptides and cationized proteins [30, 38, 39].

21.2.4 Targeting Pathways into the Brain

The principal route for macromolecule delivery across the BBB is RMT [30]. The precise mechanism of this transcytosis is not known, although extrapolation to endocytic transport in other cell types implies transport through a series of specialized vesicles. Attachment of drugs to carriers targeting receptors at the BBB potentially enables their delivery to the CNS. One of the widely reported transport pathways across the BBB is mediated by the TfR. The pre-clinical proof of concept of this approach was established almost two decades ago in rats [40] and mice [41].

In order to exploit RMT pathways using targeting ligands, such as antibodies, the binding kinetics need to approximate to those of the natural ligand. Typically, the natural ligands exhibit a high degree of selectivity but with kinetics that favor rapid association, and dissociation that favors continued binding during transcytosis but release when the complex reaches the abluminal side of the BBB. Many monoclonal antibodies achieve the first two parameters, of specificity and rapid association, but have slow dissociation kinetics, remaining bound to their target for long periods of time. Advanced protein engineering techniques can be applied to antibodies to precisely tailor their binding kinetics and overcome the slow off rate. Utilizing anti-TfR antibodies as a model system Yu *et al.* [42] investigated a series of engineered antibodies against mouse TfR to determine the affinity necessary for optimum brain exposure. They found that the parent, high affinity, antibody had measurable brain exposure only when dosed at very low amounts. When dosed at therapeutically relevant levels, the brain exposure did not appreciably increase. However, lower affinity variants of the parent antibody demonstrated progressively higher brain exposure as their affinity for TfR reduced. These antibodies have been fused to a therapeutic payload, an antibody targeting the amyloid-processing enzyme BACE-1 [42, 43], and have proven effective in effecting pharmacologically meaningful central responses in pre-clinical species [42–47].

Other work has found that valence of the antibody targeting TfR at the BBB can play an important role in CNS exposure. Niewoehner *et al.* [43] found that generating a targeting antibody with a single arm binding to TfR demonstrated significantly higher brain exposure than the equivalent bivalent antibody format. The authors also demonstrated reduced surface expression of TfR following prolonged exposure to the bivalent, but not the monovalent antibody, hinting at a potential deleterious effect on receptor function from the bivalent construct.

The same group [48] has reported that the pH dependency of the binding affinity also influences the degree of brain exposure. Antibodies that have a lower affinity for TfR at pH 5.5 than pH 7.0 achieve greater transcytosis and brain exposure than antibodies that have similar binding affinity at both neutral and acidic pH.

Unfortunately, direct comparison between studies is not possible as no data was provided to indicate the affinity of the antibodies for TfR in any of the studies by Yu *et al.* [42], Niewoehner *et al.* [43], or Sade *et al.* [48]. The key properties that determine the degree of brain exposure for anti-TfR antibodies probably lie somewhere within a combination of the three parameters of affinity, avidity, and pH dependence. Only by publishing measurements that enable a direct comparison of the data generated between all these studies will we be able to unravel how these parameters interact with each other and how they combine to influence brain exposure.

Insulin receptor (IR) has also been targeted to deliver macromolecular complexes to the CNS. A monoclonal antibody, termed HIRMAb, targeting insulin receptor in human and old-world primates has been developed. The BBB transport of HIRMAb has been exploited for the delivery of four classes of biologic

fusion proteins: lysosomal enzymes [49], neurotrophins [50, 51], decoy receptors [52], and therapeutic antibodies [53, 54] (reviewed in Ref. [55]).

Because of HIRMAb's specificity for primate and human IR, and the lack of efficacy models in primates for such fusion proteins, there are currently few examples of a measurable pharmacological response of the fusion proteins that have been developed. The one example where this has been investigated has generated data that casts doubt on the validity of such molecules for therapeutic use and is discussed below (Section 21.2.5).

Other strategies for targeting therapeutics to the brain have exploited more directly the natural ligands taken up by receptors at the BBB. Melanotransferrin (p97) is a GPI-anchored protein that was first described as expressed in melanomas. The protein is similar to transferrin in that it is an iron-binding protein [56]; however, it is proposed that rather than TfR it is low-density lipoprotein-receptor related protein (LRP1) that transports p97 across the endothelium [35]. Melanotransferrin shows enrichment in the brain following injection of the recombinant form and the targeting properties have been demonstrated when covalently linked to the small molecule drugs paclitaxel or adriamycin. The total accumulation of the p97-drug complex in the brain reached a 10-fold higher level than that of the free drug. When compared to non-targeted adriamycin, the fusion significantly decreased the progression of intracranial gliomas and mammary tumors [57]. After 24 h, p97-conjugates outperformed other, similarly designed vector-drug conjugates [58] and reached delivery levels of 1–2% of the injected dose, which is equivalent to the ratio of brain to body weight. Hence, p97 was thus claimed as the first carrier system to approximate this biological feature. Reports that Melanotransferrin can also transport larger macromolecules into the CNS are starting to emerge and it will be interesting to see how these compare to the antibody-based targeting approaches.

Derivation of smaller peptides from the alignment of natural ligands of LRP-1 was used to develop Angiopep-2, a brain targeting peptide [59]. Angiopep-2's brain delivery potential was demonstrated when it was used as a conjugate with three molecules of paclitaxel covalently attached to one Angiopep-2. Paclitaxel preserved its cytotoxic effect in this form and administration by intraperitoneal injection of the complex increased the median survival rate and prolonged the life span of mice that had intracerebral implantation of primary or metastatic carcinomas [60]. Angiopep-2 has become the most clinically advanced BBB targeting agent having successfully completed a Phase I trial, and it is now in Phase II clinical testing [61, 62].

The brain has a high requirement for lipids, and a large number of receptors for lipoproteins are present on the endothelial cells of the BBB. These receptors mediate the uptake of lipids, but also a number of other ligands, including certain drug molecules and lipid-based formulations, making them suitable targets for receptor-mediated transcytosis [63–66]. Low-density lipoprotein receptor (LDLR), a member of the LDLR family, is highly expressed at the BBB [46, 67, 68]. Using phage display of a peptide library, selections on the extracellular domain of the human low-density lipoprotein receptor (hLDLR) led to the identification

of a family of cyclic peptides. One of the lead peptides was optimized through a medicinal chemistry approach to improve its affinity and stability resulting in a peptide that crossed the BBB and could be detected within the brain using two photon microscopy techniques [69]. This peptide has not been studied and exploited to the same extent as some of the other technologies, but by using it as a targeting ligand on the surface of nano-particles, brain penetration and delivery of therapeutics have been demonstrated. *In vivo* tests showed significantly stronger brain penetration, glioma targeting, and enhanced chemotherapeutic effect of the paclitaxel-loaded nanoparticles compared with those of control groups in glioma mouse models [70].

21.2.5 Lessons from Preclinical Studies

Therapeutic delivery across the BBB is in its early stages of development and with only a small number of pathways and molecules so far in the drug discovery field there are few detailed safety studies available for scrutiny. However, two studies provide cautionary lessons. The first is as much due to the therapeutic element as to the BBB targeting element as it shows that the combination of two biologically active entities in one molecule can lead to unexpected, or at least unanticipated, effects as a result of the combined biology. As described above, the anti-insulin receptor antibody, HIRMAb, has been combined with a number of therapeutic molecules to treat CNS diseases. Two safety studies have been published examining the combination of glial-derived neurotrophic factor (GDNF)–HIRMAb, in healthy adult rhesus macaques [71] and in a rhesus macaque model of Parkinson's disease [72]. In the first study, animals received either a single i.v. dose, or repeated i.v. doses of HIRMAb–GDNF every 12 h for five consecutive doses over a 60-h period. A full toxicological analysis was performed either 24 h post the final dose, or 13 days following the final dose. The study concluded that there were no adverse events associated with the acute administration of large doses of the HIRMAb–GDNF fusion protein, and established a no-observable-adverse-effect level for future human clinical trials. However, the subsequent study came to rather different conclusions. Animals were dosed at 1 or 5 mg/kg of HIRMAb–GDNF for a period of three months in animals suffering symptoms similar to Parkinson's, induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine. The treatment did not improve symptoms, but induced a dose-dependent hypersensitivity reaction, characterized with skin flushes, eyelid edema, vomiting, urticaria, and in some cases respiratory distress, and minimal to mild non-suppurative myocarditis. Circulating antibodies against the HIRMAb–GDNF were detected in all treated animals. In addition, serious health risks were also identified in a number of animals in the form of focal pancreatic metaplasia likely caused by proliferative actions of GDNF targeted to IR in pancreatic tissues [72].

In a study examining the safety of anti-TfR antibodies for BBB transport [3] the antibodies were found to induce hypersensitivity reaction in rodents; a single dose

of a TfR bivalent antibody at doses as low as 1 mg/kg caused reversible but severe acute clinical signs as well as profound reticulocyte reduction in mice. The severe lysis of TfR-enriched reticulocytes was due to complement-dependent cytotoxicity triggered by the effector-competent Fc. The effect was reduced but not eliminated by removing effector function from the Fc and by lowering the affinity of the antibody to TfR. However, even a small level of effector function was sufficient to trigger reticulocyte lysis at higher doses and may limit the maximum tolerated dose in man.

The receptors targeted by many of the BBB vectors are not only expressed on brain endothelial cells and their presence in other tissues, and on other cell types, including cells within the CNS could create a secondary safety risk. It is therefore important to achieve a degree of BBB vector selectivity to improve safety margins. It may also be important to remove all effector function from the antibodies to avoid any risk of effector function mediated inflammatory effects within the confines of the CNS.

21.2.6 ADME in the Brain

BBB delivery is still in its relative infancy and recent advances have been focused on improving efficiency of delivery into the CNS. However, there remains a significant knowledge gap in understanding the mechanisms of transcytosis and the fate of the targeted molecules once they arrive in the CNS.

In the periphery, distribution, tissue penetration, target engagement, clearance, and metabolism of antibody drugs are all relatively well understood. In the brain there is very little understanding of these parameters, particularly when applied to molecules delivered across the BBB. Therapeutic applications of BBB delivery technologies will have double the uncertainties as they will contain the BBB arm and the therapeutic arm, both capable of independent and co-dependent engagement with their targets. Target engagement by the drug arm will lead to target-specific antibody disposition. If this is to a cell surface target this could lead to antibody internalization and intracellular degradation, whereas antibody binding of a soluble target could result in complex elimination via circulation of brain fluids, or uptake by immune cells in the brain. The target-mediated disposition of BBB-crossing bispecific antibodies is additionally complex because the BBB carrier arm itself often binds a central target; such is the case with TfR and IR antibodies. To determine the target exposure of such antibodies it is important to determine their brain residence time, governed by multiple, and still poorly understood, interacting elimination pathways. Some BBB targeting domains will function bi-directionally at the BBB enabling reverse transport and all will be subject to convection of brain interstitial fluid along perivascular routes and its exchange with the cerebrospinal fluid (CSF) [73]. Antibodies will also be subject to interaction with Fc receptors, both on immune cells, such as the Fcγ-receptors on microglia, and FcRn on endothelial and epithelial cells that make up the barriers within and around the CNS. The relative contribution of each of these pathways

to antibody residence, elimination or degradation in the CNS remains unknown. Filling in these gaps in our knowledge will be critical for understanding the true brain exposure for each BBB-enabled bispecific fusion protein and for managing its safety.

21.2.7 Path to the Clinic

Two BBB carrier technologies have entered clinical trials: ANG1005, the peptide BBB carrier-paclitaxel conjugate for primary and metastatic brain tumors [74, 75], and HIRMAb-iduronate sulfatase fusion protein [76] for the rare lysosomal storage disease, Hunter syndrome. Trials with ANG1005 have shown that it delivers paclitaxel across the BBB and achieves therapeutic concentrations in tumor tissue. The conjugate shows a similar toxicity profile to paclitaxel and appears to have activity in recurrent glioma. Importantly, there was no evidence of CNS toxicity as a result of the increased CNS exposure. Both ANG1005 and HIRMAb-iduronate are treatments for terminal diseases with no known cure and are therefore tolerant of lower, or less certain, safety profiles. The challenge for future treatments is to ensure that the combination of disease, therapeutic drug, and BBB delivery technology comes together to provide efficacy at a safety level suitable for mass market use. The stakes in developing systemically active antibody therapeutics for CNS indications are exceedingly high, given the vast unmet medical need of many neurological disorders. The potential for developing a market for a new class of therapeutics with high target selectivity and potency to treat CNS-related diseases will continue to offer hope for millions of patients. Despite the risks, the field is moving toward grasping this opportunity.

21.2.8 Future Perspectives

A search for alternative BBB transporter targets has been attempted through phenotypic approaches and also the potential of "omics" technologies. The identification, through phage display selections on human brain endothelial cells, of a series of camelid domain antibodies that are able to cross the BBB [77] indicates the possibility of the identification of BBB transporters that do not rely on predefined molecular receptors. This type of approach has been taken further, utilizing *in vivo* selection of phage particles displaying random peptide sequences [78], and has identified a series of peptides that show enrichment in CSF following peripheral delivery. The authors also showed that a peptide lead was able to promote brain exposure of a biotinylated-BACE1 peptide inhibitor coupled to streptavidin and produced a clear pharmacodynamic effect in the CNS by significantly lowering CSF levels of amyloid β.

Proteomic approaches have been used to identify the most abundant proteins at the BBB to try and determine the most abundant transporter for delivery across the BBB. Several studies have utilized cultured brain endothelial cells for this work

but have been hampered by dramatic changes in receptor expression levels when the cells are cultured *in vitro.* Using proteomic techniques on primary mouse brain endothelial cells [79] identified basigin, Glut1, and CD98hc as highly abundant antigens. The authors went on to investigate antibodies to each of these targets and found that they were significantly enriched in the brain after administration *in vivo.* They found that antibodies against CD98hc showed robust accumulation in brain after systemic dosing. Interestingly, the authors also demonstrated that there was an inverse relationship between the affinity of the antibody for CD98hc and brain uptake, as was previously found for TfR [42].

These approaches undoubtedly have the potential to uncover new pathways into the brain and allow the development of more robust, and perhaps safer, approaches to CNS delivery. The path forward may have to include the rational selection of BBB shuttles, perhaps specifically paired with individual drug molecules, and be incorporated into improved formats, tested in translational pharmacokinetic/pharmacodynamic models tailored to bispecific biologics, and very rigorous safety/toxicology studies.

21.3
Intracellular Biologics

21.3.1
The Opportunity for Intracellular Biologics

Protein therapeutics have, to date, focused primarily on extracellular targets due to the considerable challenges of delivering proteins efficiently into the intracellular space. However, new technologies are now being developed to increase the efficiency of protein uptake into the cell interior and this has led to a renewed drive toward applying proteins therapeutically against intracellular targets. The rationale for doing so is clear. Bioinformatic analyses predict that around three-quarters of the expressed human genome resides intracellularly [80], which clearly constitutes a major therapeutic opportunity in virtually all disease settings. While some would argue that it is only a matter of time before effective small molecule drugs are raised against the most prominent intracellular targets, many highly attractive targets have proved more or less "undruggable" via medicinal chemistry approaches [81]. In contrast, the exquisite specificity and high potency possible with protein drugs, could introduce an entirely new approach to many of these "undruggable" targets and potentially lead to novel and effective therapies in the future.

21.3.2
The Challenges of Intracellular Delivery

While the opportunity for intracellular biologics is great, the challenge to efficiently deliver proteins into cells is also significant. By analogy, it is worth

considering the long-standing efforts to deliver small inhibitory RNA (siRNA) molecules therapeutically to patients. When the RNA interference mechanism was first discovered it was hailed as a breakthrough [82] and offered a potential means to specifically target virtually any gene of interest, including those encoding the various intractable targets which other modalities could not reach. However, the challenge of delivery was largely overlooked in the initial excitement about the therapeutic potential of siRNA and this has impacted its translation in the clinic. In particular, the high aqueous solubility of siRNA molecules causes a fundamental inability to penetrate the protective lipid bilayers of target cells, especially the outer plasma membrane layer or internal endosomal membranes. In fact, reaching an endosome is by no means indicative of successful intracellular delivery as it has been shown that 98–99% of lipid-nanoparticle delivered siRNA does not escape from endosomes to reach the cytosol, where the siRNA must be delivered to exert its inhibitory activity [83]. These observations are worth considering for the analogous challenge of therapeutic protein delivery into cells. Small, duplex siRNA molecules have some similar properties to therapeutic proteins in that they are water-soluble and relatively large (with a molecular mass typically above 10 000 Da). While unmodified siRNA and protein are both capable of uptake into cells, the key question is really whether it can be done efficiently enough to elicit an effective therapeutic response.

If we extend this challenge to include the many other aspects of drug delivery for a systemically administered intracellular biologic, there are some useful parameters that should be considered in their optimal drug design (Table 21.1). While an

Table 21.1 Key parameters for functional intracellular delivery of therapeutic proteins.

I. Targeting	*Efficient biodistribution to the cell*: The drug needs to evade the immune system, clearance from the circulation, and sequestration by irrelevant targets in the extracellular environment *Binding to the target cell*: The drug needs to associate with the target cell surface through targeted interactions (e.g., with anti-receptor antibody) or untargeted interactions (e.g., charged residues with cell surface proteoglycans) in order to initiate the process of internalization
II. Uptake and translocation	*Uptake into the cell*: Efficient uptake must be achieved through, for example, endocytic mechanisms, mediated through clathrin *Release from intracellular vesicles*: Release from or disruption of endosomes or translocation from the endoplasmic reticulum (ER) following retrograde transport is required
III. Payload activity	*Stability of functional "payload"*: A functional protein "payload" element, such as an enzyme or inhibitory antibody, will need to be stable in both the extracellular oxidizing environment and the intracellular reducing environment in the cytoplasm *Potency of functional "payload":* The potency relative to the attainable intracellular concentration will be important, especially for proteins modulating their targets stoichiometrically rather than catalytically

intracellular biological drug that is not targeted to a particular cell type is in theory possible, and analogous to the regime for small molecule delivery, the higher manufacturing costs of protein drugs and the desire to achieve the maximal therapeutic window would tend to favor targeted uptake of the drug only in the cells of interest, such as tumor cells. This would simultaneously minimize the capacity for drug loss in inappropriate cell types and also reduce the potential for toxicity in normal healthy cells. Subsequent to targeting the drug to cells of interest is the paramount challenge of efficient uptake and release inside the cell. Success in this area primarily depends on the efficient escape from intracellular lipid vesicles, such as endosomes, as discussed above for siRNA molecules. Finally, once delivered to the intracellular space, it is important that the "payload" portion of the drug is both highly potent and sufficiently stable to perform its modulatory function prior to its degradation or release from the cell.

21.3.3 Nature's Solution to the Challenges of Intracellular Delivery: AB Toxins

The challenge of modulating intracellular biology with an extracellular protein is widespread in the biology of multicellular organisms. In these systems, signal transduction via cell surface receptors normally achieves the objective of transmitting extracellular protein "messages" to the cell's interior without the need for the extracellular protein messengers themselves to directly penetrate the cell membrane. For this reason, there are few, if any, examples of extracellular human proteins that require cell uptake and endosome release in order to transmit a signal to a target cell. It is probably no coincidence that this mechanism is disfavored because it would be energetically costly for cells to actively transport soluble proteins through lipid bilayers on a routine basis. However, there are several examples of bacterial and plant proteins whose intracellular effects on human cells are reliant on their delivery to the cell interior. For this reason they are interesting to study in order to understand their uptake mechanisms.

In particular, the bacterial AB toxins [84] have evolved strategies to directly access the cytoplasm of host cells and disrupt intracellular processes. These toxins derive their name from their enzymatically "active" A subunit and a distinct "binding" or B subunit. They also frequently contain some form of translocation domain to aid the delivery of the A subunit into the cytoplasm. The first step in their uptake requires an interaction of the B subunit with a cell surface receptor to trigger endocytosis into the cell. Once the toxin has entered the endosome, the increasingly acidic pH, generally reducing environment, and presence of highly active proteases are exploited by the toxin to enable translocation to the cytoplasm. Diphtheria toxin (DT), for example, escapes directly from endosomes by changing its conformation in response to the acidic pH and exposing a hydrophobic translocation domain which inserts into the endosomal membrane. The catalytic A subunit of DT is then able to translocate to the cytosol. Because a fully folded protein would be too large to pass through the pore in the endosomal membrane, the ability of the DT A subunit to unfold, and subsequently refold in

the cytosol, is essential [85]. By contrast, toxins such as *Pseudomonas* exotoxin A employ a retrograde trafficking strategy [86], traveling via endosomes and Golgi to the endoplasmic reticulum (ER) using an ER-localizing motif similar to KDEL before translocating through the ER membrane to the cytoplasm with the help of a host protein translocon, thought to be Sec61 [87]. Regardless of the trafficking route, the final stage of AB toxin function is to inactivate a key host cell protein or process. Without exception, the A subunits of all AB toxins employ catalytic rather than stoichiometric mechanisms to inhibit their target protein in the host cell. By employing catalytic turnover, a single toxin molecule can inactivate multiple target molecules, thereby enabling dramatic phenotypic effects with relatively few successfully delivered toxin molecules [88].

Taken together, the unraveling of the mechanisms of AB toxins has shed considerable light on what constitutes an effective strategy for functional intracellular protein delivery. It is clear that these remarkable proteins have surmounted all the challenges outlined in Table 21.1 and are able to enter the cell and modulate intracellular processes. Furthermore, their modular protein structure offers the opportunity to swap in alternative domains in order to redefine both the cell specificity and the intracellular function, as outlined in the following section.

21.3.4
Re-Engineering AB Toxins for Novel Therapeutic Functions

The predominant approach to exploiting toxin biology for therapy has been the development of immunotoxin drugs in which a toxin's catalytic activity is redirected to a tumor cell by replacement of the "binding" B moiety of the toxin with an antibody or other cell-binding ligand [89]. Over 40 different immunotoxins have been tested clinically. Most have been adapted from *Pseudomonas* exotoxin A, DT or the plant-derived toxin ricin, and some have shown impressive therapeutic benefit. One example is the immunotoxin Moxetumomab pasudotox, which comprises an anti-CD22 antibody fragment fused to the translocation and catalytic domains of *Pseudomonas* exotoxin A. This highly-potent immunotoxin inhibits protein synthesis within CD22-positive B cells, and in Phase I testing produced complete responses in 46% of patients with chemotherapy-resistant hairy cell leukemia [90]. Following the clinical proof of concept for immunotoxin therapy, the recent work in this field has focused in three main areas: reducing the immunogenicity of protein toxins [91], exploring synergies with other therapies [92], and improving our understanding of toxin translocation to the cytoplasm [93].

Following on from the application of immunotoxins, the ultimate goal of exploiting the AB toxin delivery mechanism would be to deliver a protein payload of choice to a cell which is driving a particular disease phenotype in order to achieve a therapeutic response. The immunotoxin field has already demonstrated that the cell specificity of the toxin can be re-engineered through the addition of antibodies or other ligands to replace the native B subunit and redirect the toxin to an internalizing cell surface receptor on the diseased cell. Now the focus is

turning to replacing the catalytic A domain of the toxin with alternative payloads of interest, such as antibodies or antibody mimetic proteins against intracellular targets.

One particular engineered system, which is based on two protein chain components, utilizes a re-targeted protective antigen (PA) of anthrax toxin, which forms a heptameric pore in endosomes, co-administered with the N-terminal domain of anthrax toxin lethal factor (LF_N), which achieves cell entry via the heptameric PA pore. To achieve cell specificity, PA was first mutated to ablate the binding to the anthrax receptors, and then the mutant PA (mPA) was fused to the natural ligand epidermal growth factor (EGF), which confers selectivity for EGF receptor-positive tumor cells. This specificity was demonstrated by delivery of DT catalytic domain fused to LF_N to kill EGF receptor-positive tumor cells, using mPA–EGF for targeting [94]. The concept has also been extended by replacement of the EGF ligand with other targeting moieties such as affibodies [95] and a single-chain antibody fragment [96]. At the payload end of the system, the LF_N component has been fused to an antibody mimetic monobody to Bcr-Abl kinase and an affibody to hRaf-1 into mammalian cells, leading to effects on apoptosis and signaling respectively [97].

The modularity of the *Pseudomonas* exotoxin A system has also been demonstrated by fusing the cell penetrating peptide 10R to the N-terminus of the translocation domain of *Pseudomonas* exotoxin A, and additionally fusing green fluorescent protein (GFP) as a "payload" to the C-terminus of the same domain. In this system it was possible to demonstrate via fluorescent imaging that the delivery of GFP to the cell cytoplasm was more efficient with the construct containing the translocation domain than for the 10R–GFP fusion alone, suggesting that the translocation domain was able to enhance cytoplasmic release [98]. Building on this observation, it was recently shown that designed ankyrin repeat proteins, or DARPins, can replace both the cell targeting and payload domains of *Pseudomonas* exotoxin A by genetically fusing a DARPin at either end of the translocation domain [99]. Using an N-terminal DARPin to EpCAM to facilitate cell entry and a "dummy" DARPin payload containing an avi-tag, it was possible to show cytoplasmic biotinylation of the avi-tag in target cells expressing the biotin ligase BirA. This paper in particular provides an intriguing template to follow, by using alternative constructs with "active" payloads, that is, DARPins which can functionally inhibit intracellular targets, it may be possible to achieve an intracellular response from an exogenously delivered biological drug.

A third example of an engineered AB toxin system is adapted from *Botulinum* neurotoxin. The disulphide-linked heavy and light chains of *Botulinum* neurotoxin, which confer binding and translocation or catalytic function, respectively, can be used for the delivery of alternative payloads. By inactivation of the catalytic activity of the light chain, and fusion with a luciferase protein or GFP, functional delivery of the new cargo into neurons could be demonstrated [100]. Recent work with *Botulinum* toxins has also highlighted their potential to be retargeted to other cell types, for example, by fusion with growth hormone-releasing hormone as a ligand to target the pituitary gland [101].

Taken together, these examples demonstrate the cell delivery, efficiency, and modularity of toxin domains, and underline their potential to help in the design of future intracellular biological drug therapies. Although the bacterial or plant origin of toxin components may lead to concerns over immunogenicity, the recent advances in technologies to de-immunize proteins for therapy could potentially offset such a risk [102]. Furthermore, it seems that in the case of some AB toxins, such as *Pseudomonas* exotoxin A, the translocation region and KDEL motif may be the only portions of the original toxin actually required for cytoplasmic delivery and it has been shown that the size of the translocation domain can be dramatically reduced to a short peptide of less than 15 amino acids without significantly reducing delivery efficiency [103].

21.3.5
Alternative Delivery Strategies for Intracellular Biologics

While the AB toxins provide an attractive paradigm for intracellular delivery of proteins and show much promise, as judged by the clinical success of immunotoxins, there are several other protein delivery strategies in development.

A widely used approach to deliver proteins into cells is the fusion with 10–30 amino acid amphipathic or polybasic cell-penetrating peptides (CPPs) [104]. Initially, the membrane-disruptive sequence from HIV Tat protein [105] showed promise and could be fused to enzymes including β-galactosidase and the catalytic domain of *Pseudomonas* exotoxin A to demonstrate delivery. Since then the list of proteins delivered to cells with CPPs has expanded greatly, including active proteins such as p53 [106], the BH4 domain of Bcl-xL protein [107] and antibody fragments [108, 109].

These positively-charged peptides associate with the glycosaminoglycan on mammalian cells to initiate endocytosis. However, for CPP–protein fusions, intracellular delivery is often of low efficiency, possibly due in a major part to endosomal entrapment. Mechanisms of endosomal release for CPP-cargos remain poorly understood, and cargos that require higher concentrations inside cells often fail to show significant biological effects [110, 111].

An alternative to CPP's is to introduce positively charged residues onto the surface of the protein payload itself and this has recently been used for intracellular delivery. Initially focusing on an engineered variant of GFP with a very high net positive charge, Liu and colleagues discovered that supercharged proteins were able to penetrate mammalian cells and deliver fused macromolecules [112, 113]. The principle of using positive charges to deliver proteins has also been used with zinc-finger domains and 36 amino acid miniproteins [114, 115].

The fact that CPPs and supercharged proteins bind to and enter a wide variety of cells, while useful *in vitro,* leads to relatively poor pharmacokinetic and biodistribution properties for CPPs and CPP–protein fusions *in vivo* [116, 117]. The general conclusion on this class of delivery agents is that they provide relatively low efficiency of cell entry and require additional technology development to enable

endosome release before they can provide a useful route for delivery of therapeutic proteins.

With this in mind, one approach that has been used to improve endosome release of CPP–protein cargos has been to introduce peptides that disrupt membranes at acidic pH into the fusion. For example, addition of the HA2 peptide enhanced delivery to A549 cells of red fluorescent protein tagged with R9-polyarginine [110]. Furthermore, the natural principle of using protonation of acidic residues in viral peptides to promote endosomal release has been mimicked in the use of a range of synthetic, pH-dependent polymers to deliver proteins into the cytoplasm of cells [118–121]. For example, the poly[2-(diisopropylamino) ethyl methacrylate] (PDPA)–poly[2-(methacryloyloxy) ethyl phosphorylcholine] (PMPC) co-polymer [RM41 Canton], which forms a nanovesicle at physiological pH, becomes protonated within the endosome to cause temporary osmolysis of the endosomal membrane and release of an encapsulated protein. PMPC–PDPA vesicles were used for the delivery of a functional antibody against NF-κB to HDF cells, with effects on lipopolysaccharide (LPS)-stimulated IL-6 release. All these pH-dependent systems are at early stages, but they further illustrate the potential of using the changing cellular environment to promote intracellular delivery. The pH-dependent polymers are particularly attractive because antibodies directed against internalizing receptors, such as CD19 on B cells [121], can be bound to these delivery vehicles to assist targeting and intracellular delivery of the cargo.

21.3.6 Increasing the Potency of Intracellular Payloads

Once delivered to the cytoplasm or other intracellular compartment, a protein payload must retain sufficient potency and stability to modulate cellular function. The proteins delivered to cells by toxins are highly potent and modify cell physiology at low concentrations due to their catalytic activity. To pass through membranes, the A subunits of AB toxins also require suitable thermodynamic properties, such as a lack of disulphide bridges, to enable unfolding for translocation and refolding in the cytosol. Engineered protein payloads that need to escape the endosome through membrane channels will have to undergo similar unfolding and refolding pathways. By contrast, for the cytoplasmic delivery mechanisms such as endosome osmolysis employed by polymersomes, the protein does not need to unfold to pass through the membrane, and stability to the reducing environment of the eukaryotic cytoplasm is therefore the key factor.

The requirements for intracellular protein concentration are stringent for stoichiometric inhibition, which is the typical mechanism of action for antibodies and antibody-mimetics. Intracellular concentrations up to 240 nM were estimated for delivery of antibody-mimetic payloads via anthrax toxin PA[98] and up to 590 nM for delivery via *Pseudomonas* exotoxin A translocation domain [99]. High stability and potency under intracellular conditions will therefore be required to achieve efficacy. On the question of stability it is of interest to note that many AB toxins have evolved with a significant reduction in the number of lysine residues in

their active domains. It is proposed that the lack of lysine residues is a mechanism for escaping ubiquitin-mediated protein degradation during translocation into the cytosol, given that ubiquitin attachment occurs via lysine residues [122]. Such a strategy could conceivably be employed to extend the cytoplasmic half-life and therefore potency of engineered intracellular protein payloads. An additional area of research, also reliant on ubiquitination, is concerned with boosting payload potency by providing antibodies with a catalytic mechanism of action. In this case the ubiquitin-mediated degradation pathway is enlisted for specific elimination of target molecules. The "ubiquibody" concept re-engineers the natural substrate specificity of the human E3 ubiquitin ligase CHIP (carboxyl terminus of Hsc70-interacting protein) by replacing its natural substrate-binding domain with an alternative binding domain, such as an antibody fragment. By fusing a β-galactosidase-specific antibody fragment to CHIP and expressing this ubiquibody in COS-7 cells transiently expressing β-galactosidase it was possible to show targeted degradation of the antigen, an effect which could also reproduced in other mammalian cell types [123]. Ubiquibodies therefore offer a potential route to harnessing a catalytic mechanism for the specific degradation of intracellular targets and in doing so could dramatically increase the potency of antibody payloads in cells.

21.3.7
Conclusions and Outlook for Intracellular Biologics

Engineered proteins that have to date shown therapeutic utility inside cells, namely the immunotoxins, have simply replaced the cell-binding domain of a toxin with a specific cell targeting domain of an antibody. Thus, they have not yet been engineered to address specific, disease-relevant targets within a cell. While this is still the ambition for the intracellular biologics field, the most significant barrier is the need to escape the endosomal entrapment and/or degradation pathways, where prior molecules have lacked the sophistication of native toxins. To address this it will be necessary to introduce features allowing translocation across membranes, or the disruption of these membranes triggered by naturally occurring cellular processes. Reviewing the many approaches taken to date, the most promising molecules are believed to be antibody (or antibody mimetic)-targeted polypeptide constructs that adopt the trafficking and translocation mechanisms of toxins. Provided that these can be rendered non-immunogenic, with suitable pharmacokinetics, and can be manufactured at scale, these molecules could be effective intracellular therapies in the future.

21.4
Building on the Success of Traditional Monoclonal Antibodies

Four decades have passed since César Milstein and Georges J. F. Köhler discovered the hybridoma technology which allowed researchers to isolate monoclonal

antibodies [124]. This was followed by the development of approaches for humanizing these antibodies, which ushered the dawn of antibody therapeutics and a realization of the "magic bullet" concept first espoused by Paul Erlich in the early 1900s. Additional technologies such as phage display, human IgG transgenic mice, and affinity maturation were developed in the 1990s to enable scientists to identify highly potent and specific human antibodies. These breakthroughs accelerated the pace of antibody drug development. As a drug class, therapeutic antibodies have rapidly grown and now dominate biologic drugs with about four dozen products approved in the United States and Europe by the end of year 2015 for a broad range of diseases including cancer, inflammation, autoimmune diseases, infectious disease, ophthalmological disease, and cardiovascular disease. In 2015 alone, there were nine novel antibody therapeutics that received US market approval. These were secukinumab (Cosentyx®), alirocumab (Praluent®), elotuzumab (Empliciti®), evolocumab (Repatha®), daratumumab (Darzalex®), dinutuximab (Unituxin®), idarucizumab (Praxbind®), mepolizumab (Nucala®), and necitumumab (Portrazza). In 2014, 5 of the top 10 pharmaceutical products by worldwide sales were antibodies. These products (Humira®, Remicade®, Rituxan®, Avastin®, and Herceptin®) achieved annual sales greater than $6 billion [125]. Besides antibodies, Fc-fusion proteins also play a significant role in therapeutic biologics with nine approved products on the market [125].

21.4.1 Rise of "Non-traditional" Antibodies

Will the rise of therapeutic antibody drugs maintain its momentum going forward? A survey of current clinical stage programs indicates that several hundred novel antibodies are undergoing human trials, suggesting that this class of molecule will continue to dominate the biologics field for many years to come. The majority of the current market-approved or clinical-stage antibodies are classic monoclonal IgG molecules targeting a single antigen in a bivalent fashion. However, despite their high affinity and specificity, IgGs sometimes have little or no clinical efficacy against certain disease targets, particularly in complex diseases such as cancer, inflammation, and immunological diseases. This is because complex diseases are usually caused by multiple components or pathways; therefore, targeting a single disease mediator with a monoclonal antibody may be insufficient to achieve a therapeutic outcome. In other cases, where the mechanism of action calls for the antibody to bind and mark the target disease cells for destruction, natural effector functions (antibody-dependent cellular cytotoxicity, complement-dependent cytotoxicity, and antibody-dependent cellular phagocytosis) that are elicited by antibodies of the IgG1 subclass may lack sufficient potency. For these reasons, there is an increasing need to develop novel antibodies with enhanced and/or additional properties over classic antibodies. These so-called "non-traditional" antibodies are generated by cutting-edge protein engineering approaches and many do not normally occur in nature. These include antibodies engineered to have enhanced [126] or diminished effector

functions [127] or exhibit longer circulation half-life [128], multispecific biologics, antibody–drug conjugates (ADCs), antibody mimetics (protein scaffolds), immunotoxins, immunocytokines, and so on. Among these novel modalities, two drug classes have generated considerable excitement for their potential to have a profound impact on therapeutic space. One of these is bi- and multispecific biologics, the other being ADCs.

21.4.1.1 Bispecific Antibody and Multispecific Biologics

Bispecific antibodies combine the affinity and specificity of two antibodies into a single molecule that is capable of binding to two different antigens, or two different epitopes on the same antigen. Exploiting the modular architecture of antibodies, researchers have generated a large number of bispecific antibody formats possessing a wide range of antigen-binding geometry, valency, specificity, avidity, molecular mass, and half-life. Many of these bi-specific formats have overcome the technical hurdles of discovery, manufacturing, and formulation and have moved into clinical application. Thus far, more than 30 bispecific antibodies have entered clinical development and the first two products, catumaxomab (Removab®) and blinatumomab (Blincyto®), have been approved [129]. Catumaxomab and blinatumomab are both used in the treatment of cancer, and combine a tumor-specific binding arm with a second specificity against CD3 that is used to recruit human effector T cells that can destroy cancer cells. Blinatumomab is made of 2 scFvs connected together through a flexible peptide linker (BiTE; bispecific T-cell engager) and has a short serum half-life; it is however highly potent [130]. On the other hand, catumaxomab was generated using the quadroma technology. It is an IgG-like trifunctional bispecific antibody. Besides recruiting T cells and targeting cancer cells, it can also activate NK cells, monocytes, macrophages, and dendritic cells through its interaction with Fcγ receptors [131]. Both molecules are highly potent and only require very low doses in clinical setting.

The majority of current clinical-stage bispecific antibodies fall into two categories: one is designed to recruit T cells to target cancer cells whereas the other simultaneously targets two disease mediators [129]. Most of these programs focus on cancer therapy but some are being developed for inflammation and autoimmune diseases. However, application to additional disease areas is also taking root with this novel approach. For example, a multifunctional bispecific antibody has been developed to protect against the infection of *Pseudomonas aeruginosa* which is one of the most difficult ESKAPE pathogens to treat, and a leading cause of acute nosocomial pneumonia and chronic lung infection in cystic fibrosis patients [132]. This bispecific antibody, MEDI3902, binds to virulence factor PcrV and persistence factor PsI exopolysaccharide, conferring three mechanisms of action against this pathogen through the inhibition of PcrV-mediated cytotoxicity, opsonophagocytic killing, and inhibition of host cell attachment. This antibody is currently in clinical trials for the prophylaxis of nosocomial pneumonia. The successful development of this drug may demonstrate that bispecific targeting is a promising approach to complement

and preserve shrinking antibiotic options for serious drug-resistant bacterial infections.

The bispecific approach has also been applied to the treatment of hemophilia. A recombinant bispecific molecule (ACE910) binding to both factor IXa and factor X was generated through extensive protein engineering and screening [133]. ACE910 is an asymmetric bispecific IgG antibody that mimics the function of coagulation factor VIII (FVIII). It was developed to overcome two major limitations of the current treatment which utilizes plasma-derived or recombinant human FVIII (FVIII). First, FVIII therapy requires frequent intravenous dosing (three injections per week) due to its short half-life and low subcutaneous bioavailability. Second, approximately 30% of patients with severe hemophilia A treated with FVIII develop anti-FVIII inhibitory antibodies, rendering current FVIII replacement therapy ineffective. The ACE910 bispecific antibody offers long half-life, subcutaneous administration and is effective in patients who have developed inhibitory antibodies to FVIII. This experimental therapy is currently in Phase II clinical trials, and could provide significant benefit for severe hemophilia A patients.

The bispecific concept has also been expanded beyond the fusion of two antibodies to include a variety of bi-functional biologics, such as immunocytokines [134], antibody-receptor domain fusion molecules [135], peptide–antibody fusion proteins [136], and so on. Furthermore, one of the benefits of dual targeting is to potentially increase the target selectivity via the avidity effect obtained by concurrent binding to two different cell surface antigens on a target cell. Interestingly, a recent study has shown that dual targeting alone is not sufficient to ensure selectivity for cells expressing both antigens over those expressing just one [137]. Effective discrimination required the fine tuning of the affinity of the individual binding arms, overall avidity, and valency to achieve efficient target cell selectivity.

Besides bispecifics, researchers have been exploring multispecific targeting. IgG-like trispecific antibody against EphA2, EphA4, and EphB4 [138] and tetra-specific antibody against EGFR, HER2, HER3, and VEGF [139] have been generated. Results showed that simultaneously blocking multiple targets offers a very attractive approach for cancer therapy. However, there are some potential drawbacks for such multispecific antibodies. In particular, the molecular mass is substantially large (>200 kDa), which may hinder tumor and tissue penetration. In addition, the expression yield and stability of these biologics may be significantly impacted with the increase in molecular complexity. Protein scaffold-based multispecific therapeutics may offer an alternative approach. Such scaffolds are generally much smaller in size than their antibody counterparts and have similar high binding affinity and specificity. Many bispecific protein scaffolds have been generated and one, an anti-VEGF/anti-HGF DARPin molecule, has been advanced into clinical trials [140]. Multivalent scaffold proteins have also been generated [141]. Given their intrinsic high stability, high expression and small size, protein scaffolds are likely the most ideal platform for generating multispecific biologics, in particular for targeting four (or more) antigens or epitopes simultaneously.

Following the technology maturation of a variety of bi- and multispecific approaches and their combination with other protein engineering technologies, researchers are now able to readily generate many novel therapeutics for a wide range of diseases. For instance, multispecificity could be combined with an engineered Fc region to produce therapeutic agents with an extra-long circulation half-life and with enhanced (or diminished) effector functions to target disease cells. The entire treatment regimen may just require a single dosing due to the high potency and long half-life of the drug. To summarize, bi- and multispecific molecules offer tremendous potential for treating complex diseases. This class of biologics will likely rise to challenge the dominance of traditional monoclonal antibodies as therapeutics.

21.4.1.2 **Antibody–Drug Conjugates**

Another promising "non-traditional" antibody drug format is ADC, a hybrid of small and large molecule. ADCs combine highly specific antibodies against tumor-associated antigens with potent cytotoxic chemical drugs via a stable linker. This class of molecules has gained great interest in recent years for cancer therapy. Currently, there are two ADC drugs approved on the market: brentuximab vedotin (Adcetris®; target: CD30) for hematological cancer and trastuzumab emtansine (Kadcyla®; target: HER2) for breast cancer. There are approximately 40 ADCs in clinical development [142].

Despite some clinical success and heavy R&D investment in the industry for many years, ADC development continues to face many hurdles. They are extremely potent drugs and can kill targeted cancer cells *in vitro* at a very low dose. Although the therapeutic window for ADCs is higher than for cytotoxic chemicals alone, current ADCs have not generally lived up to their promise of exhibiting a wide therapeutic window. One of the main reasons is poor biodistribution and penetration of ADCs into tumors, in particular solid tumors. This leads to a substantially higher minimum effective dose *in vivo* than one would expect based on their highly potent activity observed in *in vitro* assays. Dose-limiting toxicity, due to a combination of on- and off-target toxicity, remains one of the biggest challenges in ADC development and also accounts for a key part in their narrow therapeutic index. A leading cause of the off-target toxicity is that a majority of administered ADCs are circulating in the body for a long-period of time (on account of their antibody carriers); therefore, the prolonged exposure leads to ADC molecules entering normal cells through non-specific pinocytosis and other mechanisms [142]. In addition, ADCs are generally more hydrophobic than unconjugated antibodies due to the drug moiety, which increases their non-specific interaction with normal tissues and thus enhances toxicity. On-target toxicity usually stems from low-level expression of the target antigen in normal tissues resulting in ADC uptake and accumulation by normal cells.

Despite many hurdles in the development of ADCs, researchers have been encouraged by early clinical successes. The immense potential for cancer treatment prompted considerable efforts to resolve these issues by developing the

second generation of ADC technologies. These include newer cytotoxic warheads such as pyrrolobenzodiazepines (PBD), which are much more potent than earlier warheads (auristatin, maytansine) and can resist the P-glycoprotein efflux pump [143, 144], and the development of site-specific conjugation approaches [145] to produce homogeneous ADCs. Further improvements may also result from improved linker designs [146]. Another example of ongoing innovation with ADC design was in a novel approach that was taken to conditionally activate antibodies in the tumor microenvironment by utilizing tumor-associated proteases [147]. This so-called "Probody" approach can be applied to ADCs to potentially reduce target-mediated toxicity. There are also efforts in antibody engineering to reduce the non-specific binding of ADCs to normal tissues and to improve biodistribution and tumor penetration. It is also theoretically possible to conjugate two different payloads on single antibodies so that the ADCs can kill cancer cells with two different mechanisms of action. In addition, many other protein engineering approaches can improve the potency and therapeutic index for ADCs. A typical example is shown by a recent publication describing the generation of a biparatopic HER2-targeting ADC using a combination of multiple protein engineering approaches [148]. This bi-specific antibody targets two non-overlapping epitopes on HER2 and is conjugated to a tubulysin-based microtubule inhibitor by site-specific conjugation through two engineered cysteine residues on each heavy chain (S239C and S442C). Due to its tetravalent binding capacity, this antibody can readily cross-link HER2 and promote rapid receptor internalization and lysosomal degradation. Based on this property, the conjugated cytotoxic agent can be efficiently delivered into HER2+ cancer cells to exert its toxic effect. In addition to the bispecific nature of this ADC, modifications in the Fc region significantly reduced binding to Fcγ receptors, potentially minimizing FcγR-mediated off-target toxicity. This second generation HER2 ADC molecule has a very promising potential for treating a broad metastatic breast cancer patient population.

Given ongoing improvements in ADC technology, we can envision that the toxicity issues will be better addressed in future, leading to improvements in the therapeutic window for this class of therapeutics. ADC molecules may ultimately become one of the most effective agents for cancer treatment and potentially be applied to treating diseases other than cancer.

21.4.2 Dawn of *In Vivo* Expressed Biologics

Therapeutic proteins including monoclonal antibodies have been the backbone of the biotech industry for three decades. However, manufacturing large biomolecule therapeutics at scale is a complex and costly process. Typically, they are recombinantly expressed by microbial or mammalian cell culture, followed by chromatographic purification, formulation, fill-and-finish steps before they can be administered to patients. The entire process requires a huge upfront investment to build a manufacturing facility, generally costing several hundred

million US dollars. It would be highly desirable to simplify and reduce these downstream processes.

Researchers have recently been able to bypass conventional protein production by producing desired proteins directly *in vivo* for several applications. Oncolytic virus as cancer immunotherapy constitutes such an example [149, 150]. T-VEC (talimogene laherparepvec) was the first FDA-approved oncolytic virus against advanced melanoma. It is an engineered herpes simplex virus 1 with insertion of the human GM-CSF gene. Once T-VEC infects cancer cells, it will replicate and lyse cancer cells. In the process, GM-CSF is produced by infected cells and secreted to enhance the anti-tumor immune response by attracting dendritic cells to the tumor site. Gene therapy is another example of leveraging the concept of *in vivo* expressed biologics, and many programs are currently in clinical trial. One recent study using somatic gene therapy for treatment of hemophilia B offers the potential to replace the traditional protein-based prophylaxis treatment. A self-complementary adeno-associated virus serotype 8 (AAV8) vector was used to transfer a normal copy of the factor IX gene to patients with severe hemophilia B. In the high-dose group, a single intravenous injection resulted in persistent endogenous production of factor IX at levels approximating 5.1% of the normal factor IX concentration. This led to a more than 90% reduction in both bleeding episodes and the use of prophylactic factor IX concentrate [151]. A third example is DNA [152] or mRNA vaccines [153] for cancer therapy. In the DNA approach, circular DNA constructs are generated to encode tumor-associated antigens and administered to patients either as naked DNA or through a delivery vehicle such as nanoparticles, viruses or bacteria. Once the DNA constructs enter cells such as muscle cells or antigen-presenting cells, the tumor antigens are expressed endogenously, processed and presented to MHC molecules to trigger an anti-tumor immune response. Alternatively, electroporation has also been employed to improve the transfection efficiency for DNA vaccines. Similarly, mRNA vaccines have been used to express cancer neoantigens following administration either as naked mRNA directly injected into lymph nodes, or as a nanoparticle formulation delivered intravenously. mRNA vaccines can also be administered intradermally or intramuscularly.

Given the promises of *in vivo* expressed biologics, researchers have tried to push the technology boundary in order to express antibodies or other therapeutic proteins *in vivo,* which generally requires a larger quantity of protein expression than required for vaccines, cytokines or certain enzymes (e.g., coagulation protease factor IX). One major challenge is the sustained expression of such proteins at concentrations high enough to achieve a therapeutic effect. Adeno-associated virus (AAV) gene transfer technology is most appealing for this purpose. AAV does not cause any known human disease. Recombinant AAV vectors can persist in the episomal state and mediate long-term expression of the therapeutic transgene. AAV vectors have been utilized in a large number of gene therapy clinical trials, have demonstrated a strong safety profile and are providing encouraging signs of therapeutic efficacy for treatment of genetic diseases. Nucleic acid (DNA and mRNA) based delivery technology is also of great interest due to the ease of

scale-up manufacturing and relatively low cost. Both approaches have the ability to express virtually any protein, native or engineered, once these agents enter cells in tissues or organs. In addition, these approaches have the ability to produce secreted and intracellular proteins, the latter of which opens the possibility of using these approaches to address intracellular targets that are otherwise off-limits to protein therapeutics delivered by passive administration.

Several research groups have demonstrated the effectiveness of utilizing AAV vector-mediated gene transfer of monoclonal antibodies in animal studies. The study by Johnson *et al.* showed that two anti-SIV antibodies (directed against gp120), in the form of scFv-Fc genes, could be delivered to rhesus monkeys by an AAV vector and confer complete protection against intravenous SIV challenge [154]. The AAV gene transfer technology in this study enabled efficient expression of anti-SIV antibodies in myofibers and then distribution to the circulatory system. Four weeks after a single-dose gene transfer, which was concurrent with the time of SIV challenge, the serum concentrations of the two antibodies had reached ~100–190 μg/ml and ~40–175 μg/ml. In most monkeys, the antibody serum levels continued to rise, then tapered off and eventually plateaued between 8 and 12 months at ~200–300 μg/ml. A separate group also described the use of AAV8-based vectors to deliver genes encoding broadly neutralizing HIV full-length antibodies to mouse muscle tissues, enabling their long-term systemic production at high concentrations [155]. In this study, antibody gene expression was detectable 1 week after inoculating mice in the gastrocnemius muscle with 1×10^{11} genome copies of an AAV vector encoding antibody b12. Expression continued to rise and within 6 weeks antibody concentrations reached ~100 μg/ml. Maximal concentration of antibody was observed at 12–16 weeks, then decreased two to threefold and stabilized at more than 100 μg/ml for the duration of the 64-week study. The humanized mice expressing antibody b12 were fully protected from HIV infection even when challenged intravenously with very high doses of HIV. Results also showed that antibody expression levels were AAV dose dependent. AAV technology was also used successfully to transfer genes encoding a broadly neutralizing antibody against influenza A to the nasopharyngeal mucosa of mice and ferrets via intranasal delivery. The expressed antibodies provided complete protection of these animals against pandemic influenza [156]. Despite these very promising results, several major obstacles still exist with this technology, such as the cellular immune response to the AAV capsid, pre-existing AAV neutralizing antibodies, and the challenges of AAV vector manufacturing scale-up ability for broad application.

DNA plasmids constitute another attractive gene delivery approach to generate biologics *in vivo.* Unlike AAV vectors, they do not have AAV-associated serology, making repeated administration possible. Researchers have reported that a single injection of a 100 μg DNA plasmid encoding a human IgG antibody against Dengue virus into mouse legs, followed immediately by electroporation, can produce sufficient antibody titers to protect the mice from Dengue disease [157]. Human IgG concentrations in serum were detectable within 5 days of injection, reached peak levels of ~1 μg/ml at week 2 and ~0.7 μg/ml at week 19. This

demonstrated that DNA plasmids can enable sustained antibody expression. However, when compared to the AAV approach used in mice as described earlier, the antibody expression level via this approach is at least two orders of magnitude lower and the duration of expression might not be as long. In this study, the researchers showed that it is possible to use DNA plasmids to deliver multiple antibodies by separate injections at different sites in the same animals. Using a similar DNA delivery approach, a separate group has demonstrated a sustained high-level expression of bioactive human heterodimeric IL-15 in rhesus monkeys by repeated administration [158]. Each monkey received 8 mg of DNA treatment every 3 days for a total of five treatments. For each treatment, the plasmid DNA was injected at four sites. Plasma IL-15 levels increased about twofold over baseline (~10–20 pg/ml) 3 days after first injection and reached peak levels of ~100 pg/ml between days 7 and 12. The fourth and fifth DNA injections did not increase IL-15 levels further. The peak levels were similar to that obtained via subcutaneous injection of 1 μg/kg of purified IL-15 heterodimer. IL-15 holds immunotherapeutic potential for treating cancer. The DNA delivery approach allowed high systemic level of IL-15 and did not induce the toxicity caused by high systemic cytokine spikes that usually come with IL-15 protein injection.

The major limitation for DNA delivery technology lies in the low *in vivo* expression level of the protein encoded by the delivered gene. In recent years, researchers have improved the expression level significantly by the optimization of the promoter, codon usage, mRNA stability, and leader sequence, as well as the use of *in vivo* electroporation to enhance the delivery efficiency [159]. In particular, *in vivo* electroporation has been shown to improve plasmid delivery efficiency by 10–1000-fold compared to naked DNA plasmid delivery alone. However, the production level is still substantially lower than that of AAV technology. It does however have some advantages over AAV technology. Besides lower manufacturing cost and no pre-existing serology, DNA-mediated expression does not persist for an extended period of time, which may have certain advantages when the diseases are not chronic and the need for exposure to the expressed protein drug is only short-term.

As with the DNA approach, mRNA has also been used to express therapeutic proteins in addition to their application in the vaccine area. To evade an innate immunity through Toll-like receptor signaling pathways, and to enhance mRNA stability, chemically modified mRNA molecules are commonly utilized. Due to the nature of mRNA, expression of the encoded protein tends to be transient. In a recent report, researchers injected a synthetic modified mRNA encoding human vascular endothelial growth factor-A (VEGF-A) into mouse myocardial tissues and found that transient expression of the encoded protein significantly improved heart function of treated mice in a myocardial infarction model, leading to enhanced survival [160]. The benefit was partially due to the expansion and differentiation of endogenous heart progenitors toward cardiovascular cell types, which was triggered by VEGF-A. Modified mRNAs encoding a variety of nucleases for gene editing have also been explored for correcting genetic disorders such as surfactant protein B (SP-B) deficiency, cystic fibrosis, pediatric asthma,

β-thalassemia, and sickle cell disease [161]. For example, researchers have shown that repeated administration of modified mRNA encoding SP-B can protect mice from respiratory failure and prolong their average life span in a fatal lung disease model caused by a lack of SP-B [162]. In this study, 20 μg of mRNA was administered intra-tracheally twice a week for approximately 1 month. Continuous treatment by repeated dosing was necessary to maintain animal survival. The modified mRNA caused minimal immune activation, lower than that observed in a control group using SP-B plasmid DNA. Although chemically modified mRNA is the most commonly used approach to reduce its immune activation ability, recent work has shown that engineering an mRNA sequence to be G-C rich can achieve the same goals of sufficient protein expression and evasion of the innate immune response [163]. They showed that engineered mRNA encoding erythropoietin (EPO) could elicit meaningful physiological effects in mice, pigs, and non-human primates. For large animals, the mRNAs were formulated in lipid nanoparticles and delivered intravenously. Overall, mRNA-mediated *in vivo* expression is typically rapid and transient, and this approach is best suited to treat diseases that only require short-term drug intervention.

In summary, *in vivo* expressed biologics hold tremendous potential. Several of the technology platforms discussed here have been undergoing continuous improvement and refinement in the past decade, and are a cause for much enthusiasm. Other emerging technologies that can enable *in vivo* expressed biologics, such as cell delivery, are likely to supplement this area. For example, one can envision building a next-generation chimeric antigen receptor (CAR) T cell therapy by engineering T cells to simultaneously display surface molecules that can recognize cancer cell surface antigens while also to secrete molecules (e.g., anti-PDL-1, or anti-CTLA-4) that "reactivate" the immunosuppressive microenvironment. This CAR-T will be substantially more potent than the first-generation approach. However, for all approaches discussed here there are still many hurdles to overcome prior to their widespread applications. These include delivery, control of dosage and duration, host immune responses, and risk of genomic integration and associated tumorigenesis (for viral vectors). One desired aspect is to have controllable *in vivo* expression, for instance, via a built-in inducible on–off switch. This will allow us to shut off drug expression immediately when drugs are no longer needed or when there are safety concerns. If *in vivo* expressed biologics platforms can be developed and broadly applied, these approaches will completely transform current standard manufacturing practices and revolutionize the biotech drug industry and manufacturing sciences.

21.4.3 Oral Biologics

Since the emergence of biologic medicines ~30 years ago, researchers have dreamed of developing oral biologics that can be easily administered, much like swallowing a pill. Despite tremendous efforts, the oral delivery of biologics, such as antibodies, proteins or peptides remains a major challenge for the

biotech and pharma industry. Thus far, biologics are typically administered by intravenous, subcutaneous, or intramuscular injection, which is considerably more burdensome than oral administration, particularly for treatment of chronic diseases. Two fundamental obstacles exist for delivering biologics orally. Firstly, biologics in general do not survive the harsh acidic and protease-rich environment encountered in the digestive system. Secondly, even if they survive these conditions, it is difficult for biologics to penetrate the gut epithelial layer to enter into systemic circulation, resulting in a very low systemic bioavailability of orally delivered biologics. Even though all attempts to deliver proteins or antibodies orally have failed, there has been success in developing oral peptide therapeutics. A few products have already been approved on the market, such as cyclosporine, desmopressin, and linaclotide.

In recent years, pharmaceutical efforts in developing oral biologics have focused on the diabetes area, as current biologic treatments for this disease usually require once or more daily injection(s) of insulin or glucagon-like peptide 1 (GLP-1) analog. Novo Nordisk, a leader in the diabetes area, has demonstrated early clinical successes in developing oral insulin and GLP-1 agonists. By collaborating with Merrion on their absorption enhancer technology (GIPET), which increases the permeability of the gastrointestinal (GI) track lining, they were able to substantially increase the bioavailability of an orally administered insulin analog. This oral insulin is currently in a Phase IIa clinical trial. Novo Nordisk is also collaborating with Emisphere to apply their Eligen technology to develop oral GLP-1. Eligen technology uses carrier agents to facilitate transport of therapeutic biologics across the gastrointestinal membrane via a passive transport system. This once daily oral GLP-1 is currently in Phase III clinical trials. Besides oral peptides for diabetes, others are investigating all kinds of oral biologics (peptides, proteins, and antibodies) for treating diseases restricted to the GI tract [164]. These include metabolic disorders, inflammatory diseases, infections, and constipation. Many molecules are currently under clinical investigation. By targeting GI diseases, these therapeutics do not face the additional burden of having to transport out of the GI tract into the systemic circulation thereby substantially reducing the level of challenge.

New technologies continue to be explored to improve the delivery of biologics orally, and this has spawned a number of start-up companies testing certain novel delivery mechanisms. For example, Applied Molecular Transport is focusing on mimicking the system utilized by gut microbes to overcome gastrointestinal epithelial barriers. This approach takes advantage of the ability of some bacteria to shuttle proteins across the gastrointestinal barrier. Another company, Rani Therapeutics, is developing an engineered "robotic pill," which is made of an ingestible polymer and tiny sugar needles encapsulating biologic drugs. Once the pill reaches the intestine, the outer layer of the polymer pill dissolves, allowing the mixing of two contained chemicals, citric acid and sodium bicarbonate. These react to produce carbon dioxide, which serves as an energy source to eventually shoot the sugar needles into the intestinal wall. The sugar needles then dissolve and the encapsulated biologics are released into nearby blood vessels. This "robotic pill" is

currently in animal studies and has shown promising results. As these and other new technologies are developed to meet the challenges of orally delivering biologic drugs, the dream of having these medicines in pill form could become a reality.

21.4.4 Conclusions

Rapid advancements in biotechnology over the past 30 years have dramatically shaped the drug industry, and the pace of new innovation in this field continues unabated. As we make progress on new technologies for treating diseases, it creates excitement, opens the door to new opportunities, and presents us with challenges to overcome. Here, we have discussed several promising areas; if each one can be successfully delivered, it will result in a transformative impact to medicine and the healthcare industry.

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