Tyler J. Curiel *Editor*

Cancer Immunotherapy

Paradigms, Practice and Promise

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Introduction

For many years, immunotherapy for cancer was a sleepy backwater primarily populated by a few die-hard immunologists who did not accept the concept that relatively nonspecific cytotoxic or radiotherapeutic approaches were the optimal modalities to treat cancer. While there is no denying the beneficial and even curative effects of some chemotherapeutic, radiotherapeutic, and surgical approaches, the pessimism that has surrounded attempts at immunotherapy for cancer is no longer justified.

A colleague advised me about 10 years ago that if I ever cured even one of my cancer patients with immunotherapy, he would buy me a steak dinner. After I updated him on recent developments in cancer immunotherapy about 6 years ago, he upped the ante. Odds of curative cancer immunotherapy were so remote he informed me, that now he would buy me "the whole steak house" if I ever cured anyone with it.

Results of most cancer immunotherapy trials to date have been generally modest at best. However, until recently the majority of these trials focused on inducing antitumor immunity with little or no regard for reducing concomitant tumor-induced immune dysregulation and immune suppression[1]. For example, on April 29, 2010, the Food and Drug Agency approved sipuleucel-T (Provenge) from Dendreon to treat advanced-stage prostate cancer, which was hailed as a major breakthrough for cancer immunotherapy. While we applauded this addition to the armamentarium, many of us developing cancer immunotherapies nonetheless regarded this agent as an exemplar from the older paradigm (using adoptive transfer of an antigen presenting cell to present a tumor-specific antigen and thereby boost tumor-specific immunity). Thus, its efficacy could be limited by underlying tumor-mediated immune dysfunction not addressed by sipuleucel-T itself.

On March 25, 2011, the Food and Drug Administration approved the anti-CTLA-4 antibody, ipilimumab (Yervoy) from Bristol Myers Squibb. Ipilimumab makes antitumor T cells resist immune suppression by regulatory T cells and could impede the suppressive activity of regulatory T cells directly. Thus, it can help correct tumor-associated immune suppression and represents the first drug approved in the new paradigm, which addresses how to mitigate tumor-mediated

immune dysfunctions. Means to capitalize on the best properties of the immune activating sipuleucel-T approach with the immune dysfunction-reducing ipilimumab approach will move us closer to that ideal of highly effective anticancer immunotherapy.

In this regard, our understanding of tumor immunology and the immunopathogenesis of cancer is progressing at a rapidly accelerating rate. This knowledge is leading to a much better theoretical grasp of the essential elements required for successful anticancer immunotherapy, and thus to more effective treatment strategies. For example, the concept of tumor immune surveillance, the immune mechanisms that eliminate nascent clusters of malignant cells before they become clinically apparent[2] is no longer seriously questioned, laying a foundation for development of more effective anticancer immunotherapies[3]. Our understanding of tumor immune surveillance has been amplified by elegant work from Bob Schreiber's group showing that tumor immune surveillance is part of the larger problem of tumor immunoediting, in which the tumor becomes less immunogenic over time as it continually evades host tumor immune surveillance[4]. There is now excellent experimental evidence that tumor antigens contribute to immunoediting[5, 6]. Strategies to mitigate this important issue will be a significant component of future cancer immunotherapy development.

Equally formidable are the challenges in understanding how to optimize individual approaches, and how to combine them successfully and ideally[7, 8]. Many regulatory and logistical issues must be addressed regarding the safety of individual agents and appropriate means to test them for clinical use. There are challenges in understanding appropriate patient populations most likely to respond to immunotherapeutic interventions, in developing clinical trials capable of detecting useful strategies, in developing appropriate immune monitoring tools for these trials, in using appropriate clinical response criteria for clinical trials and in analyzing clinical data, all of which areas have seen significant progress in recent years, including the introduction of clinical response data specifically for patients in immunotherapy trials[9].

Traditional cytotoxic and radiotherapeutic approaches have been associated with significant morbidities due to their relative lack of tumor-specific targeting. The exquisite specificity of the immune system could help address these toxicity issues. Micrometastatic and residual disease contribute to late relapses after apparently successful conventional treatments. The exquisite sensitivity of immune interventions could help solve this problem as well. Cancer is primarily a disease of aging, as age is the single biggest cancer risk factor. Despite a decline in specific immune effector functions with age, and changes in other functions, it is possible to develop immunotherapy that potentially could be effective in the aged, who are least able to tolerate the toxic effects of many treatment approaches.

We are entering what promises to be a golden age in cancer immunotherapy[10] where we can identify successful treatments that can be applied widely to patients most at risk, with reduced toxicities, tractable logistics, and reasonable costs. It is now possible to sequence entire tumor genomes to understand the specific mutations underlying classes of tumors, and to understand the specific issues related

to tumors in individuals. Targeted small molecule drugs are the immediate beneficiaries of such technological advances, but these and related developments are amenable to applications in cancer immunotherapy as well to help personalize an optimal regimen for each individual[11]. Nonetheless, significant hurdles remain, although it is now possible to see how they might be overcome[12].

This text introduces the fundamentals of tumor immunology, major causes of tumor-associated immune dysfunctions, major treatment modalities that are approved by regulatory agencies, and covers promising preclinical and early clinical leads. Each chapter addresses the major issues in the field, current strategies to address them, and also speculates on the future and where the technology and new insights can take us.

We hope that the information here is useful to our colleagues in this rapidly evolving field, and to those who consider entering it.

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Part I
Overview

Chapter 1
Historical Perspectives and Current Trends in Cancer Immunotherapy

Tyler J. Curiel

Abstract The immune system is exquisitely able to identify trace antigens and eliminate cells expressing them. Tumors are quintessentially antigenic tissues as a result of their many genetic mutations. This antigenicity, however, does not generally translate into useful immunogenicity as spontaneous rejection of clinically apparent tumors is rare. Early work in tumor immunology identified tumor-specific and tumor-associated antigens and formulated strategies to bolster antitumor immunity using paradigms arising from prior successes in understanding anti-pathogen immunity. It is now clear that the inability of endogenous immune mechanisms to eradicate clinically evident cancers owes in part to tumor-driven immune dysfunction, in part to the coevolution of antitumor immunity with the ever-changing antigens of the ever-mutating tumors (immunoediting) and to the fact that antitumor immunity is a form of autoimmunity. These newer understandings caused thinking to evolve and advance. Our rapidly increasing understanding of antitumor immunity and how it can be thwarted has led to new approaches to tumor immunotherapy with great promise to be much more successful than prior generations of approaches. This chapter discusses the evolution in thinking about tumor immunity, why endogenous antitumor immunity often fails after tumors become clinically apparent and why prior tumor immunotherapy approaches have generally had only modest success at best. New paradigms leading the field and the novel therapeutic approaches based on recent insights will be introduced.

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1.1 Historical Perspective

1.1.1 The History of Immunology Is Rooted in the History of Understanding Resistance to Infections

Until recent times in the developed world, and in many parts of the modern world, infections were and are major causes of morbidity and mortality. It is thus hardly surprising that much knowledge of immunology and research into it was based on an understanding of resistance to infectious diseases. Resistance to a specific insult was first recorded in 430 B.C.E. by the Greek historian Thucydides, reflecting on the plague of Athens (a devastating infection whose identity is not known with certainty), being himself a survivor, when he remarked "The same man was never attacked twice…." The first treatise on immunology was a study of resistance to smallpox and measles by the great Persian physician Abu Bakr Mohammed ibn Zakariya al-Razi (880–932 A.D.) known as Rhazes. The first immunotherapy trial was conducted in Newgate Prison, London, on August 9, 1721. The Prince and Princess of Wales, fearing that smallpox infection could affect their children and having heard of resistance following deliberate or accidental exposure to smallpox, had six condemned prisoners and orphans infected with pus from individuals afflicted with smallpox and then exposed them to an active case. All the exposed prisoners survived and were released one month later.

In 1796, the British physician Jenner demonstrated that deliberate infection with vaccinia virus, which caused cowpox, an infection akin to smallpox, but much milder, was protective against smallpox. The first formal demonstration that immunity to one condition rendered protection against a reexposure to the same condition, without affording protection to an unrelated condition, was by Pasteur in the 1880s, experimenting with anthrax and chicken cholera in animals. These experiments also showed for the first time that artificially attenuated pathogens (in contrast to Jenner's use of naturally attenuated cowpox) could be effective agents to protect from disease. Pasteur named these protective agents "vaccines" in honor of Jenner's work with vaccinia virus. Jenner's work saved vast numbers of human lives. Pasteur's findings were of considerable economic benefit to farmers and together with Jenner's demonstrations in smallpox raised enthusiasm that vaccination and actively induced immunity could solve a great number of public health problems. The reader is referred to the marvelous book "The History of Immunology" by Arthur Silverstein[1] for many additional historical details.

1.1.2 Investigations in Infectious Disease Greatly Influenced Work in Tumor Immunology

The paradigm that shaped development of most cancer immunotherapies until relatively recently was grounded in observations based on infectious disease

experience. Although immunity to tumors and pathogens shares many similar mechanisms such as priming of antigen-specific T cells, they also differ considerably in important regards to be explained. Failure to appreciate these differences fully (among other factors) contributed to delay in development of highly effective cancer immunotherapy. It is thus useful to understand the history of thinking in tumor immunology as a prelude to understanding recent conceptual evolutions driving new treatment paradigms. As our understanding of the specifics of tumor immunology improves, we should ultimately be able to capitalize on the exquisite sensitivity and specificity of the immune system to treat cancer in highly targeted ways with a minimum of unwanted side effects.

In 1866 Wilhelm Busch in Germany noted that some patients with sarcomas had tumor regressions after surviving postoperative wound erysipelas, an infection with bacterial species of *Streptococcus*. In 1868 he deliberately infected a postoperative sarcoma patient with pus from another active case of wound erysipelas in a deliberate attempt to induce tumor regression, in what must have been one of the earliest attempts at tumor immunotherapy. Between 1892 and 1896, the surgeon William Coley in Manhattan also noted spontaneous tumor regressions after post-surgical wound erysipelas. Coley also attempted to induce remissions in some patients by deliberately infecting their wounds with pus from patients that became infected naturally after surgery and later by using cultures of *Streptococcus* derived from these wounds. He is credited with being the first to attempt systematic, large-scale trials of induced inflammation to treat cancer and reported some clinical successes, but his patients, like those of Dr. Busch, also experienced substantial morbidity and mortality from the infection[2] as these experiments predated the antibiotic era. In retrospect, endotoxin from Gram-negative bacteria in wounds or cultures likely induced endogenous tumor necrosis factor-α that contributed to clinical efficacy, among other factors. In 1899, Parke Davis licensed the rights to this treatment (using a cocktail called Coley Fluid) and marketed it as cancer therapy through the 1950s until the license was revoked for unclear reasons. Dr. Coley's work with these approaches at the Memorial Hospital in Manhattan, notably in soft tissue sarcomas, led to medical and philanthropic attention that were in part responsible for the success and development of what became known as the Memorial Sloan-Kettering Cancer Center.

By the 1950s, inbred, genetically homogeneous mice became widely available, allowing important studies of antigen-specific immunity principally in influenza that quickly led to the development of effective human influenza vaccines[3]. An important result of these early studies was the demonstration that antigen-specific $CD8^{+}$ T cells (cytotoxic T lymphocytes, CTL) could kill antigen expressing target cells and that CTLs were important mediators of protective antiviral immunity, along with virus-specific antibodies. The concept of protective CTLs quickly became incorporated into paradigms employed by tumor immunologists. Studies of how CTLs were primed in an antigen-specific manner led to the elucidation of the fundamental role for dendritic cells in this process, including the generation of influenza-specific CTLs[4]. By the 1940s and 1950s, the public health implications of cancer were apparent to forward-thinking investigators. Based on the extensive

record of successful development of vaccines against infectious agents that literally spanned centuries, testing similar approaches in cancer was an entirely reasonable strategy.

Tumor immunologists around this time developed translational goals that followed leads from research in infectious diseases: demonstrate that like pathogen-specific immunity, tumor-specific immunity also existed; identify specific rejection antigens expressed by tumors; understand if tumor rejection antigens were common or specific to given tumors; and then present appropriate tumor rejection antigens on relevant antigen presenting cells (dendritic cells in particular) to generate tumor antigen-specific $CD8^+$ CTLs that effected beneficial clinical results. Pursuing these lines of thought well over a half century ago, Prehn et al. used the relatively novel concept of studying tumor immunity in genetically identical inbred mice. These investigators used chemical carcinogens to induce distinct tumors in inbred mice and showed that immunity to one tumor did not necessarily protect against challenge with a different tumor from the same genetic background[5]. This was a clear demonstration of tumor-specific immunity. Within the next several decades, specific tumor antigens were identified[6] that could be used to generate antigen-specific $CD8^+$ CTLs that rejected tumors[7]. A major role for dendritic cells in priming tumor-specific immunity followed[8]. As these discoveries were reported and became widely known, they reinforced the notion that insights and principles derived from infectious diseases models would be sufficient to drive development of effective cancer immunotherapy. The success of the current vaccine for protection against hepatitis B virus infection, based on the generation of an immune response against a single antigen[9], furthered the concept.

1.2 The Older Paradigm

In the paradigm that prevailed until recently, tumors expressed tumor-associated antigens that were captured, processed, and presented to professional antigen presenting cells, particularly dendritic cells. Dendritic cells express immune co-signaling molecules such as CD40, CD80, CD86, and those of the major histocompatibility complex and produce soluble factors such as IL-12 that drive specific T cell Th polarization and other immune cell-activating programs. The net result of all these co-signaling and activating events is to prime tumor antigen-specific T cells, notably $CD8^+$ CTLs. These CTLs, when generated in sufficient numbers and directed against the appropriate tumor antigens, would then eliminate tumor (see Fig. 9.1 in Chap. 9 for a graphic illustration). Following this logical construct, the best steps to develop effective anticancer immunotherapy would be to identify the appropriate antigens; load antigen presenting cells with those antigens; appropriately activate the relevant antigen presenting cells; generate sufficient quantities of tumor antigen-specific effector cells, particularly $CD8^+$ CTLs; or apply various combinations of these approaches. In other words, the primal defect to be corrected was that there was *not enough of a good thing*

(tumor-specific antigen(s), T cell priming, appropriate antigen presenting cells, activation of effector cells, or numbers of antitumor effector cells). If sufficient missing elements (the good things) could be reinfused or generated through interventions in the relevant pathways, then tumor immunotherapy could elicit clinically relevant antitumor immunity as had so successfully been done with a number of vaccines for infectious agents.

Based on the essentials of this paradigm, earlier trials of cancer immunotherapies attempted to augment or replace the missing good things. Such approaches included identifying and challenging with tumor antigens, pulsing these antigens onto dendritic cells, and attempting to identify optimal antigen presenting cells or means to activate them[10–14]; extracting tumor-infiltrating T cells (TIL), growing them *ex vivo* and reinfusing them alone,[15] reinfusing them in conjunction with T cell growth and activation factors such as IL-2,[16] activating cultured tumor-infiltrating lymphocytes with cytokines *ex vivo* prior to reinfusion (LAK cells),[17] or engineering T cells to express receptors for specific tumor-associated antigens[18]; augmenting immune co-signaling pathways for greater T cell activation and infusing immune activating cytokines; identifying additional tumor-associated or tumor-specific antigens[19]; and generating transient leucopenia to foster rapid proliferation of antitumor T cells[18].

These approaches have generally been meticulously executed and well thought out. Nonetheless, they have generally delivered only modest clinical benefits at best. In considering the lack of significant clinical benefits in this paradigm, it is important to recognize that many patients receiving these treatments already express a variety of tumor-specific antigens, have large numbers of dendritic cells and other antigen presenting cells, and elicited significant numbers of tumor antigen-specific effector cells even as they fail to control, let alone eliminate their tumors immunologically[20, 21]. Thus, while increasing these good elements is likely to be necessary for effective immune control of tumors, it appears unlikely to be a sufficient stand-alone strategy. Nonetheless, recent reports identify that the effectiveness of some stand-alone therapies can be relatively good. For example, Steve Rosenberg's group engineered the T cell receptor from a patient who responded to a melanoma vaccine into the T cells of additional patients using gene therapy and were able to elicit meaningful clinical responses in new vaccinees[18]. Carl June's group engineered a chimeric T cell receptor to redirect the antigen specificity of T cells in patients with chronic lymphocytic leukemia with significant clinical responses[22].

Despite some successes, these latter two reports[18, 22] illustrate the fact that many of these approaches have significant logistical issues; require significant investments of capital, personnel effort, and time; and raise difficult regulatory and safety issues as has been noted[23]. Many of these approaches, by virtue of their utility against only a given antigen or in the context of a selected genetic (major histocompatibility complex) background, can be used in only a restricted subset of patients, aside from the issue of their modest clinical efficacy[23]. It has thus become evident that the increase-the-good-things cancer immunotherapy approaches require substantial adjustments if they are to be clinically effective, practical, and cost effective.

1.3 Shortcomings of the Older Paradigm

Insights into the immunopathology and immune defenses operative in infections have yielded impressively successful vaccines. These infections are pathologic processes resulting from rapid proliferation of dividing cells (or virions) of external origin. By contrast, cancers arise from abnormal proliferation of cells of internal (self) origin. The self-origin of cancers provides the crux of the fundamental difference between infectious diseases and cancer. No matter how abnormal a malignant cell is as a result of its oncogenic mutations, it is nonetheless of self-origin, and consequently any anticancer immune attack is an autoimmune attack.

Our immune system has evolved over millennia to monitor autoimmunity and utilizes a multitude of self-tolerance mechanisms to help prevent it[24]. However, not every tumor antigen is a normal self-antigen. Examples include the viral gene products in human papilloma virus-driven cancers and the mutated p53 self-antigen in many epithelial carcinomas[25, 26]. Nonetheless, mechanisms to promote self-tolerance represent significant obstacles to mounting clinically effective antitumor immunity. Mouse models for infection such as the powerful influenza model have helped elucidate important mediators of protective immunity including dendritic cells, protective antigens, and antigen-specific $CD8^+$ CTLs. Breaking self-tolerance, which is required to mount effective antitumor immunity, was simply not a major consideration in these infectious disease studies. Fortunately, the significance of the requirement to break self-tolerance to promote optimal antitumor immunotherapy is now garnering the consideration and attention it merits[27, 28].

It is now also clear that inefficient immune-mediated tumor eradication is not just a passive process due to lack of sufficient numbers of the good elements[20, 29]. Important work in the past 15 years has highlighted the active mechanisms deployed by tumors to thwart effective antitumor immunity such as the elaboration of immunosuppressive factors including IL-10,[30–32] transforming growth factor-β,[20, 33–35] and vascular endothelial growth factor[36]. These immunosuppressive factors and other tumor-driven mechanisms likely arise in part as antitumor immunity simultaneously elicits peripheral tolerance mechanisms defending against autoimmunity, although there are additional reasons to be discussed below. Such findings underscore the concept that *not enough of a good thing* is not the fundamental problem in tumor immunity, but that additional factors contribute to ineffective immune-mediated tumor rejection, including the fact that there is *too much of a bad thing*. It further turns out that these *bad things* fall into several major categories including dysfunctional T cells, antigen presenting cells, and immune regulation; immunosuppressive factors; pathologic inflammation; abnormal cell trafficking; and immune editing, which are discussed in detail in various chapters in this text.

1.4 Examples of the *Too Much of a Bad Thing* Paradigm in Ovarian Cancer

Our group has studied patients with ovarian cancer to help understand why immune-mediated tumor rejection fails in these patients, despite ample evidence that their tumors are immunogenic and elicit tumor-specific immunity[37–39]. Ovarian cancer patients are excellent subjects for studies of human tumor immunology as their malignant ascites contains significant quantities of tumor antigens, soluble microenvironmental factors, immune cells, and tumor cells. Tumors from malignant ascites can often be cultivated *ex vivo* to study effects of autologous tumor-specific immunity[40, 41] that are usually difficult to perform with human subjects.

In these ovarian cancer patients, we found abundant numbers of plasmacytoid dendritic cells, at the time generally associated with beneficial antiviral immunity, in contrast to the expected myeloid dendritic cells considered prime inducers of antitumor immunity. Further, these plasmacytoid dendritic cells were detrimental to antitumor immunity through induction of immunosuppressive IL-10 that inhibited tumor-specific immunity rather than inducing beneficial interferon-γ, consistent with a dysfunctional regulatory T cell phenotype[41] (see Chap. 9 for more details). We later identified myeloid dendritic cells in the tumor draining lymph nodes, thought to be beneficial to antitumor immunity based on their significant record of use in adoptive transfer strategies for cancer vaccines[12]. However, instead of being beneficial, we demonstrated that local tumor microenvironmental factors induced these myeloid dendritic cells to express dysfunctional B7-H1 immune co-signaling molecules that generated T cell IL-10 and inhibited tumor-specific immunity[40].

We also found that local tumor microenvironmental factors actively recruited dysfunctional regulatory T cells through the CCR4/CCL22 chemokine signaling axis. These regulatory T cells inhibited the tumor-specific cytotoxicity of $CD8^+$ CTL and their production of IL-2 and interferon-γ. As the number of regulatory T cells in the solid tumor mass increased, patient survival was reduced[38].

1.5 Immunoediting: Another Formidable Hurdle to Successful Tumor Immunotherapy

The concept of tumor immune surveillance, the active policing and rapid elimination of cells undergoing malignant degeneration to prevent clinically apparent cancer, has moved from theory to generally accepted reality. Major mediators of cancer immune surveillance include T cells, interferon-γ, and antigen-specific immunity[42, 43]. A profound discovery was the observation by Bob Schreiber's group that tumor immune surveillance was the first phase of a much bigger process that he termed immunoediting,[44] with its characteristic three E's[45]. The first "E" is elimination of newly formed malignant cells. This phase most closely approximates what had previously been referred to as immune surveillance. The second "E" is equilibrium.

In the equilibrium phase, the outgrowth of malignant cells that escape immune elimination is balanced by an immune response that evolves to keep pace with the ever-mutating tumor clones in their efforts to escape immune elimination. This evolution of tumor antigenicity to evade destruction under immune pressure is called immunoediting. The end result of immunoediting is the third "E," escape. Eventually, the tumor will present antigens no longer sufficiently recognized by remaining antigen-specific immune cells, and the tumor becomes clinically apparent. Evidence for immune equilibrium has been clearly demonstrated in mouse models[43]. Supporting evidence in humans comes from unfortunate natural experiments in which organ recipients from a common donor all get the same cancer that originated from donors with unsuspected cancers, most notably melanoma[46, 47]. Formal demonstration that tumor antigens drive immunoediting has recently been reported[48, 49].

1.6 Resistance to Immune Rejection Is a Fundamental Hallmark of Cancers

The basis for cancer immunotherapy rests on the immunogenicity of tumor cells. Recent work described above has led to the understanding that lack of tumor rejection is not simply due to lack of tumor immunogenicity, although immunoediting and other related tumor features demonstrate that this can be a significant barrier[44, 45, 48, 49]. In 2000, Hanahan and Weinberg reported on six fundamental hallmarks of cancer: resistance to cell death, replicative immortality, ability to invade tissues and metastasize, ability to develop new vasculature, ability to generate proliferative signals, and evasion of growth-suppressing signals[50]. This framework has proven useful to understand cancer behavior and to develop drugs targeted at cancer-specific properties. Nonetheless, these properties only included those of the tumor cells themselves, with little regard to the hosts in which the cancers grow and to which they will react, including with antitumor immunity. Lack of tumor rejection despite their striking antigenicity was summarized and reviewed in 2006–2007[21, 51]. In response to this growing awareness, Hanahan and Weinberg returned in 2011 with an updated eight fundamental hallmarks of cancer that included the original six, but now with an acknowledgement that lack of immune rejection (along with deranged metabolic features[52]) was emerging as a potentially general hallmark of cancer[53].

Aside from lack of immunogenicity, the chronic state of generalized inflammation fostered by cancers was emphasized by work from the Karin lab and others[54, 55] and is suggested as a fundamental hallmark of cancer,[56] along with genomic instability,[57] abnormal vasculature,[58] and age-dependent stem cell features[59]. Recognition that the host response to the tumor is critical in its pathogenesis will help generate novel treatment approaches, including novel immunotherapy approaches.

1.7 Novel Clinical Approaches Based on the Newer Paradigm

Armed with these new immunological insights, investigators are developing and testing novel treatment approaches to cancer immunotherapy. Eliminating dysfunctional immune regulation, such as through managing regulatory T cells (Chap. 9), is an approach being tested in several cancers including melanoma, ovarian cancer, breast cancer, and renal cell carcinoma, either as a stand-alone strategy or combined with active vaccinations[60–63]. Immunopathologic contributions of other regulatory cell populations such as $CD8^+$ regulatory T cells,[64, 65] NKT cells,[66] myeloid-derived suppressor cells,[67, 68] and other populations[69] remain to be fully established. There is considerable interest in further evaluating the immunopathologic role for myeloid-derived suppressor cells in human cancers (see Chap. 10). Given that FDA-approved agents such as 5-fluorouracil and gemcitabine are reported to deplete these cells, clinical trials to evaluate their management are being planned. Although a trial aimed to deplete myeloid-derived suppressor cells specifically has not been reported to my knowledge, their levels correlated inversely with clinical responses in a trial of the experimental agent NOV-002 (disodium glutathione disulfide) plus standard cytotoxic drugs in patients with early-stage breast cancer[70]. Investigators using adoptive T cell (Chap. 3) or dendritic cell transfers (Chap. 4) or active vaccinations (Chap. 5) now are incorporating features into their trials that can counter the immune dysfunction that must also be overcome for optimal treatment outcomes. Details are addressed in the relevant chapters.

1.8 Challenges in Developing Newer Tumor Immunotherapies

A better understanding of the immunopathology of specific tumors will help develop more specific approaches. Significant issues include understanding what immune dysfunctional mechanisms should be targeted in given tumors, with which agents and at what stage. Optimal means to combine various treatments remain largely unknown including which agents are best for combinations and in what temporal sequence. The issue of the potential efficacy of immunotherapy given the extensive immunoediting that has occurred in many tumors suggests that mechanisms aimed solely at boosting endogenous immunity could fail in certain patients and that these patients could benefit from a gene therapy or adoptive cell transfer approach. The logistical issues of such approaches remain formidable with current technologies, and we must somehow find ways to make them cost effective and practical to apply to large populations. The ideal approaches will generate relevant antigens (such as through cytotoxic agents that make tumors immunogenic[71]), mobilize adequate numbers of relevant antigen presenting cells (such as granulocyte colony-stimulating factor), use agents to boost effector T cell function (such as IL-7 or IL-15), and use agents to mitigate immune dysfunction, such as managing Tregs. These concepts suggest that cancer

immunotherapy must become more multimodal analogous to the traditional multimodal approaches of combining cytotoxic agents, radiotherapy, and surgery.

1.9 Conclusions and Summary

Immune-based therapy has potential to be the best approach to cure many types of cancer. Antigen-specific immunity is simply as or more potent and targeted as any approach known thus far. The *not enough of a good thing* paradigm helped identify and test important principles in development of cancer immunotherapy. However, addressing immune dysfunction (the *bad things*) must also be done for optimal therapeutic outcomes.

Lack of immune rejection is a fundamental cancer hallmark. Additional tumor-mediated mechanisms that degrade host immunity include generation of local inflammation and possibly the immunosuppressive effects of cancer initiating cells. As we understand these impediments to successful immune eradication of cancers, cancer immunotherapy could be greatly improved. Just as a careful general sends troops into battle only after first ensuring that all possible impediments have first been removed from the battlefield to optimize chances for victory, the *too much of a bad thing* paradigm predicts that tumor-driven impediments must also be eliminated for optimal treatment success.

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Chapter 2
T Cell and Antigen-Presenting Cell Subsets in the Tumor Microenvironment

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Abstract The development of successful antitumor immunity depends upon cross talk and collaboration between multiple T cell and antigen-presenting cell subsets. In this chapter, we review and summarize current knowledge regarding the function, interactions, and prognostic significance of each of these populations, as well as their dependence upon one another within the tumor microenvironment.

2.1 Cytotoxic T Lymphocytes

Cytotoxic T lymphocytes (CTLs) have long been the focus of antitumor immune study. The first evidence that T cells could in fact kill tumor cells came from L.R. Freedman and colleagues in 1972[1]. Nearly two decades later, investigators

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observed that high numbers of tumor-infiltrating CD8$^+$ T cells correlated with increased cancer patient survival[2]. Since that time, studies of colorectal cancer[3], hepatocellular carcinoma[4, 5], ovarian cancer[6], esophageal carcinoma[7], leukemia[8], and various other cancers have all indicated similar prognostic value of CD8$^+$ T cell infiltration[9]. Interestingly, this seems to be specific to tumor tissue as circulating tumor-antigen-specific CD8$^+$ T cells have no prognostic significance in melanoma patients[10].

CD8$^+$ T cells use multiple mechanisms to kill tumor cells. They express granzymes, perforin, and ligands of the tumor necrosis factor (TNF) superfamily, including Fas ligand. CTLs use their surface-expressed TNF family members to bind corresponding receptors on the surfaces of tumor cells, engaging an intrinsic death program[11]. Granzymes are formed in the CD8$^+$ T cell only after antigen-specific activation of the cell[12–14]. Once the enzymes have been delivered to the target tumor cell, killing can occur in as few as 20 minutes[15]. The signature cytokines expressed by CD8$^+$ T cells are also important—secretion of interferon-gamma (IFN-γ) and TNF alpha (TNF-α) mediate many antitumor effects. It is not yet clear, however, if these effects occur directly within the tumor cells, or whether they influence other mechanisms that aid in antitumor immunity. IFN-γ is well known for its antiangiogenic properties[16–18] and its stimulatory effects upon macrophages[19, 20]. It is also possible that this cytokine could prompt tumor cells to upregulate antigen-presentation machinery, increase their antigenic properties, and/or induce the expression of Fas[21–26]. Whereas CTL secretion of IFN-γ is directional (toward the immunological synapse and thus the target cell), TNF-α release is not[27]. TNF-α can therefore nonspecifically affect other nearby immune cells or vasculature[28]. There is also evidence for IFN-γ- and TNF-α-mediated destruction of tumor stroma[29]. To be sure, directed studies are required further to elucidate the antitumor effects of TNF-α. However, there are obstacles to efficient CTL and other T cell subset trafficking into tumor tissues[30, 31]. One of the major problems is the lack of a mature, properly developed vascular system within the malignancy. Recent imaging studies have contributed to our knowledge of CTL trafficking and the kinetics of killing in the tumor microenvironment[32, 33].

A discussion of tumor-infiltrating CD8$^+$ T cells cannot exclude the seminal work of Dr. Stephen Rosenberg, who was the first to harvest patients' own tumor-infiltrating lymphocytes (TILs), expand them in culture with IL-2, and reinfuse them to take advantage of their capacity for specific lysis[34, 35]. Although some patients experienced clinically measurable improvement, many did not. To evaluate why induced antitumor responses do not necessarily correlate with clinical responses, we must keep in mind both the immune-manipulating properties of the tumor microenvironment (see the antigen-presentation cell section below) and the simple property that tumor cells less susceptible to specific lysis will live and divide longer than those easily killed by TILs[36]. Any surviving tumor will likely be more resistant to such CTL mechanisms of elimination.

2.2 T-Helper-1 Th1

The Th1/Th2 paradigm was first demonstrated in 1986 by Mosmann and Coffman[37]. In their experimental conditions, T-helper-1 (Th1) and T-helper-2 (Th2) cells could be polarized with IFN-γ and IL-4, respectively. The key transcription factors to control Th1 and Th2 polarization are T-bet and GATA3, respectively. The involvement of helper T cells in the development of anticancer immunity was initially thought to include only the priming and support, through CD40/CD154 interactions with antigen-presenting cells (APCs)[38–40] and secretion of IFN-γ and interleukin (IL)-2, of a fully activated $CD8^+$ T cell response[41, 42]. However, subsequent experiments have shown that the importance of both Th1 and Th2 subsets does not end with $CD8^+$ CTL activation. An elegant mouse study from 1998 demonstrated that both Th1 and Th2 cytokines play essential roles in antitumor immunity. Cytokines secreted by Th1 cells are capable of recruiting and activating macrophages[41]. Macrophage-derived nitric oxide has multiple antitumor properties, including control of macrophage killing of tumor cells[43–45]. A key function of Th1-polarized T cells in tumor-bearing hosts is the secretion of IFN-γ, which can substantially increase the level of IL-12 production by stimulated dendritic cells (DCs)[46]. DC-derived IL-12 serves to polarize naïve T cells to the Th1 phenotype. In this way, Th1 cells can contribute to their own population growth and maintenance. Additionally, an interesting recent paper from the Corthay laboratory has suggested that Th1-derived IFN-γ in the tumor microenvironment elicits both in vivo macrophage killing of cancer cells and macrophage elaboration of the angiostatic chemokines CXCL9/MIG and CXCL10/IP-10[47]. Whether this holds true in human patients remains to be determined.

Patients with Kaposi sarcoma have what appears to be a Th1-like predominance in their TIL and blood, characterized by a high secretion of IFN-γ. These patients also had higher $CD8^+$ T cell numbers. Kaposi sarcoma is often accompanied by a concomitant infection with herpesvirus, so it is possible that this Th1-like phenotype is elicited in reaction to the virus[48]. Kusuda et al. found that a higher proportion of IFN-γ to Th2-type cytokines was strongly associated with better prognosis in patients with ovarian cancer[49]. Intriguingly, a study from the same year found that a high Th1:Th2 ratio in the peripheral blood mononuclear cells of patients with non-small cell lung cancer was actually predictive of shorter survival[50]. IFN-γ and chemokines associated with a Th1 response, including monokine induced by IFN-γ (MIG) and IFN-γ-inducible T cell α chemoattractant, identified renal cell carcinomas that did not recur after surgical resection. In addition, higher expression of MIG was correlated with a favorable prognosis[51], suggesting that the induction of a Th1-type response in kidney cancer patients is beneficial. A very recent report examining gastric cancer showed that higher initial Th1:Th2 ratio (as defined by expression of IFN-γ and IL-4) and higher Th1:Th2 ratio 14 days after surgery indicated better patient prognosis[52]. Tosolini and colleagues recently demonstrated that colorectal patients with high levels of Th1-associated gene expression (T-bet, IRF1, IL12Rb2, and STAT4) in their tumor tissue had longer disease-free survival. When the investigators paired some Th1 information with the expression of genes involved in cytotoxicity

(GNLY, GZMB, and PRF1) and Th17-related genes (RORC and IL17A), they could classify patients into four groups. Those with high Th1/cytotoxicity gene expression and low Th17-associated gene expression had the best 5-year disease-free survival.

2.3 Th2

Th2 cells are well known for their involvement in allergy and the response to helminths and other extracellular pathogens. The development of Th2 cells is controlled by the transcription factor GATA-3 and by exposure to IL-4[53–56]. Many laboratories have investigated the function of Th2 cells in the context of tumor immunity and explored how these cells impact disease development and patient survival. Th2 cells are crucial in recruiting eosinophils to the tumor site[41]. Although a definitive effect of this population on the tumor is still controversial, it has been observed that eosinophils are capable of killing tumor cells via secretion of their cytotoxic protein products[57, 58].

Myriad early reports documented an “unbalanced” or “decreased” Th1:Th2 ratio in malignancy[42, 59]. Some studies have found a predominantly Th2 phenotype in TIL populations of certain cancers[60], where they are skewed by tumor cell expression of IL-10 and serve to counteract the IFN-γ-driven Th1 and CTL antitumor response. Huang et al. demonstrated the Th2 cytokine-expressing capacity of non-small cell lung cancers in 1995[61]. Maeurer and colleagues found a similar cytokine signature in renal cell cancer[62]. A very recent report showed that Th2-type cytokines in the microenvironment of colorectal cancer had no prognostic significance for patient survival[63], which correlates well with a previous study[64]. Although early reports suggested that Th2 cells might contribute to antitumor immunity[65], it now seems that these cells fail to protect the host[66]. There is some evidence in mice, however, that the Th2-associated cytokine IL-4 serves to prime Th1-associated, tumor-specific CTL[67]. Melanoma patients who develop Th2 responses usually experience disease progression[68, 69]. Interestingly, some cancer patients do have tumor-antigen-specific Th2 cells in their blood. Melanoma[70–72] and renal cell carcinoma[73] patients have both been examined in this regard. The Rocken laboratory found that in mice, the human tumor-associated antigen EpCAM could induce Th2 skewing even under heavily Th1-polarizing conditions. Although human patient studies are required, it is possible that tumor cell EpCAM could drive a Th2 response while downregulating Th1 development. This combination of Th1/Th2 skewing could help tumors avoid the host immune response.

Pancreatic cancer, one of the most aggressive malignancies, has an intriguing relationship with Th2. Tumor stroma is typically characterized by a heavy Th2 infiltrate[74]. A recent, elegant study demonstrated that the ratio of Th2:Th1 cells in pancreatic tumors could serve as an independent prognostic marker of patient survival[75]. This study also identified cancer-associated fibroblast-derived thymic stromal lymphopoietin as capable of conditioning myeloid DC. These conditioned myeloid DCs could then produce Th2-attracting chemokines and polarize T cells to

a Th2 phenotype. It seems that Th2 cells in the tumor microenvironment can be induced by multiple tumor-derived factors and that they serve to impede or co-opt the development of antitumor responses.

2.4 Th17

Since their identification within the last half decade, Th17 cells have risen to prominence in studies of nearly every human pathology. While their role in many conditions is rather well understood, their function(s) in the context of tumor immunology remains contentious. Th17 cell effects in the tumor microenvironment are often grouped or confused with those of IL-17, IL-23, and other Th17 "signature cytokines." Data from mouse studies and chemically induced tumorigenesis have further complicated the issue. However, here we will focus exclusively on Th17 studies in the human tumor environment.

Th17 cells are defined as $CD4^+$ T-helper cells whose developmental program is controlled by the transcription factor RAR-related orphan receptor gamma T and multiple cytokines[76]. Human tumor-associated Th17 cells express minimal levels of HLA-DR, CD25, granzyme B, programmed cell death 1 (PD-1), or forkhead box P3 (FoxP3), suggesting that they are not a conventional effector or immune-suppressive cell population. Th17 cells in cancer patients produce high levels of granulocyte-macrophage colony-stimulating factor (GM-CSF), TNF-α, IL-2, and IFN-γ, but no IL-10[77, 78]. Tumor-associated Th17 cytokine products mimic those found in some instances of viral infection[79, 80]. These cytokines may be the primary mediators by which Th17 from cancer patients influence local immune responses. Interestingly, Th17 cells expanded in vitro from TIL populations in melanoma, breast, and colon cancers secrete IL-8 and TNF-α, but no IL-2[81]. Because Th17 cells isolated from both healthy donors[82] and patients with autoimmune diseases[83] produce the same cytokines, it is possible the phenotypes of freshly isolated Th17 cells and those induced in vitro from tumor-associated populations differ.

Tumor-associated Th17 express large amounts of the homing molecules CXCR4 and CCR6, c-type lectin receptor CD161, and the CD49 integrin isoforms c, d, and e, but no CCR2, CCR5, or CCR7[77]. As CCR6 and CD161 have been observed on both Th17 cells from healthy donors and on various cells in inflammatory environments[84–86], they may not serve as Th17-specific molecules.

Many laboratories have studied Th17 populations in the blood and (occasionally) tissues of patients with various cancers (**Box 1**). Throughout our work with ovarian cancer patients, we have made several key observations in regard to Th17 distribution and function. Th17 cell numbers in the tumor-draining lymph nodes and blood of these patients is comparable to that of healthy donors. Th17 cells constitute a numerically small but proportionally high population within the tumor microenvironment in comparison to other immune cell subsets. Within the tumor environment, Th17 levels correlate positively with Th1 cells, cytotoxic $CD8^+$ T cells, and NK cells. Perhaps not surprisingly, their numbers are inversely related to those of

regulatory T (Treg) cells[77, 87]. In vitro expansion data from Su et al. corroborates our findings of higher numbers of Th17 cells in TIL populations than in lymphocyte populations from non-tumor tissue[81]. IL-17 derived solely from Th17 cells in ovarian cancer ascites fluid correlated positively with patient survival and served as a negative predictor of death hazard. The average survival of patients with greater than 220 pg/ml IL-17 in ascites was 78 months, while patients with less IL-17 survived for only 27 months. IL-17 in the tumor microenvironment synergized with IFN-γ to induce the Th1-type chemokines CXCL9 and CXCL10. Ascites levels of CXCL9 and CXCL10 correlated directly with tumor-infiltrating NK and $CD8^+$ T cells, suggesting that these chemokines recruited effector cell populations to the tumor[77]. In agreement with our finding that Th17 cells are protective, Sfanos et al. found an inverse correlation between Th17 cell differentiation stage in the tumor mass in prostate cancer patients and their tumor progression[88]. Malignant pleural effusion from patients with lung adenocarcinoma or squamous cell carcinoma was chemotactic for Th17 cells, and this activity was partially abrogated by chemokine ligand 20 (CCL20) and/or CCL22 blockade. Interestingly, higher accumulation of Th17 cells in malignant pleural effusions predicted improved patient survival[89].

Intriguingly, Derhovanessian et al. demonstrated an inverse correlation between pretreatment circulating levels of Th17 cells in patients with hormone-resistant prostate cancer and time to disease progression[90]. The levels of Th17 cells are usually limited in cancer patients[77, 87]. Increased Th17 in the blood could indicate an underlying infection or other inflammatory state. IL-17 would certainly have an impact on the efficacy of immunotherapy and tumor development speed. IL-17-producing cells are enriched predominantly in the peritumoral stroma of hepatocellular carcinoma tissues, where their levels correlated with monocyte/macrophage density. Consistent with our observations[77], Kuang et al. found that tumor-activated monocytes were better than tumor-associated macrophages (TAMs) in inducing in vitro expansion and proliferation of Th17 from circulating memory T cells[91]. However, not all studies of Th17 in malignancy demonstrate a clear relation to disease progression: a recent study showed no correlation of Th17 numbers with nasopharyngeal patient clinicopathological characteristics or survival[92].

Patients with chronic inflammation have a greatly increased risk of cancer in the affected organs[93, 94]. Because inflammation resulting from infections can often contribute to the development of malignancy, it is necessary to understand the kinetics and targets of inflammation in a discussion of cancer. Our laboratory found that Th1-derived IFN-γ could rapidly induce B7-H1 expression on APCs and stimulate their production of IL-1 and IL-23. B7-H1 signaling abrogated the Th1-polarizing capacity of the APC, while IL-1 and IL-23 directed them toward a memory Th17-expanding phenotype[95]. In the course of inflammation, the acute Th1-mediated response is attenuated by IFN-γ-induced B7-H1 on APCs and is subsequently evolved toward chronic inflammation mediated by Th17 cells. Not only does this data challenge the dogma of Th17 suppression by IFN-γ, it also reinforces the notion that Th17 population kinetics depend strongly on the ongoing immune response and constituents of the cytokine milieu. Disease progression influences both of these factors.

2.5 Treg

T regulatory cells, originally termed suppressive T cells, were first described in the early 1970s as thymus-derived lymphocytes that tolerized bone marrow-derived lymphocytes to antigenic challenge[96, 97]. Subsequent research demonstrated that T cells expressing CD4 and CD25 from tumor-bearing mice abrogated tumor rejection[98–100]. After more than a decade of intense skepticism, Sakaguchi and colleagues ascertained that the IL-2 receptor α-chain (CD25) could be used to identify these suppressive cells[101]. Later studies in the same laboratory and others established the transcription factor FoxP3 as both a key intracellular marker of $CD4^+CD25^+$ Tregs and was a necessary factor for development and proper function of these cells[102–104]. Beginning with these reports, the field of Tregs has expanded and progressed rapidly. In fact, several distinct regulatory T cell populations have been proposed, including $CD8^+$ subsets. These include $CD8^+CD25^+$ T cells from the thymus that utilize TGF-β and cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4) to suppress cell activation and proliferation[105], as well as a peripheral $CD8^+CD28^-$ T cell population that targets DC immunoglobulin-like transcripts 3 and 4[106]. We have identified IL-10-secreting $CD8^+$ T cells[107, 108] in human ovarian cancer. A $FoxP3^-CD4^+$ population (termed T_R1 cells) identified by Groux et al. can also suppress through IL-10 in vitro[109]. Weiner et al. characterized a peripherally derived $CD4^+TGF\text{-}\beta^+$ population (T_H3) that exerts suppressive action in vivo through TGF-β[110]. $CD4^+CD25^+FoxP3^+$ T cells, termed "classical T regulatory cells" or T_{Regs}, differentiate in the thymus and then migrate to the periphery[111, 112]. T_{Regs} constitutively express glucocorticoid-induced tumor necrosis factor receptor-related protein, leukocyte common antigen isoform RO (CD45RO), and CTLA-4[113–117]. Recent data presents the possibility of further categorizing naturally occurring T_{Regs} into three subgroups: $CD45RA^+FoxP3^{lo}$ resting T_{Reg}, termed "rTreg," $CD45RA^-FoxP3^{hi}$ activated Treg (aTreg) cells, and cytokine-secreting $CD45RA^-FoxP3^{lo}$ non-suppressive T cells[118]. Ongoing investigations into phenotype and function will likely contribute to the appreciation of an even wider range of regulatory T cell populations in the future.

In humans, T_{Reg} cells are found primarily in the thymus, peripheral blood, lymph nodes, and spleen, where they constitute 5–10% of the resident $CD4^+$ T cells[119–121]. In bone marrow, however, they make up a remarkable 25% of $CD4^+$ T cells[122]. Bone marrow is the preferential site of metastasis for some cancers (such as breast, lung, and prostate), suggesting that the suppressive environment here is conducive to malignancy. In tumors themselves, however, there are a number of ways that T_{Reg} cells accumulate: trafficking under the influence of CCL22[123], differentiation[89, 96, 107, 108, 124, 125] or expansion[126–128] within the stroma, and conversion from other T cell populations[129–132]. Many types of tumors express tumor-associated antigens—molecules found on tumor cells and certain populations of normal cells. Multiple mechanisms of suppression enacted by tumor-associated antigen-specific T_{Reg} cells have been identified. These include the induction of IL-10 and TGF-β, which can drastically suppress APC, natural

killer (NK), and T cell function[133, 134]; competitive consumption of the T cell survival factor IL-2[119, 135, 136]; perforin and granzyme-dependent killing of APCs and T cells[137, 138]; CTLA-4 induction of indolamine 2,3-dioxygenase (IDO)-expression, which promotes tolerance[139–141]; and finally induction of B7-H4 expression on APCs, which renders them immunosuppressive[142]. In these ways, T_{Reg} cells target both T cells and APCs to create a generally tolerant tumor microenvironment.

Increased numbers of T_{Reg} cells have been observed in patients with many types of cancer, including pancreatic and breast cancer[143], colorectal cancer[144, 145], gastric and esophageal cancer[146, 147], leukemia and lymphoma[148, 149], melanoma[150, 151], lung and ovarian cancer[145, 152], and hepatocellular carcinoma[153].

Many studies have examined the prognostic significance of T_{Reg} cells in the tumor microenvironment, and these are reviewed in detail[154]. Briefly, higher T_{Reg} numbers in and around ovarian cancer negatively impact disease progression and patient survival[6, 87, 155]. Work from our laboratory has demonstrated that B7-H4 expression on TAMs and tumor cells correlated with intratumoral T_{Reg} presence[142]. Higher numbers of T_{Regs} in pancreatic cancer also predict more advanced disease and shorter survival[156]. Melanoma is similar: T_{Reg} populations were larger in patients who experienced recurrence than in those who did not. Interestingly, T_{Regs} were often found in proximity with TAMs, the presence of which is associated with poor prognosis[157, 158]. Breast cancer patients with higher T_{Reg} numbers have increased chance of relapse and shorter overall survival[159]. Finally, more liver cancer-associated T_{Regs} correlate with poorer disease-free and overall patient survival[4, 160].

T_{Regs} in other cancers are not so easy to define. Increased T_{Regs} in head or neck squamous cell carcinoma indicate better regional tumor control[161]. Studies in gastric cancer point to T_{Reg} location, rather than number, as an important prognostic factor in that patients with peritumoral T_{Regs} had better overall survival than those with a diffuse T_{Reg} pattern[162]. Another study found that larger Treg populations in the stroma of gastric cancer patients correlated positively with longer survival[163]. Colorectal cancer studies parallel gastric cancer: various studies have found associations of higher T_{Reg} numbers with poorly differentiated tumors or earlier stage and better patient overall survival[164–166]. In lymphoma, fewer T_{Regs} and more CTLs in the reactive background serve as an independent prognostic factor suggesting shorter patient disease-free survival[167]. It is possible that in these cancers, T_{Reg} cells predominantly function to minimize inflammation rather than curb the antitumor response. More careful mechanistic studies will shed light on this hypothesis.

2.6 Myeloid Dendritic Cells

Myeloid DCs are the most frequently studied of the APC subsets. They stimulate the adaptive arm of the immune system by activating naïve T cells[168]. Pulsing of DCs with killed ovarian tumor cells can stimulate tumor-specific blood-derived

T cells, which can produce IFN-γ upon autologous tumor cell encounter[169]. Various other studies demonstrate the potential of tumor-antigen-pulsed DCs to stimulate CTL responses in vitro[170, 171]. The antitumor protection observed upon adoptive transfer of appropriately primed myeloid DCs to tumor patients[172, 173] is rarely seen in the natural development of human tumors[174]. The tumor and its environment produces factors that suppress the development and normal function of DCs[107, 175], which compromises antitumor immunity. In 2003, our laboratory demonstrated low expression levels of the inhibitory molecule B7-H1 on blood- and lymph node-derived myeloid DCs in healthy individuals but observed much higher expression of the molecule on myeloid DCs from tumor-draining lymph nodes and tumors[176] from patients with ovarian cancer. B7-H1 expression on these cells was controlled by IL-10, previously shown to decrease co-stimulatory molecules on DCs[177], and vascular endothelial growth factor, known to inhibit DC differentiation from hematopoietic precursors[175]. Abrogation of B7-H1 signaling enhanced myeloid DC-mediated T cell activation, which correlated with a decrease in T cell-derived IL-10 and an increase in T cell-derived IL-2 and IFN-γ. Interestingly, this treatment also downregulated IL-10 expression and stimulated increased IL-12 expression on myeloid DCs. T cells conditioned with myeloid DCs in which B7-H1 had been blocked could inhibit autologous human ovarian carcinoma growth better than unconditioned T cells when xenotransplanted into nonobese diabetic−severe combined immunodeficient mice. A recent report from the Knustson laboratory showed that in addition to expressing B7-H1, murine ovarian tumor-associated myeloid DCs acquire higher levels of programmed death receptor-1 (PD-1) over time. PD-1 ligation on these cells impeded NF-κB activation, elaboration of numerous cytokines (IL-10, IL-6, IL-12, TNF-α, and GM-CSF) and co-stimulatory molecule upregulation[178].

Hepatocyte growth factor could stimulate papillary thyroid carcinoma cells to secrete MIP-3α (CCL20) and other chemokines to recruit immature myeloid CD1a^{++} DCs to the tumor periphery[179, 180]. By contrast, mature DCs have been documented in colon cancer, albeit at a lower density than in normal colon tissue[181]. Tumor expression of VEGF and TIL expression of TNF-α were associated with higher intratumoral DC infiltration. Interestingly, DC infiltration in metastases was approximately sixfold lower than in the primary colorectal tumors. Studies in breast cancer have revealed that immature DCs infiltrate tumor beds, while mature DCs remain in peritumoral areas[182, 183]. It seems that breast cancer tumor cells prompt intratumoral myeloid DCs to polarize local naïve T cells to an IL-13 (Th2-type cytokine)-secreting phenotype, which facilitated the progression of human tumor growth in a mouse xenograft model[184]. Culture of human multiple myeloma cell lines and primary multiple myeloma cells with myeloid DCs leads to improved survival, proliferation, and enhanced clonogenicity of the tumor cells. These effects can be abrogated by blockade of RANK ligand and APRIL[185]. In primary multiple myeloma samples, myeloid DCs are found to co-localize with tumor cells, suggesting that these interactions may occur in vivo[186].

A few years ago, Huarte et al. demonstrated that CD11c^{+}DEC205^{+} DCs co-expressing α-smooth muscle actin and VE-cadherin played an essential role in

tumor vasculature maintenance[187]. Decelerated tumor growth after depletion of myeloid DCs was associated with vascular apoptosis. Our laboratory's more recent studies demonstrated that both myeloid DCs and macrophages (but not plasmacytoid DCs) from normal donors were capable of inducing Th17 cells from memory but not naïve CD4^{+} T cells, and myeloid DCs and macrophages in the ovarian tumor microenvironment were similarly capable[77]. The relevance of Th17 induction is discussed in the next section. Altogether, myeloid DCs are thought to be the major functional DC subsets in the malignant microenvironment. Myeloid DC vaccination has been utilized in clinical trials to treat cancer patients, albeit with generally modest results at best. Functional mature myeloid DCs exist in limited numbers within the tumor, and many if not all are phenotypically and functionally altered. Myeloid DCs that are dysfunctional or mediate immune suppression are likely a reason for these thus far unsatisfying clinical observations.

2.7 Macrophages

TAMs form the major APC subset (by number) in solid human epithelial cancers. Several years ago, our group discovered that both tumor cells and microenvironmental macrophages in ovarian cancer expressed CCL22, a chemokine instrumental in attracting Tregs to the tumor environment[87]. Interestingly, because the presence of Tregs predicts poorer survival and is associated with a high death hazard in ovarian cancer patients, TAMs may contribute to their prognoses. Indeed, we subsequently demonstrated that although they are highly B7-H4 positive, ovarian cancer cells do not directly mediate antitumor T cell suppression. However, B7-H4^{+} macrophages from the human ovarian tumor microenvironment are powerful suppressors of tumor-associated antigen-specific T cell immunity[142]. B7-H4 blockade restored the stimulatory capacity of macrophages and mediated ovarian tumor regression in vivo in NOD/SCID mice. Both IL-10 and IL-6, often found in high concentrations in the tumor environment, can induce B7-H4 expression on macrophages. Contrastingly, two cytokines minimally expressed in the same environment—GM-CSF and IL-4—inhibit B7-H4 expression. Interestingly, forced expression of B7-H4 in macrophages from healthy donors conferred a suppressive phenotype on the cells. As for the prognostic significance of B7-H4^{+} macrophages in ovarian cancer, we documented an inverse relationship between the intensity of B7-H4 expression on macrophages and patient survival. Importantly, Tregs, typically predictors of poor prognoses in cancer patients[154], could induce B7-H4 expression on myeloid APCs (including macrophages) and were positively associated with B7-H4^{+} macrophage presence in ovarian tumors[188]. A later observation of Wan and colleagues showed that the mean density of TAMs is significantly higher in ovarian cancer than in benign ovarian lesions and that the average 5-year survival rate in patients with low densities of TAM was significantly higher than in patients with larger TAM populations, agreeing well with our observations. Multivariate analysis demonstrated that TAM infiltration status serves as an independent negative

predictor for overall survival of patients with ovarian cancer[189]. The presence of CCL17$^+$ or CCL22$^+$ cells in CD14$^+$ monocytes and macrophages within gastric tumors correlated directly with Treg cell presence. Tregs were also shown to migrate toward CCL17 and CCL22[162]. A study by Haas et al. demonstrated that a higher ratio of stromal CD68$^+$ (a monocyte/macrophage glycoprotein) cells to FoxP3$^+$ cells in intestinal-type gastric cancer patients correlated with shorter median survival time[163]. Another study from our laboratory examined B7-H1 expression on Kupffer cells in hepatocellular carcinoma and found that it was increased in comparison to normal tissue. This expression correlated with poor survival. Not surprisingly, B7-H1$^+$ Kupffer cells impaired the proliferation and effector function of CD8$^+$PD-1$^+$ T cells from the tumor tissue that was reversed upon B7-H1/PD-1 blockade[157]. Finally, a report from Miracco in 2007 showed that Tregs and TAMs were co-localized in melanoma tumors in human patients[157]. TAM presence in advanced melanoma has also been correlated with poor patient prognosis[158].

As for function within the tumor microenvironment, macrophages display a number of pro-tumor activities. They can modify the extracellular matrix; secrete proangiogenic chemokines such as fibroblast growth factor, monocyte/macrophage chemoattractant protein-1 (MCP-1), and VEGF; and produce the immunosuppressive cytokine IL-10[190–193]. MCP-1 expression in breast tumors and TAMs correlated significantly with the presence of other angiogenic factors and with macrophage infiltration of the tumor. Higher levels of TAMs indicated patients with a higher risk of early relapse[194]. Higher MCP-1 levels in urine correlated with more advanced bladder cancer stage[195]. MCP-1 positive invasive ductal breast carcinomas were poorly differentiated, suggesting a correlation of MCP-1 expression and tumor grade[196]. However, a subsequent study showed that MCP-1 levels did not correlate with TAM infiltration in breast carcinoma[197]. It is therefore likely that MCP-1 is not the only chemokine responsible for attracting macrophages into the tumor microenvironment.

The function(s) and prognostic significance of Th17 cells in human cancer are still under discussion[198, 199]. Although few human studies on the subject are published, it seems that Th17 in established epithelial cancers (like ovarian) act to recruit other effector T cell subsets and in doing so, support antitumor immunity[77]. As discussed, both ovarian cancer-derived myeloid DCs and macrophages are capable of Th17 induction. TAMs are more potent Th17 cell inducers than either tumor-derived myeloid DCs or blood macrophages from healthy volunteers. Th17 cell induction is additionally dependent upon TAM expression of IL-1β and IL-23. Blockade of either cytokine significantly decreases the resultant Th17 population, while concomitant blockade of both further diminishes final numbers. In the tumor microenvironment, Th17 induction is also suppressed by Treg cells[77]. In summary, macrophages are the largest APC subset in ovarian and quite possibly other types of cancer, where they may suppress antitumor immunity through multiple modes of action, including the expression of inhibitory B7 family members, the elaboration of proangiogenic chemokines, and the recruitment of Tregs.

2.8 Plasmacytoid Dendritic Cells

Our laboratory was responsible for some of the first studies of plasmacytoid DCs in the tumor environment. A decade ago, we found that human ovarian cancer cells express extremely high levels of stromal-derived factor-1 (SDF-1), which induced plasmacytoid DC trafficking to the tumor via signaling through CXC chemokine receptor-4 (CXCR4)[107, 200]. Additionally, SDF-1 induced plasmacytoid DC expression of very late antigen-5, which interacted with VCAM-1 to mediate cell adhesion and migration through vessel walls. SDF-1 also protected plasmacytoid DCs from apoptosis induced by IL-10 from TAM. Tumor-associated plasmacytoid DCs could induce interleukin-10 production from nearby T cells, which impeded T cell activation by local myeloid DC. This is evidence that plasmacytoid DCs can undermine antitumor immunity and contribute to a suppressive tumor environment. We have also demonstrated a role for plasmacytoid DCs in promoting angiogenesis in ovarian tumors[201]. SDF-1 attracted plasmacytoid DCs into the tumor, where they induced angiogenesis through the production of proangiogenic mediators including TNF-α and IL-8. Conversely, functional myeloid DCs, although numerically restricted in the tumor microenvironment, could suppress angiogenesis in vivo via elaboration of IL-12. These data suggest that malignant cells attract plasmacytoid DCs through expression of SDF-1 to augment vessel formation while excluding the presence of angiogenesis-inhibiting myeloid DCs. We subsequently observed that plasmacytoid DCs from malignant ascites could induce $CD8^+$ regulatory T cell populations[202], in contrast to macrophage-derived DCs[203] which induced tumor-associated antigen-specific $CD8^+$ T cells with effector functions. $CD8^+$ suppressor cells induced by plasmacytoid DCs were $IL\text{-}10^+CCR7^+CD45RO^+$, and could suppress myeloid DC-mediated tumor-associated antigen-specific T cell effector functions via IL-10. Plasmacytoid DC CCR7 was functional, as they migrated efficiently under the influence of the lymphoid homing chemokine MIP-3β. Suppressive populations of $CCR7^+CD45RO^+CD8^+$ T cells are found in the tumor environment of ovarian cancer patients, suggesting the in vivo functionality of tumor-associated plasmacytoid DC. Ovarian cancer-associated plasmacytoid DCs can thus induce $CD8^+$ Treg cells and promote tumor angiogenesis, inhibiting antitumor immunity.

Plasmacytoid DC detection (which occurs in approximately one-tenth of breast carcinoma samples) is correlated with poor prognosis[204]. This phenomenon may be attributed to the fact that cells of at least one type of human cancer (head and neck squamous cell carcinoma) negatively impact the ability of plasmacytoid DCs to elaborate IFN-α upon toll-like receptor stimulation[110]. Fascinatingly, investigators found that treatment of basal cell carcinoma with Imiquimod (a toll-like receptor 7 agonist) could induce myeloid DCs to express perforin and granzyme and plasmacytoid DCs to express TRAIL. Imiquimod-treated myeloid DCs and plasmacytoid DCs could kill human tumor cell lines and MHC I-expressing Jurkat cells, respectively, suggesting a new functionality of DCs in immune (and possibly antitumor) responses[203]. Plasmacytoid DCs have also been seen to accumulate in the peritumoral area of primary cutaneous melanomas, likely as a result of

melanoma cell production of SDF-1. Peritumoral plasmacytoid DCs could produce type I IFNs, but their expression of MxA (myxovirus resistance protein A, an IFN-α-inducible protein) was extremely varied and typically minimal. Intratumoral plasmacytoid DCs have an immature phenotype, suggesting incomplete development, possibly influenced by the tumor itself[205]. Salio and colleagues observed that plasmacytoid DCs from human blood could efficiently prime naïve melanoma tumor-antigen (melan-A)-specific $CD8^+$ lymphocytes to become IFN-γ-producing cells in vitro[206]. Plasmacytoid DCs stimulated with CD40L induced cutaneous lymphocyte antigen and L-selectin (CD62L) expression on primed tumor-associated antigen-specific T cells. These homing receptors could allow effector cell migration to diseased skin. This study also confirmed the presence of plasmacytoid DCs in the peritumoral area of most primary cutaneous melanomas in vivo. Plasmacytoid DC type I IFN-containing supernatant induced upregulation of CD95 and MHC class I and class II molecules on melanoma cells in vitro. Thus, tissue-infiltrating plasmacytoid DCs could have a previously unknown immune-modulating capacity.

2.9 B Cells

As noted above, tumor-infiltrating $CD8^+$ T lymphocytes typically correlate positively with improved survival of cancer patients. B cells have been observed to co-localize with T cells and are known to provide various support functions. However, the association of B cell presence or function with patient prognoses in cancer has not been well studied[207]. Milne and colleagues recently demonstrated that $CD20^+$ tumor-infiltrating B cells could be found in more than two-fifths of high-grade serous ovarian cancer samples[208]. B cell presence here was strongly associated with $CD4^+$ and $CD8^+$ T cells, the activation markers CD25 and CD45RO, and markers of T cell effector function including expression of tumor infiltrating B cells (TIA-1) and granzyme B. Intriguingly, B cells were also associated with T cell expression of FoxP3, a marker that could indicate either activated or regulatory T cells[209, 210]. Intraepithelial B cell numbers correlated positively with improved patient disease-specific survival, while fascinatingly, the combination of $CD8^+$ and $CD20^+$ TILs in the same tumor indicated significantly increased disease-specific survival over tumors that contained one or the other type of TIL. $CD20^+$ cells could support the actions of tumor-associated effector T cells through various mechanisms. In mice, B cells can produce autoantibodies directed against tumor targets[211]. It is possible that tumor-infiltrating B cells can raise the concentration of antitumor autoantibodies in the tumor microenvironment to physiologically relevant levels. Tumor-infiltrating B cells can also secrete granzyme B[212] and TRAIL[213] and induce tumor cell death through both of these mechanisms. New evidence of B cell killer potential is coming to light and will no doubt inform future studies of these cells in the context of malignancy[214].

B cell infiltration of tumors has been examined in multiple tumor settings. They are detected in approximately one-quarter of breast cancers, where they can make up nearly 40% of the TIL populations[215–217]. B cells are early infiltrators of breast

cancer[218]. Tumor-infiltrating B cell phenotypes appear driven by affinity maturation[219–222] and can also be found in tertiary lymphoid structures, where they co-localize with $CD4^+$ T cells, $CD8^+$ T cells, and/or DCs[223–225]. The expression of B cell signature genes in node-negative breast cancer was shown to have positive prognostic significance[226]. In medullary breast cancer, the presence of B cells and T cell subsets appears to be beneficial for patient survival[217, 227, 228]. A very recent study utilizing the 4T1 mouse model of breast cancer identified a subset of activated B2 cells ($CD19^+CD25^{hi}CD69^{hi}$) that proliferated poorly. Interestingly, these cells expressed B7-H1 and their principal function within the tumor environment appeared to be mediating the conversion of $CD4^+$ T cells to Treg cells via production of TGF-β[229]. Whether this conversion occurs in human cancer remains to be seen. B cells have also been examined in non-small cell lung cancer, where their presence in epithelium, tumor stroma, and tumor lysis syndrome (TLS) correlate strongly with better survival[230–232]. B cells in lung cancer are specific for antigens that include the tumor suppressor gene p53 and other molecules typically overexpressed in tumor tissue[233]. Finally, in cervical cancer, peritumoral B cell presence is associated with decreased patient relapse[234].

Interestingly, an earlier study examined $CD19^+$ cell presence in post-chemotherapy effusions from advanced ovarian cancer and found that it was predictive of poorer survival[235]. How then, can B cells be prognostically good in one investigation of ovarian carcinoma while detrimental in another? First, patients in the latter study were subjected to chemotherapy (which can profoundly affect the numbers and functionality of immune cell subsets), while those in the former study were not. Second, the populations delineated by CD19 and CD20 are not precisely the same: while CD20 is present on the surface of all mature B cells[236–238] and CD19 is predominantly expressed on B cells[239], these surface markers have slightly different expression profiles. Finally, the activation state of B cells contributes to their effector or suppressor functions in various pathologies: resting B cells inhibit the antitumor response[240], while activated B cells can aid T cell responses[241].

The roles for B cells in the malignant microenvironment are many. B cells can effect regulatory functions. They can be polarized by Th subsets into subpopulations that produce IFN-γ, IL-12, and TNF-α and promote Th1 skewing, or they can be producers of IL-2, IL-4, TNF-α, and IL-6 that support Th2 development. B cell production of these groups of cytokines feeds back into the maintenance and expansion of the Th populations that initially stimulated their cytokine expression, thereby maintaining and propagating a Th1 or Th2-type cytokine milieu[242, 243]. B cells can also influence T cell memory, survival, and proliferation[244, 245], as well as present antigen to both T cells[241, 246]. In advanced tumors, where DCs may have become suppressive or rare, B cells could serve a greater antigen-presentation role[207]. This, however, could act as a double-edged sword: presentation to helper or cytotoxic T cells might support antitumor immunity, while antigen presentation to Tregs could undermine the antitumor response. B cells can additionally mediate immunosuppressive functions via their cytokine products. The immunoregulators IL-10 and TGF-β can both be produced by B cells[246] and foster downregulation of antigen presentation, suppression of T cell activation, and maintenance of Treg

suppressor function[247–249]. However, we have recently demonstrated several new roles for IL-10 in support of antitumor immunity, including the moderation of tumor-associated suppressive cellular networks including regulatory T cells and myeloid-derived suppressor cells[250]. Further research is warranted to determine whether B cell-derived IL-10 acts solely to suppress or support antitumor immunity, or whether these cells' functions are context-dependent, like so many other immune factors. B cells themselves may serve beneficial or detrimental roles to antitumor immunity depending on their intratumoral phenotype.

2.10 Conclusions

It is evident that different subsets of immune cells infiltrate tumors in different degrees. The detailed molecular and cellular mechanisms controlling the quantity and quality of immune infiltration remain to be fully dissected. It is clear that immune infiltration is different from tumor to tumor and from different clinical stages. Therefore, the pathological relevance of each immune subset tumor infiltration may be generalized and need to be analyzed in a specific situation. It is expected that manipulation of tumor immune cell infiltration should be therapeutically important in treating patients with cancer.

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Part II
Passive Strategies to Boost Antitumor Immunity

Chapter 3
Adoptive T Cell Transfer

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Abstract The clinical use of adoptively-transferred T or NK lymphocytes with anti-tumor activity is gaining in popularity as reports of success accumulate. High specific activity with minimal off target effects contribute to a class of therapy with minimal toxicity that is transformative for cancer patients, who with their physicians have come to accept severe short and long term toxicities as the cost of a frequently small hope of cure or extension of life. However, the successes of cell therapies are as yet in the minority and scientists are developing and testing strategies to improve the function and persistence of adoptively transferred lymphocytes in the face of multiple, potent immune evasion strategies used by tumors and their accessory cells to coexist with an intact immune response. These strategies include combination with chemotherapies and/or radiation and genetic modifications that improve T cell function and tumor targeting. However many of these strategies come with a price and re-introduce toxicities including death in rare cases. This chapter will outline the development of tumor-specific lymphocyte therapies, highlighting successes and difficulties and discussing potential ways forward in this exciting field.

3.1 Introduction

Potent cytotoxicity with exquisite specificity is the *sine qua non* of novel cancer therapeutics. Nowhere in nature are these two qualities better exemplified than in the cytotoxic T lymphocyte (CTL).

Humans possess a diverse T cell repertoire with approximately 2.5×10^7 distinct T cell receptors (TCRs) recognizing unique peptide sequences or antigens[1]. Each

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T cell is capable of massive clonal expansion and potent cytotoxic activity after pathogen recognition, yet the T cell response to infection is coordinated and controlled. Infected cells are efficiently destroyed with minimal damage to surrounding healthy tissues, and T cell numbers rapidly return to homeostatic levels once the infection is resolved. Tumor immunotherapy attempts to harness the power and specificity of the cellular immune response and direct or amplify it against malignant cells. Indeed, the stimulation of tumor-specific T cells is the end goal of cancer vaccines, while the ability to recruit natural killer (NK) cells is an important component of anticancer antibodies. Perhaps the most direct way to confer antitumor cellular immunity to patients is by adoptive T cell transfer, which refers to the ex vivo culture and subsequent infusion of autologous or donor-derived T lymphocytes. Not far behind, the use of NK cells as mediators of antitumor responses in an adoptive immunotherapy setting is gaining increased attention over the last few years owing to improvements in the understanding of their function, activation and ex vivo expansion.

In this chapter, we will discuss the three major adoptive T cell transfer platforms that have been used clinically, (1) Epstein–Barr virus (EBV)-specific T cells for EBV-associated malignancies, (2) tumor-infiltrating lymphocytes (TIL) for metastatic melanoma and ovarian cancer, and (3) genetically modified T cells directed against various solid and hematological malignancies, and conclude with a brief discussion focused on NK cells as an immunotherapy platform.

3.2 EBV-Associated Malignancies

One of the first challenges to adoptive T cell transfer for tumor immunotherapy is the identification of antigens that are uniquely expressed by malignant cells and can serve as suitable targets for T cells. Antigens for T cell targeting should meet several requirements. They should (1) be presented on the cell surface by human leukocyte antigen (HLA) major histocompatibility complex (MHC) antigens, (2) be expressed on malignant cells but absent or expressed at low levels on normal cells, (3) have no expression on vital organs, and (4) ideally have some essential role in supporting tumor growth or maintenance. Malignancies associated with viruses are optimal targets for T cell therapy as many viral antigens fulfill all of these requirements. Several viruses are known to be associated with cancer, but in this section, we will focus on Epstein-Barr virus (EBV)-associated malignancies as there has been extensive experience using adoptive T cell transfer as immunotherapy for EBV-expressing tumors.

3.2.1 EBV-Associated Posttransplant Lymphoproliferative Disorder

Hematopoietic stem cell transplantation and solid organ transplantation recipients receive intensive and/or prolonged immunosuppression putting them at increased risk for developing lymphoma[2]. At least 90% of these “lymphomas” express EBV

antigens, implicating the virus as a causative agent[3]. Today this condition is referred to as posttransplant lymphoproliferative disease, reflecting the fact that this is a heterogeneous malignancy that could present as a polyclonal hyperplasia or a monoclonal, aggressive non-Hodgkin's lymphoma[4]. EBV-associated lymphoproliferative disease has also been documented in patients with acquired immunodeficiency syndrome and congenital immunodeficiency, further supporting the hypothesis that a deficient cellular immune response to EBV is a major contributor in its development[5].

One strategy to combat this complication in hematopoietic stem cell transplant recipients is the infusion of unmanipulated donor-derived leukocytes. If the stem cell donor is EBV seropositive then donor lymphocyte infusions should have protective cellular immunity against the virus, which can be transferred to the recipient by T cell infusion. This strategy proved effective, resulting in complete responses in 17 of 30 patients[6]. Unfortunately, a significant fraction of unmanipulated donor T cells are alloreactive, putting patients at significant risk for developing graft versus host disease (GVHD). In the previous study, it was reported that 17% of patients receiving donor lymphocyte infusions developed GVHD.

3.2.2 EBV-Specific CTL for Prophylaxis and Treatment of EBV-Associated Posttransplant Lymphoproliferative Disease

To reduce the incidence of GVHD associated with donor lymphocyte infusions, ex vivo expanded, EBV-specific T cells for the prevention and treatment of posttransplant lymphoproliferative disease have been evaluated. Posttransplant lymphoproliferative disease provides an excellent model in which to evaluate the efficacy of adoptively transferred EBV-specific CTL because the tumor cells express all latent-cycle virus-encoded antigens (EBNAs 1, 2, 3A, 3B, 3C and LP, BHRF1, BARF1 and LMP1, 2A and 2B), most of which are targets for virus-specific immune responses[7–10]. Furthermore, immortalized lymphoblastoid cell lines, that express the same viral antigens, can readily be generated from essentially any donor by infecting B cells with a laboratory strain of EBV. Lymphoblastoid cell lines function as superb antigen-presenting cells, expressing lytic and latent-cycle EBV antigens as well as costimulatory molecules that facilitate CTL generation. The ex vivo stimulation of peripheral blood mononuclear cells for several weeks with lymphoblastoid cell lines and interleukin (IL)-2 produces a highly enriched population of polyclonal EBV-specific CTL (Fig. 3.1).

Our group has treated 101 hematopoietic stem cell transplant recipients prophylactically with EBV-specific CTL. None developed EBV-related posttransplant lymphoproliferative disorder with up to 17 years of follow-up, compared with 5 of 42 (11%) patients enrolled on the same transplantation protocol who did not receive EBV-specific CTL[14]. Of 13 patients who received CTLs as treatment for biopsy proven or probable EBV-related posttransplant lymphoproliferative

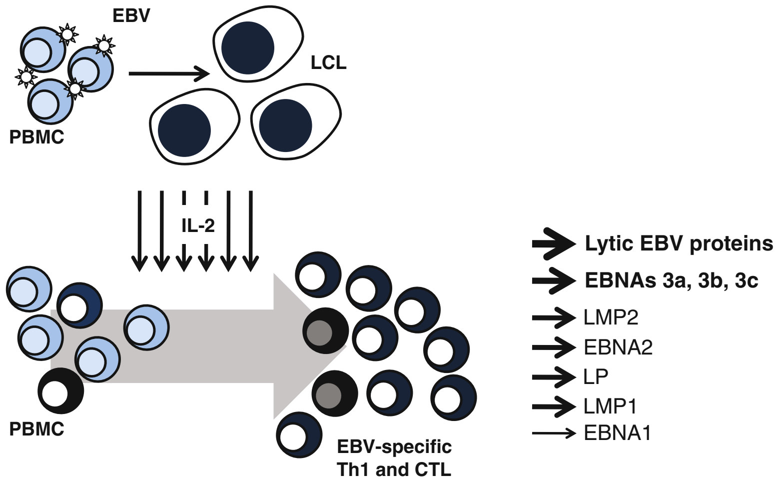

Fig. 3.1 Generation of EBV-specific cytotoxic T lymphocytes (CTLs). In the first step, EBV-transformed B lymphoblastoid cell lines (EBV-LCLs) are generated from the patient or stem cell donor for use as antigen-presenting cells (APCs). Peripheral blood mononuclear cells (PBMCs) are infected with the B95-8 strain of EBV in the presence of cyclosporin A to inhibit EBV-specific T cells. A permanently growing cell line can be established from most healthy donors within 4 to 6 weeks, but is frequently slower from patient blood. In the second step, the EBV-LCL is irradiated and used to stimulate PBMCs from the same donor to activate EBV-specific T cells. The responder T cells are restimulated weekly with the irradiated EBV-LCL from day 9 and IL-2 is added twice weekly from day 14 until sufficient T cells have been expanded. These T cell lines comprise CD4+ and CD8+ T cells specific for a range of EBV lytic cycle and latency-associated antigens. In clinical productions, the virus is drawn from a clinical grade, working virus bank and the EBV-LCLs are cultured for at least 2 weeks in ganciclovir to prevent the release of infectious virus.

disorder, 11 (85%) achieved complete remission with no recurrence. Importantly, CTL infusions were safe and not associated with *de novo* GVHD. Gene-marking studies also showed that the infused CTL could expand by several $\log_{10}$ in vivo, contribute to the memory pool (persisting for up to 9 years), and traffic to tumor sites[11–14]. The safety and efficacy of donor-derived EBV-specific CTL for the prophylaxis and treatment of EBV-related posttransplant lymphoproliferative disorder has been subsequently confirmed by other investigators[15,16].

While EBV-specific CTLs reconstitute immunity to EBV and prevent EBV-related posttransplant lymphoproliferative disorder after hematopoietic stem cell transplant, their use as therapy is limited by the time required (~3 months) for production, in addition to logistical and cost issues. Two different avenues of research are being pursued to overcome the time limitation. The first involves the use of banked, allogeneic EBV-specific CTLs, which are readily available as an “off-the-shelf” therapy. In one multicenter clinical trial, 31 solid organ transplant and 2 hematopoietic stem cell transplant recipients with EBV-related posttransplant lymphoproliferative disorder who had failed conventional therapies received allogeneic EBV-specific CTL. These infusions were well tolerated and the overall

response rate was 52% at 6 months including 14 patients with a complete response[17]. Other strategies aimed at shortening the production time of EBV-specific CTLs include the use of (1) EBV-specific peptides for overnight stimulation of donor peripheral blood mononuclear cells that can then be selected based on their secretion of interferon (IFN)-γ[18,19], (2) HLA–peptide multimers that can directly select viral peptide-specific T cells from peripheral blood mononuclear cells[20], or (3) dendritic cells nucleofected with DNA plasmids encoding immunodominant EBV antigens to expand EBV-specific CTL rapidly over 7–10 days[19,21]. Only the first strategy has been evaluated clinically for EBV. For example, Moosmann et al. treated 6 patients with EBV-specific CTL activated with peptides derived from 11 EBV antigens. They observed three complete responses in patients with early EBV-related posttransplant lymphoproliferative disease, whereas three patients with late-stage disease progressed after therapy[19]. While these results are encouraging, all responding patients in this study received additional therapies making it difficult to ascribe the anti-EBV-related posttransplant lymphoproliferative disease effects to CTL alone. The clinical safety and efficacy both of tetramers and of selection based on IFN-γ secretion have been evaluated for cytomegalovirus[22,23]. However, further studies are necessary to evaluate the efficacy and safety of rapidly generated T cells specific for EBV.

3.2.3 EBV-Associated Lymphoma and Nasopharyngeal Carcinoma

The success of EBV-specific CTL adoptive transfer to treat EBV-related posttransplant lymphoproliferative disease led to the extension of this therapy to other EBV-associated malignancies. Nearly 100% of undifferentiated nasopharyngeal carcinoma as well as 40% of Hodgkin's and about 20% of non-Hodgkin's lymphomas tumors express EBV antigens[24,25]. However, in contrast to EBV-related posttransplant lymphoproliferative disease, EBV-associated nasopharyngeal carcinoma and lymphomas develop in seemingly immunocompetent hosts and display a restricted expression pattern of EBV antigens. Whereas EBV-related posttransplant lymphoproliferative disease expresses all ten EBV proteins that are associated with the virus latent cycle (type III latency), the malignant cells of Hodgkin's lymphoma and nasopharyngeal carcinoma are typically characterized by a type II latency pattern, expressing only LMP1, LMP2, EBNA1, and BARF1[26,27]. Of these viral antigens, only LMP1, LMP2, and BARF1 are efficiently processed and presented by HLA class I molecules and thus targets for CTL therapy[28]. Although EBNA1 is rarely presented on HLA class I molecules[29], it is frequently presented on class II molecules and may also be of value[29,30].

Our group used EBV-LCL-activated EBV-specific CTL to treat 14 patients with relapsed Hodgkin's disease. Of 11 patients with clearly measurable disease at the time of CTL infusion, 2 experienced complete remissions, 1 had a partial response,

5 had stable disease, and 3 had no response to CTL therapy[30]. Chua et al.[31] used EBV-specific CTL to treat four patients with advanced nasopharyngeal carcinoma. These investigators found the treatment to be well tolerated, and they observed an increase in EBV-specific immunity for 2–3 weeks after CTL infusion. Unfortunately, the patients treated had very advanced stage disease, and the authors could not clearly evaluate an antitumor effect. Our group has also administered EBV-specific CTL to 23 nasopharyngeal carcinoma patients with relapsed or refractory disease. At the time of CTL infusion, 8 patients with relapsed nasopharyngeal carcinoma were in remission and 15 had active disease. Of those treated in remission, 62% (5/8) remain disease-free (17–75 months), while 48.7% (7/15) patients with active disease achieved a complete (33.3%) or partial response (15.4%) to therapy[31,32]. Collectively, these results were encouraging and showed that in some cases EBV-specific CTLs were therapeutically beneficial for patients with Hodgkin's lymphoma or nasopharyngeal carcinoma. Still, the clinical responses were relatively limited when compared with the responses observed in EBV-related posttransplant lymphoproliferative disorder patients.

3.2.4 Improving EBV-Specific CTL Therapy for EBV-Related Lymphoma and Nasopharyngeal Carcinoma

Work being conducted in our laboratory and others led to the hypothesis that at least two important differences between EBV-related posttransplant lymphoproliferative disorder and lymphoma/nasopharyngeal carcinoma patients might contribute to the lower clinical responses seen in the latter group. First, only a minor component of our EBV-specific CTL lines recognizes the EBV antigens expressed on lymphoma and nasopharyngeal carcinoma tumors (EBNA1, LMP1, LMP2 and BARF1). Second, in the stem cell transplant setting of EBV-related posttransplant lymphoproliferative disorder, EBV-specific CTLs are generated from healthy donors and infused into a lymphopenic environment created by the pre-transplant conditioning regimen. This provides the transferred cells immunologic space and access to antigen, homeostatic cytokines, and growth factors. In nasopharyngeal carcinoma and most lymphoma patients, the T cells are derived from the patient and T cells specific for the viral tumor antigens could be anergized within the tumor sites. Thus, EBV-specific CTL therapy for lymphoma and nasopharyngeal carcinoma patients might be improved by increasing the frequency of T cells specific for the EBV-specific LMP1 or LMP2 antigens, increasing the potency of antigen-presenting cells for T cell activation and infusing the cells after lymphodepletion of the host.

We evaluated a combination strategy of lymphodepletion prior to transfer of EBV-specific CTL in patients with refractory or relapsed nasopharyngeal carcinoma[33]. Administration of an anti-CD45 monoclonal antibody (mAb) resulted in a transient lymphopenia in all patients and an increase in serum concentrations of IL-15, an important T cell survival cytokine, in six out of eight patients. At the time

of lymphopenia, patients were infused with EBV-specific CTL, and all showed an increase in the frequency of these EBV-specific CTLs in their peripheral blood that was not seen in patients who received EBV-specific CTLs without lymphodepletion. Encouragingly, the three patients with greatest and longest lasting rise in their EBV-specific immunity had clinical benefit (one complete response and two stable disease), suggesting that continued investigation into the strategy of using lymphodepletion before CTL transfer is warranted.

We also tested the hypothesis that EBV-specific CTL enriched for LMP2 and/or LMP1 could mediate superior antitumor activity in lymphoma patients. Protocols were developed to generate LMP2 or LMP1 and LMP2-enriched EBV-specific CTL and used to treat patients with EBV-positive Hodgkin's or non-Hodgkin's lymphoma[34]. Sixteen patients received LMP2-specific CTLs and 33 received LMP1/2-specific CTLs without toxicity. The number of LMP-specific T cells in peripheral blood rose 2–70-fold and persisted for up to 3 months. Lymph node biopsies from three patients taken 3–6 months post CTL infusion showed selective accumulation of LMP-specific T cells in lymph nodes compared to peripheral blood. Preliminary results in patients who received LMP2 or LMP1 plus LMP2-specific T cells show tumor responses in about 70% of patients and complete responses in over 60%[35].

These studies suggest that the in vivo antitumor activity of EBV-specific CTL can improved by increasing the frequency of cells with specificity for the appropriate latency antigen(s). Other strategies to improve EBV-specific CTL for patients with EBV-associated lymphoma and nasopharyngeal carcinoma involve genetic modifications aimed at making CTL resistant to the immunosuppressive mechanisms of the tumor and grafting CTL with chimeric receptors to allow recognition of nonviral antigens expressed on tumor cells. These strategies will be discussed in more detail later in this chapter.

3.2.5 Summary of EBV-Associated Malignancies

EBV-associated malignancies provide an excellent platform for evaluating the feasibility and safety of adoptive T cell transfer. As prophylaxis and treatment of EBV-related posttransplant lymphoproliferative disorder, EBV-specific CTLs have proven safe and highly effective. The extension of EBV-specific CTL therapy to EBV-associated malignancies developing in immune competent hosts has been more challenging. EBV-specific CTL therapy has produced complete tumor regressions in some patients with EBV-associated lymphoma and in nasopharyngeal carcinoma, but in other cases certain limitations must be overcome to increase the overall effectiveness of adoptive T cell transfer in this patient population. Strategies to increase the antitumor activity of EBV-specific CTL therapy include lymphodepletion of the host, enriching for CTL with specificity toward particular EBV latency antigens, and genetic modifications of the CTL to improve their survival in the tumor microenvironment and to enhance tumor recognition.

3.3 Tumor-Infiltrating Lymphocytes

While EBV-specific CTLs have shown promise for the treatment of several EBV-associated malignancies, most tumors are not associated with known viruses and thus not targets for antiviral CTL therapy. Therefore, alternative strategies must be employed to generate tumor-specific T cells. In patients with melanoma, colorectal, and ovarian cancer, the presence of TIL is associated with better clinical outcomes[36–38]. Thus, investigators have attempted to use ex vivo expanded TIL as a source of tumor-specific T cells for adoptive T cell transfer. This strategy has been pioneered by Rosenberg et al., at the National Cancer Institute, who have been using TILs to treat patients with metastatic melanoma. Thus, we will focus much of our attention in this section on their results.

3.3.1 Generating TILs for Adoptive T Cell Transfer of Metastatic Melanoma Patients

The first major hurdle in developing TIL-based adoptive T cell transfer was cleared in 1987 when Muul et al. reported that TIL extracted from surgically resected metastases from patients with malignant melanoma could be expanded ex vivo in medium containing IL-2[39]. Responsive lymphocytes were cytotoxic to autologous melanoma cells and could be expanded >90,000-fold in culture while retaining tumor specificity. Shortly thereafter, a clinical trial was initiated using large doses of ex vivo expanded TIL ($>10^{11}$ cells) plus high-dose IL-2 to treat patients with metastatic melanoma. A cohort of patients was also given low-dose cyclophosphamide 36 hours prior to infusion for immunomodulation. Overall, an objective clinical response rate of 34% was reported with no significant difference in response between patients treated with TIL plus IL-2 (31%) and those given cyclophosphamide (35%) prior to infusion of TIL[40]. Unfortunately, most of the clinical responses were transient and few complete responses were observed; however, several critical findings were made that would improve TIL therapy in future studies. The investigators found that patients who responded to treatment were significantly more likely to have received TIL which (1) were from younger cultures, (2) had shorter doubling times, and (3) exhibited higher lysis against autologous tumor targets. Furthermore, patients receiving TIL expanded from subcutaneous tumor deposits had higher response rates (49%) compared with those receiving TIL from lymph nodes (17%).

3.3.2 Improving TIL Therapy: Modified Culture and Increased Lymphodepletion

In the previous study, TILs were isolated by digestion of melanoma tumors, to form a single-cell suspension, which was expanded in a single culture. A modified

protocol for growing TIL was adopted that involved mincing tumors into tiny fragments and establishing multiple cultures. Interestingly, this method generally succeeded in expanding several different TIL cultures from the same tumor specimen, often with qualitative and quantitative differences in antigen-specific reactivity. Those cultures with the highest reactivity against autologous tumor cells underwent rapid expansion using the T cell stimulating antibody OKT3 plus IL-2. Using this method, a total of 10^{10}–10^{11} T cells could be obtained in as little as 5 weeks[41]. Three subsequent clinical protocols were initiated utilizing this method of TIL preparation and focused on increasing amounts of lymphodepletion prior to cell infusion. In the first trial, 43 patients received a non-myeloablative chemotherapy regimen of cyclophosphamide (60 mg/kg) for two consecutive days followed by fludarabine (25 mg/m^2) for an additional 5 days. In the second trial, 25 patients were given the same chemotherapy regimen followed by 200 cGy whole-body irradiation the day before cell infusion. In a third trial of 25 patients, the total body irradiation was intensified by giving 200 cGy twice a day for 3 consecutive days for a total of 1,200 cGy. Hematopoietic stem cell rescue was performed by administration of autologous CD34$^+$ cells one day after TIL infusion in both trials where total body irradiation was used[42].

Overall, objective clinical responses were 49%, 52%, and 72% for the 3 trials, respectively. Of the responding patients, 12 experienced complete responses (3 in trial 1, 2 in trial 2, and 7 in trial 3) that are ongoing from 18 to 75 months[42]. Importantly, cancer regressions were observed at distant metastatic sites including the lung, liver, lymph nodes, subcutaneous tissues, and brain, suggesting that T cells migrate across the blood–brain barrier. Interestingly, the data also suggests that more aggressive lymphodepletion prior to TIL infusion could lead to an improvement in overall survival, though that conclusion can only be definitively drawn after a randomized trial. Still, preclinical data supports the assertion that, at least in melanoma, increasing amounts of total body irradiation are directly correlated with increased treatment efficacy. Moreover, the ratio of tumor-specific CD8$^+$ T cells to endogenous host cells with inhibitory potential was increased in animals receiving the highest doses of total body irradiation, suggesting that a severely lymphodepleted host provides the optimal environment for transferred T cells[43] as previously observed for EBV-specific T cells in the stem cell transplant setting. However, lymphodepleting regimens come with a significant risk of toxicity, and therefore, the potential benefits must be appropriately weighed against the risks.

3.3.3 TIL Therapy for Ovarian Cancer

While much of the pioneering work with TIL therapy has been performed in patients with metastatic melanoma, TILs can also be found in ovarian tumors and have been expanded ex vivo for adoptive T cell transfer of ovarian cancer patients. An early study conducted in 1994 used ex vivo expanded TILs, isolated from solid metastases or malignant effusions, to treat eight patients with advanced epithelial

ovarian carcinoma[44]. The generated lines were primarily CD4$^+$ T cells and these were infused into patients who also received recombinant IL-2. Unfortunately, no objective antitumor responses were observed in this trial, though the investigators reported some signs of clinical activity including ascites regression in two patients. In a study by Fujita et al., 13 ovarian cancer patients treated with surgical resection and cisplatin-containing chemotherapy who showed no detectable disease after treatment were given TIL to prevent relapse[45]. A similar control group was established who did not receive TIL. With an average of 3 years of follow-up (36 months in TIL group and 33 months in the control group), the estimated 3-year disease-free survival rate was significantly ($p < 0.05$) higher in the TIL group (82.1%) versus the control group (54.5%). Thus, this study concluded that in ovarian cancer patients with minimal residual disease after surgery and chemotherapy, TIL could significantly extend disease-free survival.

Current work suggests a critical factor affecting patient outcome is the ratio of CD8$^+$ effector T cells to CD4$^+$ regulatory T cells[46]. However, the factors that affect these ratios from one patient to the next are largely unknown. A better understanding of the immune response in patients with a high effector to regulatory T cell ratio might help to improve future adoptive T cell transfer strategies for ovarian cancer.

3.3.4 Summary of TIL Studies

Where available, ex vivo expanded TILs provide an excellent source of tumor-specific T cells for use in adoptive T cell transfer. TILs have proven particularly successful in the treatment of patients with metastatic melanoma, and preliminary evidence suggests that an intensive lymphodepleting regimen of chemotherapy and total body irradiation with stem cell rescue could enhance the antitumor activity of the transferred cells. Ovarian and colon carcinomas have also been treated with TILs (TILs), though the clinical experience with adoptive T cell transfer for these cancers is limited. While TILs were found to be successful in extending disease-free survival in ovarian cancer patients with minimal residual disease, they did not produce objective clinical responses in patients with advanced stage disease. Still, patients with ovarian cancer who have a high effector to regulatory T cell ratio have significantly better outcomes, suggesting further investigation into T cell-based immunotherapy is warranted.

3.4 Genetically Modified T Cells

While TILs have produced antitumor responses in melanoma patients, the broader application of this strategy is limited by the fact that TILs are not available or difficult to isolate from most tumors, and even when TILs are available, it is not always possible to expand a large number of tumor-specific CTLs[47]. However,

advances in immunology and vector biology have allowed the development of tools, including the genetic modification of T cells to redirect their specificity toward tumor antigens, or increase their resistance to inhibitory ligands produced by tumors and their stroma, which might overcome some of the limitations of TIL and EBV-specific CTL therapy. In this section, we will discuss two strategies being used to redirect T cell specificity, TCR transfer, and chimeric antigen receptors (CARs), with a particular emphasis on those studies that have entered phase I clinical trials.

3.4.1 T Cell Receptor Transfer

Over 25 years ago, it was discovered that T cells derive antigen specificity from a heterodimeric complex of two immunoglobulin-like proteins that form part of the TCR complex[48,49]. From early on, investigators recognized that cloning and transferring these TCR genes into T cells offered the potential to redirect T cell specificity toward any antigen of interest. However, it was not until several years later that advances in vector technology have made redirecting T cell specificity through TCR gene transfer possible.

Retroviruses, in particular the Moloney murine leukemia virus, have revolutionized gene therapy approaches by allowing for high transduction efficiency of primary cells and a relatively high and stable expression of the transgene[50]. However, even with this technology, the transfer of TCR genes has proved difficult. Since a functional TCR requires both the α and β TCR chains, these genes must be transferred into T cells either by two different retroviral vectors, requiring two separate transductions, or on a single vector containing an internal ribosomal entry site or a viral 2A sequence capable of producing high-level expression of both chains. Further, mispairing between transgenic and endogenous TCRs can create unwanted specificities and reduce expression of the transgenic pair resulting in T cells with low avidity for tumors.

3.4.2 Adoptive T Cell Transfer for Metastatic Melanoma Using TCR Transfer

The first clinical trial to use TCR transfer was conducted in patients with metastatic melanoma[51]. The genes for a MART-1-specific TCR were cloned from TIL with proven antitumor activity and transferred into peripheral blood T cells of the study patients. While this study was the first to demonstrate the feasibility of this strategy in man, only 4 of 31 patients (13%) experienced any clinical response and none achieved a complete response, despite the fact that these MART-1-specific T cells engrafted and persisted for several months after infusion[51,52]. The investigators

noted that none of their patients receiving genetically modified T cells experienced side effects such as skin rash or melanocyte toxicity in the eye or ear, which had previously been associated with robust antitumor responses in TIL studies. After extensive in vitro study, they concluded that a TCR with a higher avidity for the target antigen might be necessary to achieve clinical response rates similar to those achieved with naturally occurring TIL. In a follow-up study, Johnson et al. cloned a high-affinity TCR from a human T cell that recognized an HLA-A2-restricted MART-1 epitope[52]. Using HLA-A2 transgenic mice, they also cloned a high-affinity murine TCR recognizing the gp100 154–162 epitope, which is the most highly presented peptide from the gp100 protein in the context of HLA-A2. Six of 20 (30%) patients receiving the high-affinity MART-1 TCR achieved clinical regression of melanoma. While the numbers were low, it appeared that the high-affinity TCR was associated with better clinical response rates than the low-affinity TCR. When the high-affinity murine TCR to gp100 was transferred into patient T cells, they observed clinical responses in 3 of 16 patients (19%). While encouraging, these response rates are still well below the >50% response rate observed when using naturally occurring TIL to treat metastatic melanoma; further, high-affinity TCRs destroyed normal melanocytes in the skin, eye, and ear requiring local steroid treatment to treat uveitis and hearing loss[52]. More recently, severe off-target effects associated with the use of enhanced, high affinity TCRs specific for MAGE-A3 have been reported to the Recombinant DNA Advisory committee (RAC).

Another problem brought to light in the wake of these clinical studies is the frequent mispairing of introduced α and β TCR chains with the endogenous α and β chains[53,54]. This mispairing can result in two major problems: the cloned TCR failing to achieve wild-type expression levels, thus lowering the overall avidity of transgenic T cells and inhibiting their effector functions, and the generation of a novel TCR with autoreactive potential. Strategies currently being investigated to decrease mispairing of introduced α and β chains include modifying the constant regions with disulfide bonds, using hybrid TCRs that consist of murine constant regions fused to human variable regions, and silencing of the endogenous TCR[55–57].

While the problem of TCR mispairing may be solvable, the use of cloned TCR genes means that tumor killing is HLA restricted, thus limiting this strategy to patients with common HLA types for which a high-affinity TCR has been cloned. Additionally, tumors have been found to downregulate class I MHC molecules as means of escaping TCR recognition[58]. Many of these limitations may be overcome with another technology that utilizes a hybrid TCR known as the CAR (chimeric antigen receptor).

3.4.3 Chimeric Antigen Receptors

First-generation CARs consist of an extracellular antigen recognition domain, a short hinge, a transmembrane domain, and an intracellular signaling domain derived from the TCR CD3-ζ chain[59] (Fig. 3.2). The extracellular antigen recognition domain is

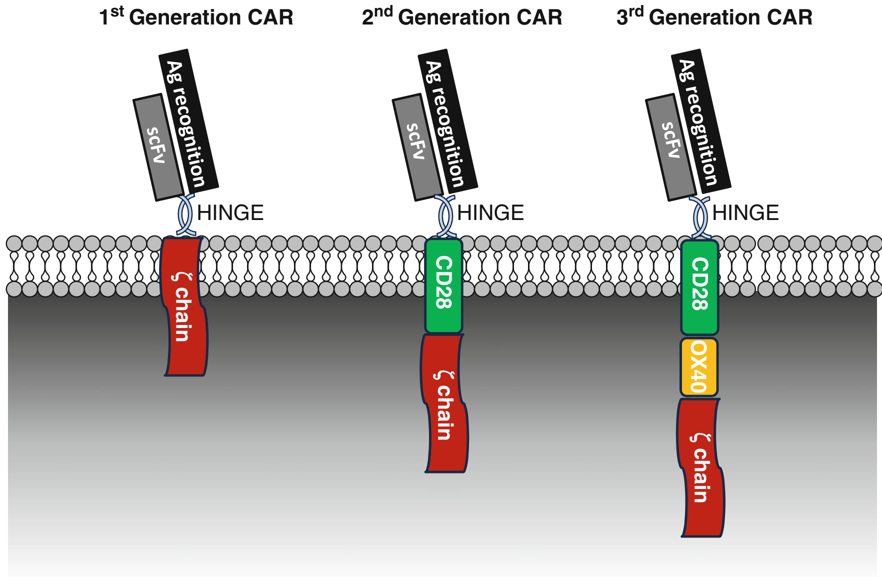

Fig. 3.2 Enhancing the activity of chimeric antigen receptor (CARs). First generation CARs linked the single chain variable fragment of an antibody to the intracellular signaling domain of the TCR CD3-ζ chain, via a hinge region and a transmembrane region of various origins. When expressed on T cells, this molecule mediates killing of tumor cells recognized by the antibody domain, but unless the tumor cell expresses costimulatory molecules, it does not mediate proliferation. Since most tumors do not express costimulatory domains, second generation CARs introduce the intracellular signaling domain of CD28 to provide second tier costimulation and added to that in third generation CARs are costimulatory domains from third tier costimulatory molecules such as OX40 or 4-1BB.

typically composed of a single-chain variable fragment from a mAb. When the single-chain variable fragment binds its cognate ligand, a signal is transmitted through the CD3-ζ chain resulting in T cell activation[60]. Since antigen recognition is through an antibody–ligand interaction, CARs can be used for any patient regardless of HLA type. Additionally, CARs can recognize tumors which have downregulated class I MHC, and they can be generated against virtually any tumor-associated antigen that is expressed on the cell surface, including carbohydrates and glycolipids[57], and that is minimally expressed on essential normal tissues.

Owing to the many advantages that CARs offer over traditional TCRs, substantial interest has surrounded their use in adoptive immunotherapy with several phase I trials currently underway to assess the safety and efficacy of this approach. To date, five phase I clinical trials have been completed using CARs to treat cancer, and the results have been published (Table 3.1). Unfortunately, in most of the initial studies, clinical responses have been modest, and limited persistence of the CAR-expressing T cells is hypothesized to be one of the major obstacles to antitumor efficacy.

Table 3.1 Completed phase I clinical trials using first generations CARs for cancer

Tumor-associated antigen	Targeted malignancy	Clinical response (# of pts.)	Ref.
α-Folate receptor	Ovarian cancer	NR (14)	Kershaw et al. [101]
Carbonic anhydrase (CAIX)	Renal cell carcinoma	NR (3)	Lamers et al. [102]
CD20	Lymphoma	PR (1); SD (4); NED (2)	Till et al. [103]
CD171	Neuroblastoma	PR (1); NR (5)	Park et al. [104]
G_{D2}	Neuroblastoma	CR (3); PR (1); SD (1); PD (4); TN (2)	Pule et al. [61]

CR, complete response; PR, partial response; NR, no response; SD, stable disease; PD, progressive disease; TN, tumor necrosis by biopsy

3.4.4 Costimulatory Domains to Improve CAR Function

When T cells are activated by professional antigen-presenting cells, they not only receive stimulation through the TCR but also receive essential costimulatory signals through CD28 and tumor necrosis factor receptors like CD27, 4-1BB, and OX40[62–64]. See chapter 8 for details of T cell co-signaling. Since T cells expressing a first-generation CAR receive only CD3-ζ stimulation in the absence of a costimulatory signal, their activation is incomplete, and this is thought to be one of the major reasons for their limited persistence in vivo since most tumors do not express costimulatory molecules and inhibit the activation of local professional antigen-presenting cells that do. Therefore, several investigators have made improvements to the original CAR by including a CD28 signaling domain in addition to the CD3-ζ chain, now known as the second-generation CAR[65–68]. T cells expressing second-generation CARs show increased proliferation and important cytokine secretion (e.g., IFN-γ, IL-2, and TNF-α), after stimulation with target cells expressing the cognate antigen, compared to T cells expressing a first-generation CAR. Furthermore, T cells with second-generation CARs persist longer in vivo when used in immunodeficient (SCID) mouse xenograft tumor experiments and displayed superior in vivo antitumor activity when compared with first-generation CAR-expressing T cells[67,68]. A clinical study describing an intrapatient comparison of activated T cells expressing first- and second-generation CD19-specific CARs also demonstrated increased persistence of the CD28-containing CAR[69], but clinical responses were not produced.

Researchers have therefore added even more costimulatory domains into CARs. Thus, the third-generation CAR was developed, which contained an additional signaling domain (OX40 or 4-1BB) sandwiched between the CD28 and CD3-ζ domains. Several preclinical studies have now shown that third-generation CARs mediate superior in vivo tumor regression because of their enhanced cytokine secretion, expansion, and persistence[70–72]. Recently three complete tumor remissions were obtained in response to T cells expressing a CD19-specific CAR expressing the 4-1BB endodomain when infused after lymphodepletion into

patients with chronic lymphocytic leukemia. However, severe toxicity associated with cytokine storm was also reported[73,74]. Thus, increasing the number of costimulatory domains is not without risk, and two deaths have recently been reported in patients receiving T cells genetically modified with second- or third-generation CAR[61,75]. While it is unclear exactly what caused the deaths of these two patients, there is concern that second- and third-generation CARs could be easily triggered, such that even low avidity off-target binding could cause potent activation, again leading to cytokine storm[76]. Thus, while adoptive T cell transfer has an excellent safety record overall, these two cases highlight the need to tread cautiously when testing new-generation CARs in humans.

3.4.5 Expressing CARs on Virus-Specific CTL

Another strategy to increase the persistence of CAR-expressing T cells is to modify virus-specific CTL with CARs, rather than using T cells nonspecifically activated with anti-CD28 and/or anti-CD3 antibodies (that activate the TCR)[75,77,78]. As discussed earlier, EBV-specific T cells persist for up to 9 years in some patients, and we hypothesized that EBV-specific CTL expressing CARs could be stimulated through their native EBV-specific TCRs in vivo after transfer into EBV-seropositive hosts, thus allowing CAR-expressing T cell to persist longer and expand to greater numbers thereby increasing their potential for antitumor activity.

We recently infused neuroblastoma patients with both EBV-specific CTL and anti-CD3-stimulated T cells, each expressing a G_{D2}-specific CAR (distinguished only by a unique DNA barcode in each vector that allowed for PCR detection of transgenic EBV-specific CTL or transgenic $CD3^{+}$ T cells). Indeed, tenfold more G_{D2}-CAR EBV-specific CTLs than $CD3^{+}$ T cells were detected in the peripheral blood of infused patients. Furthermore, G_{D2}-CAR EBV-specific CTL could be detected for more than 6 weeks after infusion, whereas G_{D2}-CAR $CD3^{+}$ T cells could only be detected for 2–3 weeks post infusion[61]. The latest clinical data from this study show that of 11 patients with detectable disease at the time of CTL infusion, 3 achieved complete responses, 1 had a partial response, 1 had stable disease, 4 had progressive disease, and 2 had detectable tumor necrosis. This was the first study to report complete clinical regression of solid tumors after treatment with CAR-expressing T cells and suggests that the increased expansion and persistence of G_{D2}-CAR EBV-specific CTL could contribute to more robust antitumor activity in patients.

3.4.6 Overcoming Tumor Immune Evasion Strategies

T cells expressing CARs are an effective way to redirect T cell specificity to tumors as well as overcome the downregulation of class I MHC molecules. Still,

tumors have a plethora of other immune evasion strategies to avoid recognition and destruction by T cells, including secretion of inhibitory cytokines (e.g., TGF-β and IL-10), upregulation of the inhibitory ligand PD-L1, and the recruitment of regulatory T cells[79]. Additional genetic modifications of T cells to overcome immune evasion are in preclinical and clinical development. These strategies include modifying T cells with chemokine receptors for increased tumor homing[80,81], transgenic expression of cytokines/receptors that support T cell proliferation and effector function[82,83], constitutive activation of Akt in T cells to resist regulatory T cells[84], as well as the expression of mutant proteins allowing T cell function in the presence of TGF-β and the therapeutic drug rapamycin[85]. Finally, combining T cell transfer with DNA demethylating agents or histone deacetylase inhibitors to modify the tumor microenvironment and increase antigen presentation on tumor cells might improve clinical outcomes in patients[86].

3.4.7 Summary of TCR Transfer and CARs

The ability to redirect T cell specificity using TCR transfer or CARs is a powerful tool that makes it possible to target virtually any tumor with T cell immunotherapy. Thus far, the only clinical trial to use TCR transfer has been in patients with metastatic melanoma. Clinical tumor regression was observed, though the response rate was lower than in patients receiving unmodified, expanded TILs. While a potentially useful strategy, the use of TCRs means that tumor killing is HLA restricted and thus only available to patients with common HLA types. This limitation can be overcome by redirecting T cell specificity with a CAR. CARs use monoclonal antibodies to recognize surface molecules in an HLA-unrestricted fashion and can therefore be used to treat any patient whose tumor expresses the appropriate antigen. Although first-generation CARs were largely unsuccessful in phase I studies, strategies to improve the persistence of CAR-expressing T cells, including the addition of costimulatory domains and expressing CARs on virus-specific T cells, should improve the clinical efficacy of CAR-expressing T cells. Further genetic modifications to overcome elements of the immunosuppressive tumor microenvironment should also lead to more effective T cell therapies.

3.5 Adoptive Therapy Using Natural Killer Cells

Our discussion throughout this chapter has focused on T cells because they are widely used in clinical adoptive transfer studies. However, NK cells represent another important population of cells with potent antitumor potential. Indeed, interest in NK cells to treat cancer grew substantially after the discovery that IL-2 activation of NK cells could result in cytotoxic activity against previously NK-resistant tumors[87]. Various protocols have been developed for isolating NK

cells from peripheral blood, and more recently new methods have been described for expanding NK cells *ex vivo*[88]. While a detailed review of clinical adoptive NK cell transfers is beyond the scope of this chapter, we will conclude with a brief discussion of the current progress and challenges in the field of NK cell transfer[89].

3.5.1 Adoptive Transfer of Allogeneic NK Cells

In retrospect, the use of NK cells in adoptive immunotherapy should have been evident from the outset. After all, the existence of NK cells in immunodeficient SCID mice all but resurrected the theory of tumor immune surveillance, after apparently receiving a significant blow following the findings that immunocompromised mice are no more at risk of getting spontaneous tumors[90] (although that view has been updated now to demonstrate the importance of T cells and IFN-γ in cancer immune surveillance; please see chapter 1 for details). As their name implies, NK cells have an innate ability to mediate target cell destruction without any previous priming event (as required by T cells)[91]. While most of the conceivable applications of tumor immunotherapy with these cells center on this particular effector function, their ability to mediate immunoregulatory effects between the adaptive and innate immune systems also lends them increased applicability in the clinical setting[92].

NK cells recognize self from nonself through a system of activating and inhibitory ligands, as well as receptors that mediate antibody-dependent cytotoxicity. The balance of the corresponding ligands on the target cells determine whether the NK cells choose to exert their cytolytic function or to retreat into immune tolerance[93].

Results from different groups all seem to suggest that NK cell transfer is more effectively accomplished in the allogeneic setting, where the presence of KIR mismatches (among other things) allows for unimpeded NK cell activity against the tumor. A retrospective study by Ruggeri et al.[94] has shown the positive effects of allogeneic NK cell transfer in a haplotype-mismatched hematopoietic stem cell transplant setting. Miller et al. reported complete remissions in cancer patients given haploidentical NK cells[95], an observation they did not find when they previously infused autologous NK cells[96].

To date, several studies demonstrate the feasibility of using allogeneic NK cells in such a therapeutic approach. Shi et al.[97] infused KIR–ligand mismatched NK cells depleted of T cells in patients with advanced multiple myeloma. Besides demonstrating that the therapy is safe and well tolerated, the authors reported an encouraging (near) complete remission rate of 50%.

On the other hand, Dillman et al.[98] utilized autologous NK cells as part of a population of cells termed lymphokine-activated killer cells that are comprised of approximately 77% T cells and 23% NK cells. These lymphokine-activated killer cells were a leukapheresis product and injected intralesionally during surgery. A median survival of 20.5 months from diagnosis was observed following the use of this therapy as an adjunct in glioblastoma multiforme, higher than the 15-month

median survival seen in standard temozolomide therapy[98] and the 12-month survival associated with the disease.

3.5.2 Hurdles in the Use of NK Cells for Immunotherapy

Despite these and many other promising results, several significant hurdles are actively being addressed to improve the chances further of using NK cells as cancer immune therapy.

Their poor number has been a constant concern, since NK cells represent only 3–20% of circulating lymphocytes. To expand NK cells, several new approaches have been proposed, among them the use of feeder K562 cells genetically modified to express 41BBL and IL-15[99].

Related to their limited numbers is the need for IL-2 coadministration in vivo. Because IL-2 administration is associated with a host of adverse events, several investigators are looking at the possibility of delivering IL-2 locally—along with the NK cells—using genetic modification[93]. NK cells were engineered to express IL-2 via retroviral transduction. In vitro studies using the NK cell line NK-92 showed that expression of IL-2 increased cytotoxicity against tumor lines and IL-2 independence[100].

Not all the functions of NK cells are currently understood, most of their activating ligands are unknown, and it is not clear how they might be induced to persist in vivo. A more comprehensive review of their functions is required to harness better the potential of this therapy in combating malignancy.

3.5.3 Summary on NK Cell Transfers

Adoptive transfer of NK cells is an emerging immunotherapy for patients with malignant disease. While it has long been known that NK cells possess an innate ability to recognize and kill tumor cells, their use has been limited by difficulties in expanding large number of cells needed for adoptive transfer and the ability of these cells to persist in vivo. Recently discovered methods for expanding NK cells in culture and genetic modifications that improve NK cell persistence and function in vivo will undoubtedly increase the use of these cells in future clinical adoptive transfer studies.

3.6 Conclusions

Adoptive transfer of antigen-specific T cells presents a highly specific means to eliminate tumors with minimal toxicity. The efficacy of adoptively transferred T cells has been linked to their ability to proliferate massively after infusion to

numbers able to eliminate large tumors. Unfortunately, most tumors have low immunogenicity, poorly present weak tumor antigens, and have multiple ways to inhibit every stage of T cell activation, proliferation, and effector functions. They also inhibit local antigen-presenting cells that might otherwise cross present tumor antigens, so that infused T cells rarely proliferate sufficiently, except under conditions of extreme lymphopenia. These characteristics of tumors likely explain the disappointing clinical effects of tumor vaccines. Fortunately, T cells are readily modified with immunomodulatory transgenes that can redirect their specificity and alter their migration and response to tumor-derived inhibitory ligands. These modifications will likely be required to ensure the optimal antitumor activity of T cells that can be further improved by combination with small molecules that change the tumor microenvironment in favor of T cells and increase tumor antigen presentation. The problems of NK cells may also be solved by genetic modulation and combination with small molecules.

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Chapter 4
Dendritic Cell-Based Cancer Immunotherapy: Achievements and Novel Concepts

K.F. Bol, G. Schreibelt, E.H.J.G. Aarntzen, I.J.M. de Vries, and C.G. Figdor

Abstract Dendritic cells (DCs) are the most potent professional antigen-presenting cells of the immune system. They acquire and process antigen and migrate to the lymphoid organs where they present the antigen and control the activation of B and T cells, the mediators of specific immunity. DC-based immunotherapy is explored worldwide in clinical vaccination trials with cancer patients aiming to induce or augment an antitumor immune response. The majority of clinical trials up to the present have vaccinated patients with ex vivo-generated monocyte-derived DCs, matured using cytokines and loaded with tumor antigen via peptides, protein, or lysates.

Thus far, DC-based immunotherapy has proven to be feasible, safe, and potently able to induce immunological responses, particularly if the DCs have been appropriately matured. Nevertheless, only a limited number of clinical responses have been observed. Although the evidence on clinical responses is still scarce, expectations are high because the clinical responses that are induced are often long lasting. To improve clinical responses of DC vaccination further, a number of variables are already being tested in clinical trials, including DC maturation via toll-like receptors, mRNA transfection to load antigen, and the use of naturally occurring DC subsets instead of monocyte-derived DCs. Future aspects of DC vaccination that are being explored include combination treatment to counteract tumor escape mechanisms and in vivo targeting of DCs. The full potential of DC-based immunotherapy has not yet been fully exploited, which in combination with data to date supports a promising role for DC-based immunotherapy.

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4.1 Introduction

As professional antigen-presenting cells, dendritic cells (DCs) are the central players of the adaptive immune response. They acquire and process antigen and migrate to the lymphoid organs where they present the antigen to the specific arm of the immune system, resulting in the induction of primary T and B cell responses. Because of these unique qualities, they represent an interesting tool in cancer immunotherapy.

It has been proposed that when a tumor reaches a certain size and causes damage to the surrounding tissues with release of products into the microenvironment, local DCs become activated and subsequently the immune system is alerted[1]. Depending on the size of the tumor and its immunomodulatory characteristics, the immune system might be able to eradicate the cancer. Often, however, malignant growth is a slow and silent process that fails to elicit a “danger signal” necessary for the activation of the immune system. The goal of DC vaccination is to mend this inattention of the immune system by providing it with ex vivo “educated” DCs, that is, DCs appropriately activated and loaded with tumor antigen.

The first clinical study of a dendritic cell vaccine was reported in *Nature Medicine* in 1996[2]. At present, DC-based immunotherapy is explored worldwide in clinical vaccination trials with cancer patients aiming to induce or augment an anticancer immune response.

4.1.1 Dendritic Cell Immunobiology

Dendritic cells are the most potent professional antigen-presenting cells of the immune system. They instruct and control the activation of B and T cells, the mediators of specific immunity. DCs are derived from hematopoietic bone marrow progenitor cells. From bone marrow, they migrate into the peripheral tissues where they reside as resting immature DCs, mainly in parts of the body that are in close contact with the outside world, such as skin and mucosal tissue. They act as the sentinels of the immune system, continuously patrolling the environment in search of antigen. At this stage, they possess an immature phenotype that is mainly characterized by a low surface expression of major histocompatibility complex (MHC) class I and II molecules and co-stimulatory molecules[3]. These immature DCs are very efficient at antigen uptake and processing, mediated by high endocytotic activity and expression of an array of cell surface receptors capable of capturing antigens that could harm the host[4, 5].

Exogenous antigens, derived from extracellular pathogens such as bacteria and yeasts, are internalized and processed by DCs, and the antigenic peptides are presented in the MHC class II complexes on the cell surface. Endogenous antigens, either self proteins or viral proteins, are cleaved into peptides by proteasomes

and assembled into stable MHC class I–peptide complexes in the endoplasmic reticulum, which are subsequently transported to the cell surface. Of importance for DC-based vaccines in cancer immunotherapy is the finding that internalized antigens from exogenous sources, such as apoptotic or necrotic tumor cells, may also be present in MHC class I molecules. This process, called cross presentation, by which exogenous tumor antigens can be presented to $CD8^+$ T cells, is a unique feature of DCs[6]. The immunological outcome of this entire process strongly depends on the activation state of DCs. Resting DCs or immature DCs receiving inhibitory signals, such as interleukin-10 (IL-10) or corticosteroids, induce immune tolerance via T cell deletion or induction of regulatory T cells (Tregs), whereas mature DCs induce active immunity.

To convert the DC into a cell that is exceptionally well equipped for antigen presentation and T cell activation, a maturation step, a tightly controlled series of events, is necessary. For maturation to begin, a "danger signal", derived from tissue damage or microbial products, is required[7]. The maturation process includes down-modulation of endocytic and phagocytic receptors and upregulation of chemokine receptors CCR7 and CD62L, which leads to DC migration to secondary lymphoid organs. Furthermore, surface expression of MHC class I and II and co-stimulatory molecules such as CD40, CD58, CD80, CD83, and CD86 are upregulated, and changes in morphology of the DC lysosomal compartment occur[3].

In lymphoid tissues, DCs present pathogen-derived peptides to resting T cells via MHC molecules. This interaction between the MHC–peptide complex and the T cell receptor (signal 1), stimulation via co-stimulatory molecules from the DC to the T cell (signal 2) and cytokines in the microenvironment (signal 3) together lead to the activation of T cells. The activated T cells subsequently proliferate, leave the lymph nodes, and circulate through the body in search of cells that express antigen.

In addition, DCs are also able to activate natural killer (NK) cells directly[8] and can produce large amounts of interferon (IFN) upon encounter with viral pathogens[9], thus providing a link between the adaptive and innate immune system. The unique capacity of DCs to initiate and modulate immune responses is currently exploited by many investigative groups to fight infectious diseases and cancer.

4.1.2 Dendritic Cell Subsets

DCs comprise a heterogeneous population of cells. In human peripheral blood, two main populations of DCs can be distinguished: myeloid DCs (mDCs) and plasmacytoid DCs (pDCs)[10]. These DC subtypes differ in function, localization, and phenotype. mDCs mainly migrate to or reside in the marginal zone of the lymph nodes (a primary entry point for blood-borne antigens), whereas pDCs mainly reside in the T cell areas of lymph nodes[3, 11]. Both subsets express distinct toll-like receptors (TLRs; Fig. 4.1) and therefore respond differently to pathogenic

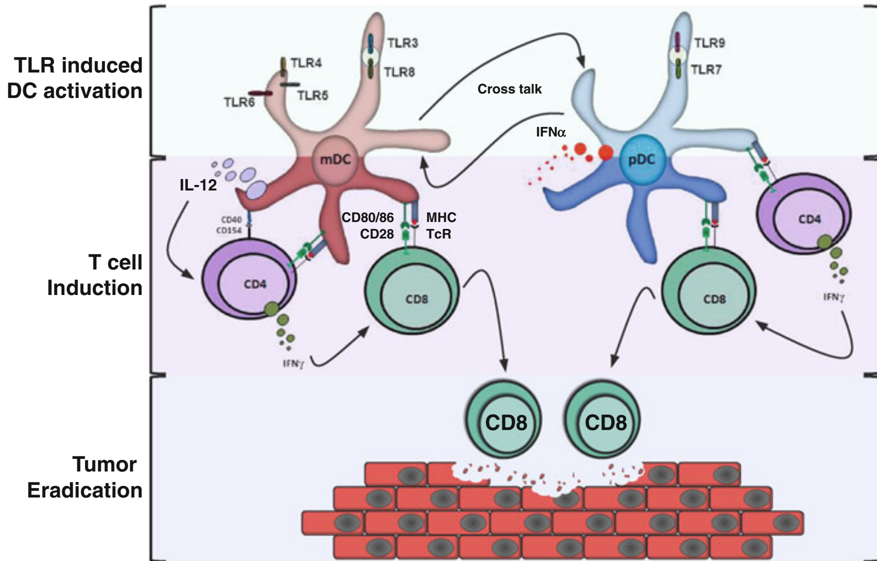

Fig. 4.1 Toll-like receptor (TLR)-activation of human DC subsets can enhance antitumor responses in vivo (Reproduced with permission from G. Schreibelt et al.[12]). DC subsets express a wide repertoire of TLRs, which upon triggering induce DC activation. Whereas pDCs predominantly express TLRs in endosomal compartments, mDCs have a broader TLR expression pattern, both at endosomal and extracellular membranes. By cross talk between mDCs and pDCs, either by cell–cell contact or soluble factors such as type I interferons, TLR-induced activation of one subset can lead to the activation of the other subset. Type I interferons appear to yield more potent mDCs in terms of IL-12 secretion, induction of tumor-specific cytotoxic T cells, and T helper 1 responses in vitro. Upon direct TLR activation, mDCs gain the ability to secrete large quantities of IL-12, which is beneficial for the polarization of a T helper 1 response. Both mDCs and pDCs have the capacity to evoke T helper cell responses. Moreover, pDCs can promote the ability of mDCs to cross prime $CD8^+$ T cells. Consequently, TLR activation of mDCs and pDCs and the cross talk between those two subsets can strongly enhance antitumor responses in vivo.

stimuli, suggesting that each subset has a specialized function in directing immune responses[12]. A large body of data suggest that mDCs mainly recognize and respond to bacterial and fungal antigens, whereas pDCs seem specialized for viral antigen recognition. More recent observations suggest that both pDCs and mDCs might be of importance for the induction of antitumor responses with and without DC-based immunotherapy.

Since natural DCs constitute only about 0.2 % of peripheral blood leukocytes, several ways to generate DCs from precursors have been investigated for DC vaccination purposes. In 1994 this resulted in the discovery that DCs can be generated from monocytes or $CD34^+$ progenitors by culture in the presence of IL-4 and granulocyte–macrophage colony-stimulating factor (GM-CSF), allowing the procurement of these otherwise scarce cells in the considerable numbers essential to conduct clinical trials[13].

4.1.2.1 Plasmacytoid Dendritic Cells

Human pDCs are a rare subpopulation of cells. pDCs are devoid of lineage markers and myeloid antigens and do not express CD11c. They express BDCA2 and BDCA4[10]. In the steady state, they are round, nondendritic, and relatively long-lived cells. After receiving inflammatory stimuli, pDCs develop a dendritic cell morphology and function. They express TLR7, which recognizes single stranded RNA, and TLR9, which recognizes unmethylated CpG DNA. Both are intracellular TLRs that are located in the endosomal compartments (Fig. 4.1). Most notably, pDCs produce large amounts of type I IFNs in response to viruses and are therefore thought to be crucial to antiviral immunity[14].

Initially, pDCs were thought to be of lymphoid origin[15]. However, several human and mouse studies pointed out that the cytokine FMS-like tyrosine kinase 3 ligand (Flt3L) is of importance for pDC development and that pDCs can develop from myeloid precursors under the influence of Flt3L[14, 16, 17]. pDCs reside in blood as well as in several lymphoid organs and some recent studies suggest functional differentiation between different tissue-residing pDCs[18].

4.1.2.2 Myeloid Dendritic Cells

mDCs found in peripheral blood are defined by the expression of myeloid markers, such as CD13 and CD33. They lack lineage-specific markers (CD3, CD14, CD19, and CD56), but express MHC class II and CD11c. The mDC population can be further subdivided into three classes based on differential surface expression of BDCA1/CD1c, BDCA3/CD141, and CD16[12]. The mDC subsets differ in their expression of cell surface markers and potency to stimulate T cells[19–21]. For instance, the recently identified C-type lectin receptor (CLR) CLEC9a is expressed only by BDCA3-expressing mDCs[22]. mDCs express two extracellular TLRs on the cell surface that recognize exterior components of bacteria and fungi, for example, cell wall components such as lipopolysaccharide (TLR4) and peptidoglycan (TLR2; Fig. 4.1). TLR 3 and 8 are expressed intracellularly to respond to viral RNA. Upon activation, mDCs mainly produce IL-12 to regulate the differentiation of naive T cells into T helper 1 cells to augment a cellular immune response. One interesting possibility is to combine mDC together with pDC as it has been suggested that pDCs and mDCs cooperate and act synergistically[23, 24]. Future studies will address whether mDC–pDC cross talk can indeed improve antitumor responses in cancer patients.

4.1.2.3 Ex Vivo-Generated Dendritic Cells

As discussed above, most clinical studies carried out to date have been used ex vivo-generated monocyte-derived dendritic cells[5]. Monocytes are pre-DCs that

originate from myeloid progenitor cells and are easily obtained by leukapheresis. In vivo, monocytes are capable of transforming into DCs after sensing inflammatory signals and are important for the replenishment of DCs in the host. Ex vivo, a cocktail of GM-CSF and IL-4 differentiates monocytes into immature moDCs over a period of 3–5 days[5, 13]. Subsequent maturation can be achieved by addition of cytokines such as tumor necrosis factor-α (TNF-α), prostaglandin E2, IL-1β, and IL-6 or monocyte-conditioned medium, the supernatant of activated autologous monocytes[25, 26]. This allows the generation of large quantities ($>500 \times 10^6$) of clinical grade DCs from a single leukapheresis[5, 13]. Although ex vivo-generated monocyte-derived DCs share many phenotypic and functional characteristics with circulating mDCs, it remains unclear to what extent they resemble natural blood DCs.

In addition to monocytes, $CD34^+$ progenitors in blood are also used to generate DCs for vaccination of cancer patients. $CD34^+$ progenitors are cultured in the presence of GM-CSF, Flt3L, and TNF-α for about a week[27]. They consist of two populations: one with Langerhans cell-like properties and another called interstitial/dermal DCs with properties resembling monocyte-derived DCs. Yields from leukapheresis after in vivo Flt3L expansion and negative selection ex vivo are much lower than from monocytes[28].

To date most clinical DC vaccination studies use monocytes to generate DCs ex vivo. However, immunological and clinical responses have been observed in cancer patients vaccinated with monocyte-derived DCs as well as with $CD34^+$ progenitor-derived DCs[27, 29, 30].

4.2 Dendritic Cell Maturation

The term “mature” DC has generally been used to describe T cell-stimulatory DCs. Immature DCs are considered to be primarily involved in the recognition and uptake of antigen. Upon receiving maturation signals, these immature DCs then change their chemokine receptor repertoire, down-modulate endocytic and phagocytic receptors, and upregulate their co-stimulatory molecules, thus acquiring the phenotype and functionality of mature DCs that are capable of migration to the lymph nodes and activation of T cells. In the absence of maturation signals, DCs will not upregulate their co-stimulatory molecules and thus remain anergy- or tolerance-inducing antigen-presenting cells.

Besides taking up, processing, and presenting antigens, DCs need proper activation by adjuvants to elicit a productive immune response. DC maturation is highly complex and should be regarded as a flexible process of which the outcome depends on the type of signals the DC receives in the periphery. While these maturation signals primarily come from contact with pathogens or tissue injury in vivo[31, 32],

ex vivo maturation can be achieved by coculturing DCs with several stimuli such as cytokines[33], pathogen-associated triggers[7], or endogenous danger signals such as heat shock proteins[34].

In the majority of clinical studies, immature or semimature monocyte-derived DCs have been used[30]. Studies that have compared the immunogenicity of immature versus mature DCs show that maturation is essential for the induction of immunological responses in cancer patients[35, 36]. Moreover, the use of mature DCs appears to be associated with a better clinical outcome compared to immature DCs[36, 37]. This difference may partly be explained by the better migratory capacity to the draining lymph nodes of mature DCs compared to immature DCs after intradermal or subcutaneous injection. Also within the lymph node, mature DCs show a pronounced migration into the T cell areas where antigen presentation takes place, whereas immature DCs remain at the periphery[38]. Besides their enhanced migratory capacity, mature DCs also have a higher expression of MHC and co-stimulatory molecules. Together this leads to superiority of mature DCs in antigen presentation and therefore in inducing T cell responses.

4.2.1 Tolerogenic Dendritic Cells

While clinical DC vaccination studies in the field of cancer immunotherapy are aimed at stimulating immune responses, the finding that immature DCs play a critical role in the continuous induction of peripheral tolerance and thereby preventing both autoimmunity and hyperreactivity[39] suggests a potential role for clinical DC applications in management of transplantation, allergy, autoimmunity, and chronic inflammatory diseases. Several factors such as IL-10, vitamin D3, and corticosteroids can skew the DC into a more suppressive T cell type of inducer[40]. Indeed, a number of trials have been initiated aimed at silencing the immune system in diseases like rheumatoid arthritis and Crohn disease.

4.2.2 Cytokine Maturation Cocktails

Maturation of DCs can be induced by proinflammatory cytokines such as IL-1β or IL-6. Several maturation methods have been applied with maturation being defined by a high expression of mature DC-specific surface markers such as CD80, CD83, CD86, and MHC molecules. Since the maturation stage of DCs cannot be fully characterized by the expression of co-stimulatory molecules and surface MHC, it is therefore critical that the phenotypic and functional characteristics of the used

DCs be carefully described when reporting clinical DC vaccination trials[30]. For example, functionality of DCs can be measured by the production of IL-12, a proinflammatory cytokine that plays an essential role in the differentiation of T cells in T helper 1 cells. The most widely used method to mature immature DCs is a cytokine cocktail that includes TNF-α, with any of the following cytokines in any combination: IL-1β, IL-6, prostaglandin E2, or monocyte-conditioned medium that was used in early clinical studies[25, 26, 33, 41, 42]. There is some evidence that culturing DC with IL-15 may lead to a type of mature DC that induces stronger T helper 1 type of immune responses[43]. However, no comparative studies have yet been reported. Lastly, CD40 ligation has also been used as a method of activation of DCs in a clinical setting[44, 45].

In addition, another level of complexity is added by the timing and duration of the maturation signal. Different cytokine cocktails require different lengths of maturation periods and can induce some differences in expression of co-stimulatory molecules and cytokine production of the DCs[46, 47]. None of these different maturation methods has shown to be clearly superior, which is mainly due to the fact that there are no direct comparative studies, although the use of prostaglandin E2 for maturation may negatively affect DC function because of reduced IL-12 production[48, 49].

4.2.3 Maturation via Toll-Like Receptors

More recently, TLR ligands that trigger TLRs on DCs are being explored to mature DCs. TLRs are part of the pattern recognition receptors by which DCs can detect pathogens[50]. Triggering of these TLRs might be a more natural route to induce DC maturation.

During evolution, the immune system has acquired various receptor families that recognize several crucial molecular components of pathogens. This set of pathogen-associated molecular patterns (PAMPs) recognized by the immune system is limited and constituted mostly of general molecular patterns that are absent on host cells and are essential for survival of the microbe. On DC membranes, two main pattern recognition receptor families are present, C-type lectins and TLRs, of which the TLR family is best characterized and recognizes the most diverse group of PAMPs. Fifteen mammalian TLRs are now known (TLR1-15), of which ten are found in humans[51, 52].

The better-described TLR1-9 can be divided in two main groups: extracellular TLRs that are found on the cell surface (TLR1, TLR2, TLR4, TLR5, and TLR6) and the intracellular TLRs that are located in endosomal compartments (TLR3, TLR7, TLR8, and TLR9). In general, intracellular TLRs recognize nucleotide-containing structures. For example, RNA molecules are recognized by TLR3, TLR7, and TLR8, and unmethylated CpG DNA originating from viruses and bacteria is recognized

by TLR9. Extracellular TLRs recognize exterior components of bacteria and fungi, for example, cell wall components. Besides PAMPs derived from pathogens, TLRs have been proposed to recognize endogenous ligands such as heat shock proteins or necrotic cells[53, 54]. The signaling pathways associated with ligation of the different TLRs are not identical, and therefore, distinct biological responses are initiated. Ligand binding of TLRs recruits one or more adaptor molecules. The difference in signaling outcome (e.g., variation in cytokine production) can be explained in part by the use of different adaptor molecules by the TLRs. The binding partners of the recently discovered TLR10-15 are less well known.

Recently it became apparent that subsets of DCs naturally circulating in the blood express different TLRs and respond distinctly to TLR ligands. Human monocyte-derived DCs and mDCs show very similar TLR expression profiles. These DC subsets express the extracellular TLRs TLR1, TLR2, TLR4, TLR5, and TLR6 and the endosomal TLRs TLR3 and TLR8. In addition, both monocyte-derived DCs and mDCs respond to specific ligands of these TLRs, leading to a mature phenotype and production of proinflammatory cytokines[55–63]. However, some significant differences between monocyte-derived DCs and mDCs in TLR expression and ligand reactivity were found. Most notably, monocyte-derived DCs show negligible TLR10 expression, whereas blood mDCs do express TLR10. Unfortunately, the ligands and functionality of TLR10 are still unknown.

Through the formation of heterodimers with TLR1 or TLR6, TLR2 gains the capacity to bind a wide variety of bacterial and yeast-derived ligands. Consequently, this plays a central function in pathogen recognition by DCs. TLR1/2/6 activation leads to DC maturation and secretion of several cytokines important in immune system activation, especially IL-6, IL-8, IL-10, IL-12, and TNF-α. Human monocyte-derived DCs and mDCs do not express TLR9 and do not respond to TLR9 ligands[55, 58]. TLR9 is only expressed by pDCs in the human setting and is responsible for the very high type I IFN response[64]. In summary, both monocyte-derived DCs and mDCs express TLR1-8 and not TLR9, and only mDCs express TLR10. The mRNA expression of these receptors was confirmed by DC reactivity studies using TLR ligands.

pDCs show a more distinct pattern of TLRs compared to monocyte-derived DCs and mDCs. They abundantly express TLR7 and TLR9 in their endosomal compartments. In addition, triggering of TLR7 and TLR9 on pDCs leads to high type I IFN secretion and a typical mature DC phenotype. Interestingly, it has been suggested that in human pDCs, TLR9 exhibits a unique feature not shared by the other described TLRs: depending on the stimulus, activation of TLR9 on human pDCs can have different outcomes. The dual function of TLR9 is attributed to the distinct intracellular locations where TLR9 can be triggered. They can either activate an innate immune response via IFN-α secretion after encountering nucleic acids via early endosomes or activate an adaptive immune response through IL-6 and TNF-α secretion in late endosomes[65].

Both the timing of the activation signal and the exposure to antigen are of crucial importance for optimal antigen presentation; only the simultaneous presence of apoptotic cells and TLR ligands to DCs results in efficient antigen presentation and subsequent T cell activation[66]. With respect to the type of TLR ligands, it has been

shown that combinations of different TLR ligands can have a synergistic effect on the immunogenic potential of DC ex vivo[67] and in vivo[68].

The combination of clinical grade TLR ligands and prostaglandin E2 resulted in the generation of mature DCs that secrete high levels of IL-12, IFN-γ, and TNF-α[69]. With the discovery of those promising ex vivo data, the potency of these TLR ligand-activated monocyte-derived and naturally occurring blood DCs is being explored in clinical trials. Despite the low number of naturally occurring blood DCs, preliminary data indicate that these cells are extremely potent in initiating immune responses in cancer patients[12].

4.3 Dendritic Cell Antigen Loading

To induce an immune response in cancer patients, the MHC molecules of a mature DC must be loaded with relevant tumor antigens. Preferably, tumor antigens are presented by DCs to both $CD4^+$ T helper cells (via MHC class II) and $CD8^+$ cytotoxic T cells (via MHC class I), since there is convincing preclinical evidence that targeting both cytotoxic T cells and T helper cells is of crucial importance for the induction of a strong and sustained antitumor T cell response. Several methods of loading DC with relevant tumor epitopes have been examined, of which the most widely used will be discussed in the following paragraphs.

4.3.1 Peptide- or Protein-Pulsed Dendritic Cells

Several techniques have been developed to load human DCs with tumor-associated antigens, the most widely used being incubation of DCs with human leukocyte antigen (HLA) class I-binding peptides that can bind directly to MHC molecules on the cell surface[27, 33, 36, 70–83]. In some clinical vaccination studies, HLA class I-binding peptides are combined with class II-binding peptides to also allow the activation of $CD4^+$ T helper cells[41, 84]. Please also see Chaps. 5–6 for additional details on peptides and DC targeting strategies.

Tumor antigen-derived peptides have the advantage that many peptides are commercially available, but the antigens have to be known for each specific tumor and the peptides are restricted to a given HLA type. Unfortunately, the half-life of MHC–peptide complexes is relatively short due to low affinity and MHC turnover. Further immune response, if any, is restricted to the epitope(s) used. On the other hand, a phenomenon known as antigen spreading can occur. In antigen (or epitope) spreading, killing of tumor cells after vaccination against a single epitope results in release of tumor antigens from killed tumor cells. These antigens can subsequently be taken up by DCs and presented to T cells, resulting in T cell responses against antigens that were not included in the vaccine[76].

Aside from HLA-binding peptides, peptides can be endogenously loaded onto MHC molecules after proteolytic processing of recombinant protein or endocytosed tumor lysates. The DC processes the protein into peptides, which has the advantage that multiple epitopes are presented in both MHC class I and II and that it is not limited to the HLA restriction of smaller peptides. Unfortunately, only a few clinical grade recombinant proteins are available[85].

Autologous[72, 86–91] or allogeneic[44, 92–94] tumor cell lysates have also been applied as a source of antigens. This has several advantages: the antigen expression by the tumor does not need to be defined, and a wide array of both MHC classes I and II epitopes are presented including tumor-specific antigens. Possible drawbacks of this approach are the presentation of autoantigens, the requirement of a sufficient volume of tumor tissue for preparation of the lysate and difficulties in monitoring tumor-specific T cell responses since the antigens relevant to T cell responses are not known.

Very novel thoughts also include sequencing of the tumor and to focus on those mutated proteins that contain MHC-binding peptides, thus increasing the number of potentially immunogenic tumor-specific antigens[95].

4.3.2 mRNA-Transfected Dendritic Cells

Transfection of DCs with RNA comprises an alternative antigen loading technique[96], with either tumor-derived RNA[97, 98] or synthetic RNA encoding full-length tumor antigens being used[99]. The most widely used technique to transfect DCs with RNA is RNA electroporation, the transient permeabilization of the plasma membrane during application of an electric field at which point the RNA can enter the cell. A benefit of this technique lies in the presentation of several MHC class I epitopes and sometimes also MHC class II epitopes, depending on the presence of an endosomal targeting sequence[100]. It could also lead to a more prolonged presentation of the antigen as compared to peptide loading, which appears to be short lived[101]. Disadvantages of RNA transfection include a variable expression and a low yield of viable cells after transfection, although without loss of phenotype and maturation potential of the viable cells. mRNA electroporation is more efficient compared to plasmid DNA electroporation, and since it is a nonviral method of transfection, the RNA lacks the potential to integrate into the host genome, thereby obviating the safety concerns associated with clinical gene therapy trials.

Although tumor-derived RNA potentially harbors tumor-specific epitopes of mutated genes, it has the additional disadvantage that an unknown number of autoantigens will also be presented. However, several studies have shown that this technique is feasible and results in highly efficient DC transfection[99, 102–105].

Furthermore, antitumor T cell responses and some evidence for clinical activity have been reported in patients vaccinated using DCs electroporated with tumor-derived RNA[106, 107].

Another technique consists of using DC–tumor cell fusion hybrids. Inactivated tumor cells are fused with mature DCs. Ex vivo, this method has shown to be feasible and results in effective antigen presentation[108]. As with tumor lysates, the advantages of this technique are that the antigens expressed by the tumor do not need to be defined and a wide array of epitopes are presented. On the other hand, cultured tumor cells are needed and the inactivated tumor cells might still exhibit tumorigenicity in vivo[109].

Furthermore, RNA technology can be exploited not only to improve antigen presentation but also to improve DC maturation and T cell stimulation. For example, it has been shown that the T cell-stimulatory capacity of peptide-pulsed DCs can be greatly enhanced by providing them with three different molecular adjuvants through electroporation with mRNA encoding a so-called TriMix of CD40 ligand (CD40L), CD70, and a constitutively active form of TLR4. The combination of CD40L and TLR4 electroporation would mimic CD40 ligation and TLR4 signaling of the DC and generates phenotypically mature, cytokine-secreting DCs. Further, the introduction of CD70 into the DC provides a co-stimulatory signal to $CD27^+$ naive T cells by inhibiting activated T cell apoptosis and by supporting T cell proliferation[110].

At present, all of the above DC-loading methods have been tested in preclinical models or already used in DC vaccination trials. All have their advantages and disadvantages, but the optimal method for antigen loading with any strategy remains unknown.

4.4 Dendritic Cell Vaccination Trials

During the past decade, DC-based immunotherapy is explored worldwide in clinical vaccination trials, predominantly in cancer patients[111]. Most clinical studies use autologous ex vivo-cultured, antigen-loaded monocyte-derived DCs or $CD34^+$ progenitor-derived DCs that are administered to patients with the aim of inducing tumor-specific effector T cells that can reduce the tumor mass specifically and that can induce immunological memory to control tumor relapse (Fig. 4.2).

In recent years, over 100 clinical studies have been or are being carried out in cancer patients. Most studies carried out were small exploratory studies aimed at optimizing vaccines and measuring immune responses. In short, we can conclude that DC immunotherapy has been introduced into the clinic and has proven to be feasible and safe and potently induces immunological responses, particularly if the DCs have been appropriately matured. Nevertheless, thus far, only a very limited number of long-term clinical responses have been observed.

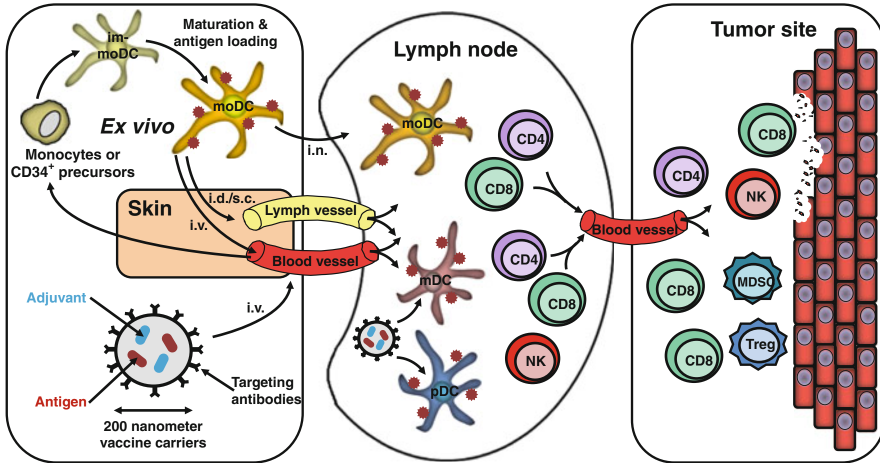

Fig. 4.2 The induction of tumor antigen-specific T cells via ex vivo or in vivo dendritic cell vaccination. DCs cultured from monocytes or $CD34^+$ progenitor cells can be loaded with tumor antigen ex vivo and administered to cancer patients via different routes, after culture in the presence of maturation stimuli such as proinflammatory cytokines. Within the lymph node, DCs present antigens to T cells, in combination with a co-stimulatory signal to initiate an immune response. The activated tumor antigen-specific T cells proliferate and migrate out of the lymph node toward the site of the antigen, the tumor site. At the tumor site, MDSCs and Tregs are able to create an immunosuppressive microenvironment, inducing peripheral tolerance and complicating tumor clearance by T cells. Exploiting natural DC subsets can be performed either by isolating pDCs or mDCs and stimulating them ex vivo with adjuvant and antigen (not shown) or by targeting them in vivo exploiting nanoparticles that carry antigen and adjuvant and are coated with antibodies against DC-specific surface receptors. Both pDCs and mDCs can stimulate T cells. Cross-talk between both DC subsets can also stimulate other immune cells such as NK cells. *Im-moDC*, immature monocyte-derived dendritic cell; *mo-DC*, mature monocyte-derived dendritic cell; *i.d.*, intradermal; *s.c.*, subcutaneous; *i.v.*, intravenous; *i.n.*, intranodal; *mDC*, myeloid dendritic cell; *pDC*, plasmacytoid dendritic cell; *CD4*, $CD4^+$ T helper cell; *CD8*, $CD8^+$ cytotoxic T cell; *NK*, natural killer cell; *MDSC*, myeloid-derived suppressor cell; *Treg*, regulatory T cell.

4.4.1 DC Maturation Status and Antigen Loading

The majority of the initial vaccines used immature or semimature rather than mature DCs, which might have affected the immunological and clinical outcomes[30]. Studies that compared the immunogenicity of immature versus mature DCs showed that maturation is essential for the induction of immunological responses in cancer patients[35, 36]. Moreover, the use of mature DCs appeared to be associated with a better clinical outcome compared to immature DCs[36, 37]. Vaccination with immature DCs might even promote antigen-specific tolerance[112].

This superiority of mature DCs in inducing T cell responses is probably not only related to their high expression of MHC and co-stimulatory molecules but also to their enhanced migratory capacity. Compared with immature DCs, mature DCs

migrate much better to draining lymph nodes after intradermal or subcutaneous injection, although this migration process is still rather inefficient. Within the lymph node, mature DCs show a pronounced migration into the T cell areas where antigen presentation takes place, whereas immature DCs remain at the periphery[38].

To date, the optimal mode of DC maturation for clinical use has still not been established completely. Although a wide range of cytokine maturation cocktails have been tested in clinical studies, DC maturation via triggering of TLRs has been explored in great detail only recently. This not only holds for monocyte-derived DC and $CD34^+$ progenitor-derived DCs but also for naturally circulating DC subsets, which are now also being considered for therapy.

Besides the optimal maturation method, mature DCs have not been used in clinical studies comparing different antigen loading techniques. Most of the early vaccine studies focused on MHC class I-restricted antigens as targets for cancer-specific $CD8^+$ T cells. Identification of MHC class II-restricted antigens as targets for $CD4^+$ T cell responses allowed concurrent immunization with class I and class II epitopes to generate more potent immune responses. In addition, exploitation of mRNA transfection even led to presentation of multiple MHC class I and II epitopes on the cell surface and prevented the need for HLA selection of patients. There is consensus that mRNA is preferred over peptide loading because of the multiple epitopes presented, although mRNA application is not always possible. For instance, because of the rather fragile nature of natural DCs, mRNA transfection is not possible with them, and peptide loading is preferred. Also the number of RNAs that can be applied is limited.

4.4.2 Clinical Trial Overview

Clinical trials with DC vaccines are being carried out in a wide variety of human cancers. In theory, DC-based immunotherapy should be applicable to all cancer types, although several practical limitations mean that it is mostly investigated in certain types of tumors. Limitations include the lack of appropriate tumor-associated antigens or the absence of sufficient tumor material when tumor lysates are used for loading of DC. In general, two types of antigens have been used: (1) tumor cell-associated antigens, which are also expressed by their nonmalignant counterpart, and (2) tumor-specific antigens including the MAGE antigens, which are only expressed at immune privileged sites or only during embryogenesis. Nowadays, genomic and proteomic approaches are also used to identify tumor-specific antigens that are derived from mutated genes.

Of all cancer types, melanoma is by far the most studied cancer type in DC immunotherapy. This is mainly because in melanoma a large variety of tumor-associated antigens have been characterized, consisting of tumor differentiation antigens such as gp100 and tyrosinase and tumor-specific antigens such as MAGE-3[113]. Other reasons are that melanoma is considered of one of the most

immunogenic tumors and that no first-line treatment is available which improves overall survival in the case of metastatic disease[114–116].

Hematological malignancies form another group of cancer types that are studied more extensively, mainly the lymphomas and myelomas. For example, malignancies of B cell origin express monoclonal immunoglobulins carrying unique tumor-specific antigenic determinants in the variable regions, called idiotypes[117]. These idiotypes can be isolated from B cell malignancies and subsequently be used as DC-loading antigens for the induction of specific $CD4^+$ and $CD8^+$ T cells. In contrast to the antigens used in solid tumors, these antigens are thus not only tumor specific but also patient specific.

Other tumor types that have been investigated using DC vaccines include colon cancer, renal cell carcinoma, breast cancer, ovarian cancer, nasopharyngeal carcinoma, hepatocellular carcinoma, pancreatic tumors, adrenal carcinoma, cholangiocarcinoma, parathyroid carcinoma, non-small cell lung cancer, head and neck cancer, sarcoma, bladder cancer, glioma, and pediatric malignancies[118–125]. In most of these studies, DCs were cultured without maturation stimuli. In colorectal cancer, the most widely used antigen for loading on DCs is carcinoembryonic antigen (CEA)[126]. Patients with CEA-expressing malignancies have also been treated with Flt3L expanded DCs pulsed with a modified CEA peptide or with monocyte-derived DCs transfected with RNA encoding CEA.

Because a new vaccine has to be prepared for each patient, this has hampered the development of DC vaccines by the pharmaceutical industry. The first dendritic cell-based vaccine that has been brought to the market by a private company is sipuleucel-T (Provenge; Dendreon), a vaccine used in men with metastatic castration-resistant prostate cancer. Despite the discussion about the DC characteristics of this vaccine, the impact of this first FDA-approved cancer vaccine has been significant and certainly boosted the field. Furthermore, because of the highly individual DC vaccines, there is also a large interest in developing strategies to target DCs in vivo instead.

4.4.3 Immunological Monitoring Methods and Results

The ability to measure the expected immune response of a given immunotherapy reliably, including in DC vaccination, is of major importance. Further, correlation with clinical outcome remains notoriously difficult and laborious. Although there is an abundance of different assays that are being used to measure tumor antigen-specific T cell responses[27, 41, 127], many of these assays have not shown consistent results, and none have been validated in prospective clinical trials. A major reason is the extremely low frequencies of high-affinity tumor antigen-specific T cells in the peripheral blood. These frequencies can be as low as 1 in 40,000 T cells[128]. These low responses are often not detected by the most frequently used techniques such as enzyme-linked immunosorbent spot analysis or direct MHC tetramer staining of peripheral blood lymphocytes.

Further, the general immune status of a patient is not necessarily indicative of the clinical outcome. For example, control antigens such as the foreign protein keyhole limpet hemocyanin are frequently used to assist monitoring for immune monitoring purposes. While reactivity against keyhole limpet hemocyanin is often seen after vaccination, no correlation between the reactivity against keyhole limpet hemocyanin and the clinical outcome has been observed. Also, cloning of the antigen-specific T cells and subsequent T cell receptor sequence analysis of the clones has only been limited successful[128]. A correlation between clinical outcome and circulating antigen-specific T cell response is found in only a minority of studies.

Another approach is analyzing T cell responses from biopsies of delayed-type hypersensitivity reactions in patients vaccinated with antigen-loaded DCs[127, 129]. The rationale for biopsying the skin comes from the observation that measuring induration upon delayed-type hypersensitivity challenge is not predictive of vaccine-related T cell responses[127]. By contrast, the presence of antigen-specific and functional T cells present in biopsies from delayed-type hypersensitivity was significantly correlated with a prolonged progression-free survival in metastatic cancer patients[127]. One argument for why this assay correlates better with survival is the fact that in this case, antigen-specific T cells have been identified that were able to extravasate and migrate into the tissues, similar to what is expected from effector T cell infiltration into the tumor. Perhaps only a fraction of the antigen-specific T cells are capable of that function.

Although correlations between tumor regression and T cell responses are sometimes observed[79], the use of immune responses as a surrogate end point in clinical trials still remains limited. Furthermore, the immunological studies performed so far are unfortunately too diverse in their setup to allow for a meta-analysis[130]. Nevertheless, some lessons can be drawn from these studies. For example, an intact and proper functioning immune system seems to have a higher potential to react to immune therapy, and tumor antigen-specific T cells are less frequently induced in patients with distant metastatic disease compared to patients with solely local (non-metastatic) disease[131]. In addition, more emphasis should be put on monitoring immune responses at the effector site, or at surrogate effector sites as discussed above, given that T cell responses in peripheral blood and the tumor microenvironment can show markedly different patterns[132]. Please see Chap. 15 for additional details on immune monitoring issues.

4.4.4 Clinical Results

The first proof of principle studies exploring DC vaccination were performed in the late 1990s, showing the feasibility and the potential efficacy of DC vaccination in cancer patients[2, 33, 72]. DC vaccines have proven to be safe with only minimal side effects in multiple phase I and/or II trials in adults[27, 35, 36, 41, 71, 133–135] as well as in children[136]. Side effects seen with the majority of DC vaccination protocols were

mostly limited to transient chills, fever, fatigue, nausea, and headache. Although immunological responses are often reported using several immune monitoring methods and different culture protocols, objective clinical responses remain anecdotal with objective response rates not exceeding 5–15 %, with disease stabilization and mixed responses being observed more often[29]. Interestingly, however, in cases where clinical responses were induced, these were often long lasting[137].

Several of the early studies published were inadequate in their design and interpretation. Indeed, quality control of the DC vaccines and information on phenotypic differences between DCs of individual patients have been largely lacking in many studies. Rather, the miraculous cure of a single patient is highlighted without proper discussion of the potential reasons for treatment failure in other patients.

To date, only very few phase III trials have been performed with DC-based immunotherapy, mainly because it is thought that the current vaccines have not yet reached their full potential and also because financial support is hard to obtain as most companies are not interested in producing laborious patient-specific vaccines. However, less than 2 years after one of the first publications on dendritic cell therapy was published[72], a prospective phase III trial was initiated in 2000 that compared standard dacarbazine chemotherapy with a dendritic cell vaccine as first-line treatment of patients with metastatic melanoma[84]. The trial was prematurely discontinued at the first interim analysis after the inclusion of 103 patients owing to lack of efficacy. The authors identified several possible negative contributing factors, including a variable quality of the dendritic cell vaccine among participating centers and a suboptimal maturation state, dose, and route of administration of the DCs. In retrospect, this trial was carried out too soon and was performed at a time when DC vaccination was too early in its development. Although this trial could be interpreted as a negative trial for DC vaccination in melanoma patients, equality with the standard therapy for the last 30 years is perhaps not a bad starting point, given the fact that there are many parameters regarding DC vaccination that have been optimized over the last decade and still can be optimized in the following years.

More recently sipuleucel-T, a DC-based vaccine used in men with metastatic castration-resistant prostate cancer, a patient population without any other available effective treatment options, has been brought to the market by a private company. This cell-based vaccine consists of autologous peripheral blood mononuclear cells obtained by leukapheresis, which include professional antigen-presenting cells that have been activated with a fusion protein (PA2024) of the prostate antigen prostatic acid phosphatase and the immunostimulant GM-CSF. Sipuleucel-T was approved based on results from two placebo-controlled phase III randomized trials. No significant difference in time to biochemical failure, defined as serum prostate-specific antigen $\geq$3.0 ng/ml[138], or improvement in progression-free survival could be shown[139, 140]. However, median overall survival was prolonged by approximately

4 months compared with the placebo group, from 21.4 to 25.9 months[139] and 21.7 to 25.8 months[140]. A relative reduction of 22 % in the risk of death was also shown[140].

The finding of a discrepancy between lack of benefit in progression-free survival and an advantage in overall survival could possibly be explained by the measuring technique. The classic World Health Organization (WHO)[141] and Response Evaluation Criteria In Solid Tumors (RECIST)[142, 143] criteria that are applied to measure the efficacy of cytotoxic chemotherapy depend on tumor shrinkage, and any increase in tumor size beyond a certain level as well as the appearance of new lesions is considered as a treatment failure. However, there is now ample evidence that these criteria do not apply to immunotherapy. Immunotherapy-induced tumor regressions have been well documented after initial progression and even after the appearance of new lesions, which are presumably caused by the infiltration of lymphocytes into tumors[144].

These observations have led to the proposal of novel immune-related response criteria, as response evaluation according to conventional response criteria (such as WHO and RECIST) can lead to unwanted early cessation of treatment owing to initial tumor growth[145]. These observations reflect the different dynamics of the immune response compared with the direct effects of cytotoxic drugs on cancer cells[146]. Please see chapter 14 for additional details of these issues in clinical immunotherapy trials.

At present, the majority of studies have investigated the therapeutic effects of DC vaccines in late-stage cancer patients with metastases, despite the finding that more potent immune responses are induced in patients without end-stage disease[131]. Part of the use of late-stage patients can be attributed to the notion that immunotherapy is less effective in patients with a large tumor burden, owing to the presumed correlation of immune suppression and tumor burden, and the delay in time taken to translate immune responses into a survival benefit, which is not possible in most patients with advanced disease. We thus might take better advantage of the unique capacity of DCs to direct the immune response by exploiting DC-based cellular therapy earlier in the disease course.

It has been demonstrated that melanoma-specific T cells are present together with antigen-presenting cells in sentinel nodes at initial evaluations of melanoma patients without known metastases. In this window between primary tumor and metastasis, immunological processes can be crucial. It might be at this turning point in the development of metastatic disease that ex vivo-generated DCs can best assist the immune system, such as in prophylactic studies in patients suffering from ocular melanoma where the primary tumor is removed have been initiated, as well as studies in high-risk patients for developing cancer because of familial genetic predisposition. A further interesting observation was made in advanced melanoma patients vaccinated with monocyte-derived DC that were pulsed with a single melanoma peptide. The only clinically responding patient showed evidence of spreading of T cell reactivity against other antigens as well, suggesting that determinant spreading is of importance for the induction of clinical responses[76].

4.5 Novel Concepts and Future Perspectives

4.5.1 *General Considerations*

The immunological and clinical responses in clinical trials thus far support the concept of using DC-based immunotherapy successfully to treat cancer. Nevertheless, a number of variables need to be evaluated and controlled to improve clinical outcomes further in DC vaccination in more patients. Among these are optimization of the ex vivo generation of DCs; the use of different, naturally circulating DC subsets; route of DC administration; maturation stimuli for DCs[69]; and antigen loading of DCs[30]. These variables still provoke an ongoing debate, but one can clearly conclude that the full potential of DC-based immunotherapy has not yet been fully realized or exploited.

Second, upon induction of tumor antigen-specific T cells, the next hurdle to overcome is the local immune suppressive environment created by the tumor. No matter how effective a DC vaccine is, an immunosuppressive tumor microenvironment can inhibit its efficacy. In particular in end-stage cancer patients, this misbalance is already in favor of the metastasizing tumor. It is reported that in some patients, tumor antigen-specific T cells are present after DC vaccination, but they still experience disease progression. It became clear that these tumor antigen-specific T cells did not produce relevant cytokines nor exhibit cytotoxic activity upon tumor challenge[131]. Apparently, these effector cells were not capable of overcoming the local suppressive tumor environment. To circumvent or tackle these hurdles, novel concepts are under development to further improve DC-based immunotherapy. These concepts are extensively addressed in other chapters in this textbook.

4.5.2 *Natural Dendritic Cell Subsets*

Whether DCs differentiated ex vivo from precursor cells are the optimal source of DCs for the induction of potent immune responses remains unclear. The extensive culture period (7–9 days) of ex vivo-generated DCs and compounds required to differentiate them into DCs might negatively affect DC function, especially their capacity to migrate toward the site of T cell interaction by exhaustion of the cells[147, 148]. Therefore, it is attractive to consider alternative DC sources, such as natural blood DCs: mDCs and pDCs. While natural blood DCs might not require extensive culture, to be effective, they must be activated through molecularly defined triggers of DC activation, such as TLRs or CD40 ligand, prior to reinfusion[58]. This is of particular importance as nonactivated or improperly activated DCs might result in T cell tolerance rather than productive T cell immunity[39]. Also, since the frequency of naturally occurring circulating blood DCs is very low, it was thought that multiple leukapheresis would be needed to obtain sufficient numbers of DCs. However, it has

proven feasible to obtain more than 10 million pDCs and even higher numbers of mDCs after a single leukapheresis. Furthermore, to collect even more DCs, Flt3L is a potential candidate for expanding human DCs in vivo without activating them[149, 150].

Recently, a first clinical trial with tumor antigen-loaded, TLR ligand-matured pDCs in stage IV melanoma patients was completed, which appeared feasible and safe. In the majority of patients vaccinated with pDCs, responses against the monitoring protein were found, demonstrating that even small numbers of naturally occurring DCs can induce immunological responses. Although this phase I study was primarily aimed at determining potential toxicity, the clinical results were extremely promising with significantly extended survival in the majority of the patients.

Several ex vivo and preclinical studies suggest that pDCs and mDCs might cooperate and act synergistically. Human mDCs and pDCs activate each other after specific stimulation of only one of the DC subsets with appropriate TLR ligands ex vivo[24]. Importantly, when mDCs and pDCs are used in combination, both DC subsets will need stimulation with carefully selected TLR ligands, due the fact that they express different repertoires of TLRs (Fig. 4.1). Since TLRs act in synergy[151], the combination of different TLR ligands might even be more potent. Therefore, it is of crucial importance to find TLR ligand combinations that either induce optimal maturation of both mDCs and pDCs or at least do not interfere with maturation of either DC subtype. Ex vivo findings with human cells suggest that in a clinical setting, vaccination with both pDCs and mDCs might also generate stronger antitumor responses than vaccination with monocyte-derived DCs alone.

4.5.3 Targeting Dendritic Cells In Vivo

Another recent approach to exploit natural DCs for cancer immunotherapy is to target DC subsets in vivo. To this end, instead of isolating the various subsets, laborious culturing, and antigen loading ex vivo, antibodies are used to target DCs with activating agents and antigens directly in vivo[152]. Early studies have shown that when antigen is bound to antibodies directed against surface receptors of DCs that are implicated in endocytosis, that this leads to uptake of antigen. Antigens loaded through these receptors are efficiently channeled into endocytic compartments for loading of MHC class I and II molecules and the subsequent induction of immune responses[153]. However, if the antibody–antigen conjugates are not accompanied by adjuvant to stimulate the immune system, tolerance rather than immunity might occur[152, 153].

Therefore, several investigators have embarked on the development of nanoparticles[154] that are coated with antibodies to target natural DC subsets and that are loaded with both antigen and adjuvant (Fig. 4.2)[155, 156]. The advantage of this approach is that adjuvants only activate those DCs that are targeted by the antibodies, thereby preventing systemic activation and toxicity. Co-targeting of TLR ligands in nanoparticles as an adjuvant has shown substantially enhanced

DC maturation and production of immune-stimulatory cytokines. In addition, corresponding antigen-specific activation of CD8$^+$ T cells in mice was observed, without a so-called cytokine storm and related toxicity that may be associated with the administration of soluble TLR ligands[156].

Another major advantage of in vivo targeting strategies is that they can be produced in bulk quantities, whereas vaccines based on DCs loaded with antigens ex vivo require extensive tailor-made procedures for each individual. However, ex vivo culture conditions allow careful control of maturation and activation, while due to lack of direct control in in vivo targeting strategies, the duration and stability of the vaccine following administration will be difficult to determine[152].

Many of the receptors that are studied in targeting strategies belong to the CLR family. These CLRs comprise a family of calcium-dependent lectins that share primary structural homology in their carbohydrate domain. Through this domain, CLRs bind to specific self or nonself sugar residues and are implicated in antigen capture and endocytosis. For example, the CLR DC-SIGN is predominantly expressed on immature DCs and at lower levels on mature DCs and macrophages[157–160]. Anti-DC-SIGN antibodies efficiently target mDCs in vivo and reach saturation with one single dose. The binding of a humanized antibody to DC-SIGN showed high-affinity and facilitated endocytosis.

Furthermore, targeted human monocyte-derived DCs with delivery of antigen conjugated to the humanized DC-SIGN-specific antibody leads to presentation of the antigen by both MHC class I and II molecules and elicits both naive and memory T cell responses ex vivo[160]. DEC205 (CD205) is another CLR that is widely explored as a potential targeting antibody in mice. DEC205 expression levels in human are highest in mature DCs, but it is also expressed by B cells, T cells, monocytes, macrophages, and natural killer cells[161]. In mice, DEC205 seems to be more potent in mediating cross presentation ex vivo compared to the other CLRs. Several studies are ongoing to compare and determine the efficacy of different targeting receptors.

To conclude, although it will probably still take several years before direct targeting of antigens via nanoparticles to DC surface receptors in vivo will be applied in clinical trials in cancer patients, it might replace laborious and expensive ex vivo culturing in time and facilitate large-scale application of DC-based vaccination therapies. Please see Chap. 6 for additional DC targeting strategies.

4.5.4 Combating Tumor Escape Mechanisms

The immune system has several ways to tune down immune responses in order to prevent autoimmunity or excessively long or vigorous inflammatory reactions. In addition, tumors have evolved various mechanisms to evade immunological surveillance or to counterattack the immune response to facilitate their own progression, so-called tumor escape mechanisms[162]. The immunosuppressive strategies used by tumors interfere with multiple steps and pathways in the

generation of an effective immune response. They include the evasion of immune recognition, the secretion of immunosuppressive cytokines, the expansion and recruitment of Tregs, and the activation of negative regulatory pathways. The degree to which the immune system is compromised by the tumor presence is variable, the most aggressive tumors appear to be more successful in creating an optimal microenvironment suppressing antitumor immune responses to favor tumor progression. It is evident that when developing novel vaccines, we must deal with and revert local immune suppression.

4.5.4.1 Overcoming Reduced Tumor Immunogenicity

To evade immune recognition, tumor cells frequently modify, downregulate, or even lose expression of tumor antigens on their cell surface. Modification of tumor antigens can result in peptides that do not fit into the MHC class I groove or form a MHC class I–peptide complex that cannot be recognized by T cells. Besides altered antigen expression, downregulation or loss of MHC class I expression can occur independently and is frequently seen in various cancer types, particularly in metastatic lesions[133, 163–165]. Both processes allow tumor cells to avoid recognition by $CD8^+$ cytotoxic T cells[166]. For example, in melanoma it has been shown that downregulation of both antigen and MHC class I has a negative effect on prognosis[167, 168].

Besides alterations in MHC class I and antigen expression, another aberration frequently seen in tumor cells involves downregulation of the expression of co-stimulatory molecules on the tumor cell surface[169]. This leads to an insufficient co-stimulatory signal (signal 2) that is necessary for the induction of an effective T cell response in combination with MHC class I antigen presentation. In its absence, T cells are rendered anergic[170]. Evidently, novel vaccines cannot compensate for this deficiency, but one could attempt to design vaccines that also boost natural killer cell activity besides stimulating T cell-mediated immunity.

4.5.4.2 Soluble Factors Influencing the Immune System

Tumor cells produce a variety of cytokines and small molecules to promote tumor progression, mainly by increasing tumor invasiveness and angiogenesis. Some of these cytokines also possess immunosuppressive properties that, together with the lack of immunostimulating cytokines, create a cytokine imbalance responsible for immune deviation seen at the tumor site and distantly. The best characterized immunosuppressive factors to date involved in the tumor microenvironment and/or systemic immunosuppression are transforming growth factor β (TGF-β), vascular endothelial growth factor (VEGF), IL-10, and prostaglandin E2[171–177]. It has been shown that TGF-β induces IL-10 secretion that negatively affects the maturation and activation of DCs and causes a shifts toward T helper 2 responses[178].

The immunosuppressive function of VEGF is mainly dependent on the alteration of the function of DCs by blocking DC maturation[179].

The expression of the small molecule prostaglandin E2 is correlated with impaired DC and T cell function[180].

4.5.4.3 Regulatory T Cells and Myeloid Suppressor Cells

The accumulation of immunosuppressive cells at the tumor site is another mechanism contributing to tumor escape (Fig. 4.2). The most well-known type of cell that can suppress the immune system and plays a key factor in peripheral tolerance is the Treg. Tregs are not only capable of infiltrating a site of infection; it is also a well-known phenomenon that they can infiltrate tumors[181–188]. Besides Tregs, myeloid-derived suppressor cells also have a suppressive effect on host immunity and consist of a group of cells including macrophages, granulocytes, and DCs[189, 190]. For further information on these immunosuppressive cell types, please see Chap. 9 on Tregs and Chap. 10 on myeloid-derived suppressor cells. It is evident that new vaccines should also be aimed at reverting the T regulatory/T effector cell balance, for instance by inducing strong T helper 1 type of cytokines.

4.5.4.4 Therapeutic Options to Counteract the Tumor Escape Mechanisms

It is clear that the most fantastic vaccines can be developed, but if we are not able to manipulate immunosuppression at the site of the tumor at the same time, DC vaccinations will have little or no long-term effect. Approaches to tilt the balance toward more effective DC-based immunotherapy involve ways to counteract the tumor escape mechanisms discussed above, either by optimizing the DC vaccine or by combining treatment modalities.

To counteract the evasion of immune recognition, one can think of several strategies. Vaccines containing multiple tumor antigens can partly bypass the emergence of antigen loss; however, this solution will probably not be effective enough since multiple tumor antigens can be downregulated simultaneously. Instead of using standardized tumor peptides, proteins, or RNA to load DCs with tumor-specific antigen for vaccination purposes, the development of RNA sequencing technologies allows the determination of the complete range of mutated antigens from the primary tumor and metastases of a patient, thereby possibly enabling tailor-made therapeutic vaccines to the patient's tumor in the near future[191]. Furthermore, novel vaccines might also be directed at activating innate cells such as natural killer cells that can recognize tumor cells that have downregulated MHC expression, in addition to stimulating the adaptive immune response. Finally, it is suggested that histone deacetylase inhibitors are able to upregulate MHC class I expression on tumor cells by increasing the expression of many components of the antigen-processing machinery[192] and thus enhance their susceptibility to killing by cytotoxic T cells. However, there are also studies

showing an immunosuppressive effect of histone deacetylase inhibitors that necessitates further research before clinical application in combination with DC vaccination.

Soluble factors secreted by the tumor create a suppressive environment, including preventing differentiation and maturation of natural infiltrating DCs[193, 194]. Several studies have now demonstrated that a topical TLR7 agonist (imiquimod) led to enhanced pDC recruitment and type I IFN production by resident pDCs at the tumor site, which then generated an inflammatory environment resulting in tumor regression[195, 196]. Furthermore, activation of tumor-resident pDCs can also be achieved by intratumoral injection of CpG motifs inducing TLR9 triggering. This strategy has therapeutic potential in patients with basal cell carcinoma and melanoma skin metastases[197]. Since TLR9 is thought to be expressed only by pDCs, CpG injection will not directly activate mDCs. TGF-β inhibitors, anti-VEGF antibodies, and selective inhibitors of cyclooxygenase 2, an enzyme involved in prostaglandin E2 synthesis, also have potential to counteract tumor escape by blocking the suppressive effects of secreted soluble factors[198–201].

Reducing the negative effects of cytokines produced by tumor cells or creating an immunostimulatory microenvironment by adding cytokines that stimulate the immune system to DC vaccination is being applied in the clinic but has shown little clinical benefit thus far.

Breaking peripheral tolerance mediated by Tregs theoretically potentiates the naturally occurring antitumor immune response or the induced effects of immunotherapy. In line with this concept, depletion of Tregs by anti-CD25 antibodies, targeting the α-chain of the IL-2 receptor, in murine models demonstrated an improved immune-mediated tumor rejection[202–205]. Furthermore, it resulted in enhanced therapeutic efficacy of immune-based therapy[206, 207], including DC vaccination[208–211]. Also other treatments to counteract tumor escape, such as anti-PD-L1 antibodies or indoleamine-2,3-dioxygenase inhibitors, might enhance the efficacy of various immunotherapies, including DC-based immunotherapy[212, 213]. Further research is needed, also to combine these approaches with DC vaccination.

4.5.5 Toward Combination Treatment

4.5.5.1 Tumor-Debulking Therapies

Given that immunotherapy is considered to be less effective in patients with a large tumor burden, owing to the presumed correlation of immune suppression and tumor burden, it is tempting to speculate on the possibility of tumor debulking as one treatment modality, combined with immune surveillance and immune memory induction by DC-based immunotherapy to clear small residues and to prevent relapses.

Tumor debulking, depending on the tumor type, could, for example, be accomplished with chemotherapy, targeted therapy, radiotherapy, or surgery[214]. Beside a

positive effect on the immune system when tumor burden decreases, local tumor-destructing therapies induce tumor cells to undergo apoptosis, with release of tumor antigens, which might elicit additional tumor-specific immune responses via in vivo loading of DCs[215]. However, since these modalities in themselves rarely induce a potent antitumor immune response, the immunogenicity of these methods need further enhancement by the local delivery of DC-activating signals[216, 217].

4.5.5.2 Adoptive T Cell Transfer

Adoptive T cell therapies (detailed in Chap. 3) are based on the infusion of large numbers of tumor-specific T cells[218, 219]. It is currently under investigation whether DC vaccination can enhance the graft-versus-tumor effect of stem cell transplantation and donor lymphocyte infusions in hematological malignancies[220]. Adoptive T cell transfer generates a high but short peak of antigen-specific T cells, whereas DC vaccination induces T cell responses more gradually that endure longer[221], providing a rationale to combine the two treatment modalities. In preclinical models, DC vaccination indeed boosted and sustained antitumor T cell responses after adoptive T cell transfer[221, 222].

4.5.5.3 Concomitant Cytokine Therapy

In contrast to the above-discussed immunosuppressive cytokines involved in the tumor escape mechanisms, immunostimulatory cytokines play a key role in regulation of lymphocyte survival[223] (and detailed in Chap. 7). It is thought that DC vaccination, among other immunotherapies, is likely to favor concomitant cytokine therapy with the goals of protecting effector $CD8^+$ T cells from tumor-mediated dysfunction or death and of restoration of normal lymphocyte homeostasis. Most experience with combined cytokine treatments is obtained with IL-2, which not only prolongs survival of transferred $CD8^+$ T cells but also enhances their antitumor activity[224]. Along with IL-2, GM-CSF and IL-12 are also being explored in preclinical models and in the clinical setting as well, either alone or in combination with other treatment modalities[225]. Thus far, clinical successes are limited, partly due to the restricted systemic administration because of toxic effects or due to unwanted immunosuppressive side effects. Type I IFNs have been well documented to suppress growth of tumor cells through inducing apoptosis and cell cycle arrest and have been clinically applied for treatment of certain tumor types, although various adverse effects impede optimal clinical application. Results of the combination of IFN-α with DC vaccination shows that it is safe, but it only elicits limited immune responses[70].

4.5.5.4 Chemotherapy

For a long time, the dogma has been that the myelosuppressive effects of chemotherapy would prevent its combined use with immunotherapy. However, in recent years more evidence is accumulating that some forms of chemotherapy may not harm T cell responses[226] and may in fact have a synergistic effect together with immunotherapeutic approaches[227–233]. More recently, this notion of a possible synergistic effect of chemo- and immunotherapy has been supported through studies showing that treatment of tumor-bearing mice with gemcitabine results in enhanced cross presentation and T cell activation[229]. Subsequent studies have demonstrated that chemotherapy-induced cell death can indeed invoke an immune response, depending on the biochemical cell death cascade that is induced by the drug[230]. These data clearly show that chemotherapeutic agents can have a beneficial effect on the antitumor immune response and may even imply that at least part of the clinical effect of chemotherapy depends on its immunological effects. In addition, recent data from clinical studies in patients with cancer have shown that T cell induction is not hampered by chemotherapy treatment[234, 235].

The optimal sequences of immunotherapy–chemotherapy combination treatments remain to be established. As different cytotoxic drugs have different immunological effects, it is conceivable that optimal treatment strategies will differ depending on both the cytotoxic compound chosen and the immunotherapeutic approach. Clinical data are lacking; however, trials combining chemotherapy and DC vaccination are in progress.

4.5.5.5 Anti-CTLA4 Antibodies

Considering that CTLA-4 is constitutively expressed on Tregs, blockade of CTLA-4 was thought to deplete Tregs. However, data indicate that CTLA-4-blocking antibodies did not result in depletion or decreased suppressive activity of Tregs, but execute their immune-stimulatory effect by preventing normal downregulation of activated T cells by blocking the CTLA-4/B7 interaction[236–239].

Since treatment with anti-CTLA-4 is antigen nonspecific, the combination with a vaccine could potentially direct the T cell response in a more specific manner, thereby diminishing autoimmune side effects. There is anecdotal information that anti-CTLA-4 treatment after DC vaccination may indeed enhance DC vaccine-induced T cell responses[240]. Further, the combination of anti-CTLA-4 plus DC vaccination could be synergistic rather than additive. However, clinical trials that are specifically designed to answer this question have not yet been published.

To conclude, one can speculate of countless currently available and combined treatment modalities which may have a positive effect on the immune system. Trials in the near future will have to answer the question of whether DC vaccination can elicit sustainable clinical responses in a substantial percentage of treated patients or can add to the clinical efficacy of other anticancer treatment modalities.

With the wealth of information currently available on the molecular mechanisms that control the immune system, there is no doubt that these are exciting times for immunotherapy.

4.6 Summary and Conclusions

In conclusion, DC vaccination has proven to be safe in multiple phase I(/II) trials. In the early days, trials were mainly performed with immature or semimature DCs which is now known to have a negative effect on immunological and clinical responses. The first phase III trial in melanoma patients showed no survival advantages of DC vaccination over first-line chemotherapy, but also suffered from the early stage of the DC vaccination field. Thus far, sipuleucel-T is the only DC vaccine tested in phase III trials and proven to induce a survival benefit. To date many different mature DC vaccination studies in patients with various types of cancer are in progress, continuing to optimize the vaccines before starting a meaningful phase III trial. Naturally circulating DC subsets are now also being clinically explored as are the first studies on in vivo targeting of DCs. Although observations of meaningful clinical responses are still scarce, expectations remain high, because when clinical responses have been induced, they are often long lasting. Thus, DC vaccination research has now entered a stage somewhere between "proof of principle" and "proof of efficacy" trials. Crucial questions to answer at this moment are why clinical responses generally remain limited and what can be done to improve the efficacy of vaccination. The answers to these questions probably lie in the preparation and administration of the DC vaccines. While DC vaccines have made significant advances over the last decade, multiple hurdles still remain. Within the next decade, the field will have to demonstrate maturity and not only yield a higher percentage of clinically responding patients but preferentially also develop means to predict at an early stage which patients will likely benefit and which not (please also see Chap. 14 on patient selection). The implementation of DC vaccination as the preferred form of standard therapy for cancer cannot become a reality until all these issues are fully addressed. We will not only have to improve our vaccines but also our clinical and immune monitoring tools. The latter will not only be beneficial for DC vaccination but also for other forms of immunotherapy such as antibody therapies against immune checkpoint molecules such as CTLA-4 and PD-1.

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Part III
Active Strategies to Boost Antitumor Immunity

Chapter 5
Peptide and Protein-Based Cancer Vaccines

Marion Braun, Rachel Perret, Godehard Scholz, and Pedro Romero

Abstract Vaccination has become one of the most successful public health achievements, yet its efficacy remains limited to prophylactic vaccination for infections and early mobilization of the immune system. In cancer, tumor cells have evolved to overcome immune surveillance or grow in the presence of exhausted immune cells. However, the identification of tumor-infiltrating lymphocytes as good prognostic indicator of increased survival in cancer disease[1–2] has profoundly encouraged the design of immunotherapeutic strategies aiming at the induction of tumor-specific T cell responses. Numerous past and present studies therefore focus on the re-education of a tumor-controlling immune system via therapeutic vaccination. In particular, the high numbers of T cell-defined tumor antigens identified thus far[3–7] form the basis for the design of therapeutic cancer vaccines. In this chapter, we review current knowledge on the promise and pitfalls of defined antigen vaccination strategies for cancer based on synthetic peptides and proteins and discuss novel approaches for combination treatments with novel immunomodulatory agents.

5.1 Antigenicity and Immunogenicity of Tumors: Tumor-Associated Antigens

Most tumors, both experimentally induced and naturally occurring, are antigenic. Results from decades of research have conclusively shown that the immune system can recognize and prevent tumor development. The main mediators of these two key processes are T cells, in particular the subset of cytolytic T lymphocytes (CTL)

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which express cell surface-associated αβ T cell receptors (TCR) and the co-receptor CD8. This subset of $CD8^+$ T cells is also crucial in recognition of, and protective immunity against, intracellular parasites such as viruses. To recognize their targets, CTL use clonotypically distributed TCR which are specific for short antigenic peptides. Unlike antibodies, TCR cannot directly recognize antigenic peptides in solution. Instead, these peptides must be presented to T cells in non-covalent association with major histocompatibility complex (MHC) class I molecules on the surface of antigen-presenting cells. The extremely polymorphic nature of MHC class I molecules allows capturing of a large variety of cytosolic peptides for presentation to T cells. In turn, TCR are generated by the somatic recombination of gene segments in the *TCR alpha* and *beta* loci during the late stages of fetal life and the neonatal period. The very large diversity of TCR provides a T cell repertoire able to recognize virtually any given antigen present in nature. However, in order to exclude autoreactive T cells in the periphery and prevent autoimmunity, T cell selection in the neonatal thymus deletes approximately 95 % of TCR rearranged thymocytes displaying too high an affinity for self-MHC-peptide complexes. Despite this stringent selection, a considerable fraction of self-antigen reactive T cells escape thymic tolerance and are a stable component of the mature T cell repertoire in adult individuals. It is this pool of $CD8^+$ T cells that forms the repertoire of T cells that can recognize self-antigens expressed by tumor cells. At the same time, thymic selection generates a relatively small subset of MHC class II-restricted $CD4^+$ T cells with the ability to control T cell reactivity of both $CD4^+$ and $CD8^+$ T cells. These cells, known as natural regulatory T cells (Tregs), are thought to be selected in the thymus based on their high affinity for self-MHC class II-peptide complexes.

Thus, T cell selection processes in the thymus have a profound influence on tumor immune surveillance because tumor-associated antigens are mostly self-antigens. On the one hand, $CD8^+$ T cells available in the mature T cell repertoire bear TCR of intermediate or low affinity, and only rarely of high affinity, for tumor antigens. On the other hand, dominant tolerance mediated by Tregs limits antitumor immunity to a great extent.

In comparison to healthy tissues, the expression levels of many proteins are deregulated in tumor cells (over- or under-expressed, or lost). A relatively large proportion of tumor antigens derive from tissue-restricted proteins (differentiation antigens) and/or from gene re-expression (cancer-testis antigens). They may also arise from alternative open reading frames, frameshifts, or isoforms from alternatively spliced genes. Recent genomic studies of tumors in humans have shown that each tumor can contain a few hundred non-synonymous somatic mutations resulting in altered proteins (mutated antigens). In theory, these would be an excellent source of nonself tumor antigens for which it is reasonable to expect the existence of T cells with high-affinity, specific TCR that could be exploited for immunotherapy[8]. However, they are not only tumor-specific but often individual-specific and therefore not applicable for vaccination of large patient cohorts.

Nonself tumor antigens also include oncoproteins expressed after viral infection of persistent viruses (oncoviral proteins). They are more immunogenic and have recently proved to be a promising target for prophylactic vaccination to protect from human papilloma virus infection, thereby preventing the virus-associated development of cervical cancer.

5.1.1 Identification of T Cell Epitopes

The first T cell-defined tumor antigens were identified with the aid of T cell clones isolated from cancer patients capable of reacting to tumor cells in assays of antigen recognition. One source of such clones was the mixed lymphocyte-tumor cell culture setting[9] involving the coculture of blood-derived lymphocytes with cells from the autologous tumor established in in vitro culture. This experimental system eventually led to the first antigen to be identified, the so-called MAGE-A1 antigen using molecular cloning of genomic DNA fragments from a melanoma patient[10]. The other source of tumor-reactive T cells has been the tumor-infiltrating lymphocyte (TIL) populations themselves. Historically, melanoma has been the tumor type that has lent itself for frequent TIL isolation and detailed characterisation. These TIL populations contain a relatively large number of $CD8^+$ T cells directed against a multitude of tumor antigens. In fact, many of the tumor antigens defined at the molecular level to date have been the result of analysis of the specificity of $CD8^+$ T cell clones isolated from melanoma TIL[11, 12].

While many of the tumor antigens were identified following molecular cloning techniques and monitoring of tumor antigen expression by specific CTL clones, another approach consisted in the direct biochemical isolation and sequencing of the antigenic peptide. In this case, the process was also monitored by functional assays of antigen recognition with the tumor-reactive CTL[13]. Such techniques have been refined and combined with genomics techniques for fast high-throughput tumor antigen identification[14]. The latter has led to the identification of panels of antigenic peptides from tumors such as renal cell carcinoma, colorectal carcinoma and glioblastomas[15, 16].

Another widely applied strategy to identify antigenic tumor-derived peptides is the so-called “reverse immunology” approach. This approach uses the knowledge accumulated on the structural details of antigen peptide–MHC interactions. Structural studies of peptide–MHC interactions identified the peptide side chains that confer the major binding energy, and specificity, to the peptide-binding groove in about 60 different peptide-MHC (pMHC) complexes. The amino acids in these peptide side chains have been called peptide anchor residues. There are generally two anchor residues for any given MHC class I allele. They are highly conserved in what are identifiable as distinct structural peptide-binding motifs. For instance, HLA-A*0201 molecules, expressed by 20–40% of humans of different ethnic

groups, generally bind nona- or decapeptides with a Leu, Met or Ile residue at the second position relative to the peptide N-terminus and a Val residue at the C-terminus[17]. A few other amino acid residues in the antigenic peptide can also provide binding energy and are identified as secondary anchor residues. Tyr or Phe residues at the N-terminus form part of the extended HLA-A*0201-binding motif[18]. Thus, the MHC allele-specific peptide-binding motifs have been incorporated in algorithms for in silico predictions of the binding affinity of peptides. Instead of using T cell clones from patients, the "reverse immunology" approach relies on the use of algorithms to scan protein sequences for the presence of peptides that could specifically bind to a given MHC molecule. Following verification of peptide binding to the MHC molecule by classical biochemical methods, the high-affinity binder peptides are tested for their ability to trigger the expansion of specific $CD8^+$ T cells in the blood lymphocytes of healthy individuals. The ability of specific $CD8^+$ T cells to recognize and kill appropriately selected tumor cells provides the ultimate validation of the predicted peptide as a *bona fide* tumor antigen[19]. This method has been expanded and used widely for the prediction and eventual identification of tumor antigens for vaccine development. Various web-based platforms and databases have been generated[20–22]. Importantly, the natural processing and presentation of identified epitopes needs to be confirmed, preferably on HLA-matched tumor cell lines, before the peptides are eligible as vaccine candidates.

5.1.2 Tumor Peptide Analogues and Heteroclitic T Cell Responses

Since tumor peptides are mostly self-peptides that form pMHC complexes with rather low affinity for T cells, manipulations to enhance the affinity of the tripartite interaction between MHC molecule, peptide and TCR have been the focus of many studies. Amino acid exchanges at antigenic peptide positions that either increase binding to MHC or directly increase the strength of the interaction with the TCR have been generally pursued. The MHC allele-specific peptide-binding motifs help in guiding the choice of amino acid replacements.

Even though there are conserved positions within peptides that are responsible for MHC anchor residue binding and others for TCR interaction, mutations of anchor positions can still alter TCR recognition[23]. This is most likely due to conformational changes of the peptide backbone and minor displacements of TCR-interacting residues. It has been demonstrated for the Melan-$A_{26–35}$ A27L peptide analogue that anchor residue modification does not impede peptide recognition of T cell clones specific to the native peptide. However, functional studies revealed that vaccination with this peptide analogue generates a T cell pool with a smaller TCR repertoire and a more differentiated T cell phenotype[24,25]. Other peptide analogues with mutated MHC anchor residues have even been shown to increase T cell recognition significantly, generating a heteroclitic T cell response[26]. Such improved TCR-binding qualities are usually sought when mutating those

amino acids known to represent direct TCR interaction residues. These peptide analogues have also been termed variants or mimotopes, since they only mimic the protein against which they induce a T cell response. Even though such heteroclitic peptides increase TCR affinity or T cell avidity very efficiently, they harbour the dangerous potential to induce cross-reactive T cell responses[27], or to behave like superagonists that could promote autoimmune responses[28].

5.1.3 Adjuvants: Conferring Immunogenicity to Pure Polypeptides

Since peptides or proteins by themselves are poorly immunogenic, the initiation of adaptive immune responses largely depends on the danger context under which the peptides are displayed. The discovery of pathogen-recognition receptors (PRRs) expressed on a variety of innate immune cells formed a basis for the understanding of the molecular signals involved in recognition of nonself or "danger" contexts. PRRs represent evolutionarily conserved membrane-bound receptors, some at the cell surface, some in endosomes that recognize pathogen-associated molecular patterns (PAMPs) commonly found on bacteria, viruses and fungi[29]. PRR family members include Toll-like receptors (TLRs), C-type lectins, as well as the intracellularly expressed RNA-sensing RIG-like helicases and the DNA sensors DAI and AIM2. Cytoplasmic nucleotide-binding domain and leucine-rich repeat-containing receptors build another family of receptors that add to the group of cytoplasmic PRRs.

"Danger" signals originating from injured cells that have been exposed to pathogens, mechanical stress or toxins, can also lead to the efficient activation of antigen-presenting cells and the initiation of immune responses[30]. The mediators involved have been collectively termed danger-associated molecular patterns (DAMPs). Three nucleotide-binding domain and leucine-rich repeat-containing receptors, namely Nucleotide Oligomerization Domain (NOD)-like receptor family, pyrin domain containing 1 (NLRP1), NOD-like receptor family, pyrin domain containing 3 (NLRP3) and INOD-like receptor family, CARD domain containing 4 (IPAF) as well as AIM2 are able to sense host-derived "danger" signals resulting in the assembly of the molecular platforms inflammasomes and the initiation of potent inflammatory responses via the production of IL-1β[31].

Activation of antigen-presenting cells via PAMPs and DAMPs induces a signalling network that results in the maturation of the antigen-presenting cells, the expression of co-stimulatory receptors such as CD80 and CD86, and the release of key cytokines such as type I interferon IFN, IL-12 or IL-23. Fully mature dendritic cells are pivotal for the optimal activation of T cells recognising their cognate antigen and the initiation or priming of immune responses. The design of potent vaccines therefore includes adjuvants, which are substances that trigger PAMP and/or DAMP signals in order to expose the vaccine antigen in a defined natural danger context.

While specificity is imparted by the target antigen, adjuvants are compounds that potentiate their immunogenicity nonspecifically. They can be composed of microbial products, mineral salts, emulsions, microparticles and liposomes[32]. Recent reviews on adjuvants in cancer immunotherapy have been published[33,34].

5.1.4 Adjuvant Emulsions

One of the oldest adjuvants, incomplete Freund's adjuvant (IFA), is in principle independent of direct TLR stimulation and consists of an oil-in-water emulsion. Currently used emulsions in vaccine formulations include Montanide, adjuvant 65, MF59 and lipovant. Such emulsions are thought to provide a tissue depot effect that results in prolonged availability of antigen by slow release[35]. Moreover, this class of adjuvants highly potentiate immunogenicity of subunit vaccine antigens by tissue disruption and stress. Factors released upon tissue damage include ATP, uric acid and high mobility group box 1 (HMGB1), which are DAMPs that are implicated in innate immune sensing via the inflammasome and can induce dendritic cell activation. It is still unclear the extent to which both mechanisms of adjuvanticity are required or whether one of them is enough to confer strong immunogenicity.

5.1.5 Alum

The most widely used adjuvants currently in human vaccines are aluminium derivates. In particular, aluminium hydroxide makes part of the formulation of a large spectrum of vaccines including tetanus, diphtheria, hepatitis A and pertussis. They induce important danger signals that are sensed by the NLRP3 inflammasome that initiates potent inflammatory responses[36]. Protection is associated with the induction of humoral immunity and priming of cellular immune responses that consist mostly of Th2 helper cells[37,38]. This goes in line with the recruitment of eosinophils, which are an early source of IL-4[39,40], indicating the priming of Th1- and CTL-antagonizing responses. Such Th2-polarized responses could run counter to effective antitumor immunity.

5.1.6 TLR Agonists

Microbial products have potent immunostimulatory capacity and perform their adjuvant effect via activation of TLRs. Numerous studies make use of a broad spectrum of TLR ligands with increasing success for cancer immunotherapy. The mycobacterium bacillus Calmette–Guérin contains multiple ligands for innate immunoreceptors, including TLR2, TLR4 and NLR2[41]. Other adjuvants based on TLR4 stimulation are lipopolysaccharide derivatives. The lipid A compound in

lipopolysaccharide mediates its adjuvant effect but is itself too toxic for clinical use. Hydrolised monophosphoryl lipid A is a lipopolysaccharide derivative that contains the structural element for TLR4 stimulation but has reduced toxicity and has been successfully used in several vaccine formulations including in some prophylactic vaccine formulations against hepatitis B virus and human papilloma virus[42,43]. Since their mechanism of protection is antibody mediated, this adjuvant is useful to induce powerful humoral immune responses. Monophosphoryl lipid A is also included in cancer vaccines targeting tumor-associated antigens, but mostly in combination with other adjuvants. Vaccination with a synthetic Melan-A peptide in a mixture of monophosphoryl lipid A and a component of the saponin Quil-A (QS-21) or in IFA showed poor induction of peptide-specific $CD8^+$ T cell responses of the monophosphoryl lipid A adjuvant formulation in comparison to Montanide ISA51[44]. However, the same combination of monophosphoryl lipid A and QS-21 in an oil-in-water emulsion resulted in prolonged disease-free survival and led to the development of a phase III clinical trial in non-small cell lung cancer patients vaccinated with a recombinant MAGE-A3 fusion protein[45]. The final adjuvant formulation to be used in the phase III trials with this fusion protein in metastatic melanoma (DERMA) and in non-small cell lung carcinoma (MAGRIT) included yet another TLR agonist, CpG-oligodeoxynucleotides that specifically signal through TLR9.

Other TLR ligands eligible as adjuvants are synthetic analogues of viral double-stranded RNA that stimulate TLR3. TLR3 agonists induce high levels of type I IFNs and have shown promising results in preclinical and clinical trials for cancer treatment[46–49]. A recent phase I trial in patients with glioblastoma combined synthetic peptide antigens loaded onto alpha type 1 polarized dendritic cells and the TLR3 agonist poly(I:C) stabilised by lysine and carboxymethylcellulose[50]. TLR7/8 ligands have also been considered as adjuvants in the treatment of cancer. Imiquimod as a TLR7 agonist has been approved for the topical treatment of basal cell skin cancer, and its application in a peptide-based vaccine trial resulted in an elevation of peptide-specific T cells[51].

One of the most promising targets for the mobilization of cellular immunity in cancer patients is TLR9. Ligands for TLR9 are unmethylated oligodeoxynucleotide stretches of cytosine and guanine (CpG-oligodeoxynucleotides), which are commonly found in bacterial and viral DNA. TLR9 is expressed in endosomes of human plasmacytoid dendritic cells, macrophages and B cells. It was recently shown that plasmacytoid dendritic cells are essential for the initiation of inflammation, and, via type I IFN signalling, for the induction of $CD8^+$ T cell responses and immune rejection of tumors[52–54]. The fact that TLR9 stimulation in plasmacytoid dendritic cells leads to production of significant levels of type I IFNs might hence explain its potential as an adjuvant in cancer immunotherapy. Interestingly, a combination of Montanide and CpG-oligodeoxynucleotides has shown superior potential in the generation of antigen-specific $CD8^+$ T cell responses in melanoma patients than vaccines using Montanide alone[55]. Similarly, a randomized open-label phase II study using a recombinant MAGE-A3 fusion protein and two adjuvant systems clearly revealed enhanced vaccine efficacy for the adjuvant formulation

that contained CpG-oligodeoxynucleotides, suggesting superiority of added TLR9 stimulation in the induction of antitumor immune responses[56].

An intriguing possibility in polypeptide vaccine formulation is the combination of two or more TLR agonists which would be expected to enhance the accompanying innate immune cell activation and hence the immunogenicity of the vaccine. A rationale for combination of TLR agonists stems from the obvious fact that most pathogens bear several TLR agonists, although such a rationale has less meaning in cancer vaccine development. Moreover, different TLR-triggered signalling pathways engaging MyD88 or TIR domain-containing adapter molecule 1 (TRIF) might synergise. Indeed, it was reported that in both human and mouse dendritic cells, TLR3 and TLR4 did synergise with TLR7, TLR8 and TLR9 in inducing the transcription of selected sets of genes and production of cytokines such as IL-12 and IL-23 leading to enhanced and sustained Th1 immune responses[57]. Using a human immunodeficiency virus envelope peptide vaccine, it has been shown that the triple combination of TLR2/6, TLR3 and TLR9 led to induction of protective immunity which correlated not with increased frequency of specific $CD8^+$ T cells but with increased avidity for antigen reactivity and increased IL-15 production by activated dendritic cells[58]. However, not all combinations are synergistic for immunogenicity and can actually lead to enhanced immunoregulation[59]. Thus, TLR agonist combinations must be carefully designed and tested. Another strategy to marry antigen and TLR ligands for potent vaccination is the covalent linking of antigenic peptides to TLR ligands. Such conjugates, particularly to TLR7 agonists, have shown promise in various preclinical models of peptide or protein vaccination[60–62].

5.1.7 Cytokines and Chemokines

An important adjuvant effect of those PRR-targeting adjuvants is the induction of immune stimulatory cytokines. Therefore, more direct approaches to enhance immunogenicity of peptide vaccines could be the use of recombinant cytokines as adjuvants[63]. Studies testing combinations of cytokines with tumor-associated antigens to booster antigen-specific immunity revealed that IL-2 improved vaccination outcomes and led to objective cancer regressions[64–66]. In contrast to IL-2, systemic administration of granulocyte-macrophage colony-stimulating factor (GM-CSF) or IL-12 did not induce clinical responses in the same clinical center[65]. However, IL-12 should not be administered locally due to its instability and serious systemic toxicity, and different delivery strategies have been tested[67]. In different phase I clinical trials in patients with metastatic melanoma, the direct delivery of IL-12 to the tumor resulted in lymphocytic infiltrates, marked tumor necrosis and a decrease of the treated lesion up to complete regression of all metastases[68,69]. It is therefore likely that the route of administration is critical for the outcome of the adjuvant efficacy and requires optimization in order to evaluate the potential of each adjuvant to break immune tolerance. Finally, GM-CSF has the potential to recruit dendritic cells, and clinical data argue for the continued development of these cancer vaccines[70].

The use of high-dose IFN-α2b also been approved for the adjuvant therapy of melanoma. The efficacy of the treatment remained controversial until more detailed analysis revealed a survival benefit in patients with ulcerative melanoma[71].

5.1.8 Tensoactive Adjuvants

Saponins are secondary metabolites particularly found in plants. They are tensoactive (meaning that they can change surface tension) and can enhance the induction of strong antibody and CTL responses[72]. Quil-A saponins extracted from the *Quillaja saponaria* tree induce strong cellular responses but have significant toxicity. Therefore, derivatives with less toxic properties have been isolated and include QS-21 and ISCOMATRIX. The latter are immunostimulating complexes of cholesterol, phospholipids and Quil-A fractions that assemble into nanospheres of around 40 nm, and can be combined with non-hydrophobic antigens for slow and constant release. They are being used in NY-ESO-1 cancer vaccines in phase II clinical trials[73,74].

In contrast to infectious or cancer diseases, many autoimmune diseases or allergies are characterized by an over reactive set of immune cells. Major efforts to induce tolerant immune responses by vaccination have been made, and those results represent an equally important source of knowledge for the design of vaccines that could induce immune surveillance. For example, successful conversion of naïve $CD4^+$ T cells into Tregs is achieved via suboptimal activation of dendritic cells during continuous exposure to sub-immunogenic levels of antigen[75,76]. TGF-β exposure during stimulation further decreased T cell proliferation, correlating with an increased Treg conversion rate and absence of autocrine IL-2 signalling.

In peptide vaccination for cancer immunotherapy, it is therefore essential to achieve full dendritic cell activation by choosing a powerful adjuvant and by applying optimal antigen dose with the right timing to achieve full T cell proliferation and differentiation into effector T cells. Many peptide vaccination trials have aimed at inducing such T cell responses in the past, and the subsequent section will discuss the promises and pitfalls from those studies in order to highlight improvement strategies to be investigated in the future.

5.2 Clinical Experience from Molecularly Defined Peptide/Protein Vaccines

5.2.1 Antigen Dosage and Vaccination Schedule

The success of vaccination depends on the choice of the target antigen, the right combination of the adjuvant(s), and the regimen of immunization[77]. Since the exploration of newly available and molecularly defined adjuvants has only begun

recently, many past clinical trials were based on the formulation of the target antigen in mineral oil solutions. In addition, most of those studies were done in patients suffering from metastatic melanoma due to the lack, until recently, of therapeutic options for this aggressive cancer. Additionally, melanoma is one of the most immunogenic tumors, and immunotherapy represents a promising strategy for its treatment. This now allows a fairly good comparison of previous clinical trials to evaluate factors that contribute to successful immunization. Many early vaccination trials have used an analogue gp100 (amino acids 209–217) immunodominant $CD8^+$ T cell epitope designed for better binding to HLA-A2 molecules for peptide vaccination. Those studies have shown that an increase of the antigen dosage directly correlated with higher immunological response rates and higher frequency of circulating, peptide-specific $CD8^+$ T cells. Comparisons with other studies using the Melan-A 26–35 27L peptide analogue revealed a plateau for such a dose response at around 0.1 mg peptides per cycle[64]. Concerning the vaccination regimen, trials comparing a 2- and 3-week injection cycle gave similar results[78].

5.2.2 Recombinant Proteins and Synthetic Peptides

Successful induction of T cell responses requires stable presentation of T cell epitopes on MHC complexes. The antigen of interest can be introduced by vaccination with the whole protein, or with short or long synthetic peptides derived from this protein. Figure 5.1 illustrates the possible outcome of an immune response using either of those formulations. The advantage of vaccination with whole tumor proteins is not only that they could provide peptides for binding to a large array of MHC allelic products therefore available to virtually all patients but also that no prior knowledge of T cell epitopes is required (Figure 5.1a). Whole protein vaccines have been successfully applied to generate immune responses leading to the induction of neutralizing antibodies, for instance, against the tetanus, pertussis and diphtheria toxoids[79]. Protein antigens have also shown promise when used in cellular-based vaccines. A recent clinical trial in hormone refractory prostate carcinoma patients receiving autologous blood lymphocytes enriched in antigen-presenting cells loaded with a fusion protein of recombinant prostatic acid phosphatase and human GM-CSF (called sipuleucel-T) resulted in an increased survival of the patients[80]. Its efficacy in reducing the risk of death in men with metastatic castration-resistant prostate carcinoma was confirmed in a second randomized phase III trial, IMPACT[81]. These studies led to the approval of the first therapeutic cancer vaccine by the US Food and Drug Administration in 2010.

However, a shortcoming with the use of protein-based therapeutic vaccines is that they mainly induce responses to dominant epitopes, resulting in a rather narrow T cell response. Analysis of T cell responses to protein-derived tumor antigens can give important information on the dominance of certain epitopes. Processed peptides with higher affinity for the MHC molecule might be preferentially loaded onto MHC molecules and persist as long-lived complexes on the cell surface due to

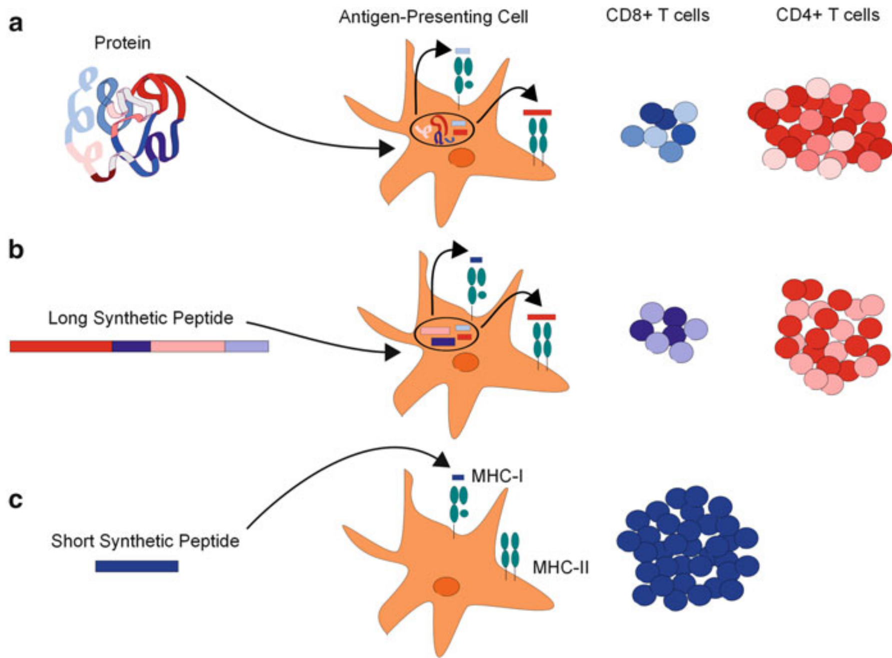

Fig. 5.1 Differential outcomes in epitope-specific CD8$^+$ and CD4$^+$ T cell responses to therapeutic vaccination either with whole proteins, long synthetic peptides or short synthetic peptides. **(a)** Tumor proteins include numerous CD8$^+$ (blue) and CD4$^+$ (red) T cell epitopes. Vaccination with a whole protein induces a weak antigen-specific CD8$^+$ T cell response, and a larger CD4$^+$ T cell response. Even though proteins induce mainly immunodominant responses, they have the capacity to induce CD4$^+$ T cells with specificity for multiple epitopes. The poor efficiency of antigen uptake, processing and MHC-loading by the antigen presenting cells limits the magnitude of the resulting immune response. **(b)** Long synthetic peptides of a defined sequence within the protein may already contain several CD8$^+$ and CD4$^+$ epitopes. These peptides also require optimal uptake, processing and MHC-loading of the appropriate T cell epitopes in order for an effective immune response to be generated. The adjuvants and formulations that are currently used in long synthetic peptide vaccines often result in a good CD4$^+$ T cell response but in a poor CD8$^+$ T cell response, suggesting a failure to induce efficient processing and cross-presentation of CD8$^+$ T cell epitopes. **(c)** Short synthetic peptides generally contain a single immunodominant CD8$^+$ T cell epitope, which can bind directly to MHC-I on the antigen presenting cell surface, without requiring phagocytosis and entry into the cross-presentation pathway. Short peptide vaccines thus trigger robust specific CD8$^+$ T cell responses but generally lack the ability to generate a CD4$^+$ T helper cell response. The process of antigen uptake into intracellular vesicles by antigen-presenting cells, followed by processing and loading on to MHC-I and MHC-II versus direct antigen loading on to MHC molecules on the cell surface is depicted in the middle section of the figure.

their higher stability and thus give rise to immunodominant T cell responses. However, T cells specific to subdominant epitopes have also been shown to participate in antitumor immune responses[82]. For CD4$^+$ T cells, immunodominance is thought to be due, at least in part, to early loading of the preliminarily processed, only partially unfolded protein to the MHC class II complex, followed by an extensive proteolytic trimming. If the peptide binding at this point remains stable,

the MHC complex remains loaded, and presentation of late processed peptides becomes less prominent[83].

Induction of $CD8^+$ T cell responses to soluble proteins may be variable and generally weak. A reason for this is the requirement for efficient uptake and cross-presentation by dendritic cells. Generally, only particulate antigen (e.g. dying cells or proteins adsorbed to inert particles) is efficiently taken up by phagocytosis or macropinocytosis and cross-presented on MHC class I molecules[84,85]. Therefore, vaccination of soluble proteins without any protein-carrier system is likely suboptimal for induction of CTL responses. The capability of cross-presentation further varies among different dendritic cell types, and recent studies provide evidence that only the $CD141^+$ conventional dendritic cell subset is superior in cross-presenting soluble protein antigen in comparison to the other $CD1c^+$ conventional dendritic cells, $CD16^+$ conventional dendritic cells and $CD304^+$ plasmacytoid dendritic cell subsets[86,87]. Despite these difficulties, there are promising protein vaccines currently being tested in advanced phase III clinical trials, such as the recombinant MAGE-A3 vaccine in combination with a liposomal formulation containing monophosphoryl lipid A, QS21 and CpG[77]. Vaccination of recombinant NY-ESO-1 with ISCOMatrix adjuvant has also proven to induce potent concomitant $CD4^+$ and $CD8^+$ T cell responses[73]. In addition, approaches are being tested to increase protein translocation directly to the cytoplasm. Membrane-traversing peptides or toxin subunits are eligible as vehicles with such shuttling properties. Covalently linked proteins would thereby directly be guided into the MHC class I-processing compartment and would circumvent the cross-presentation pathway[88,89].

Most early peptide vaccines tested in the clinic were composed of synthetic peptides covering exact epitopes recognized by $CD8^+$ T cells. They have the advantage that they do not require further processing by antigen-presenting cells and can be efficiently presented to $CD8^+$ T cells (Figure 5.1c). Numerous clinical trials using melanoma-derived antigens led to the induction of peptide-specific T cell responses that correlated with local skin reactions but improved the clinical outcome in only a small fraction of patients[90]. Higher immunogenicity and correlation between immune responses and clinical outcome was observed when melanoma patients were vaccinated with a peptide pool containing 12 peptides from melanocytic differentiation proteins and cancer-testis antigens[91]. This approach would further prevent the selection of escape variants through antigen loss. Higher response rates were also achieved by using peptide analogues with improved MHC class I-binding properties. For example, the A27L substitution in the Melan-A peptide 26–35 leads to one $\log_{10}$ improved binding affinity to HLA-A2, and two to three $\log_{10}$ gain in relative antigenic activity[92]. However, a recent study reported on the expansion of a more restricted T cell pool with a more terminally differentiated phenotype when vaccination was performed with the peptide analogue. Most important, only about half the induced T cells recognized tumor cells efficiently. In comparison, vaccination with the native peptide only recruited $CD8^+$ T cells with fully tumor-reactive TCRs. These $CD8^+$ T cells also exerted stronger effector functions and displayed a more polyfunctional phenotype[24]. Therefore, higher T cell responses might not necessarily confer better

antitumor functions. Nevertheless, the first clinical trial that reported on a survival benefit for melanoma patients was performed using a gp-100 peptide analogue in combination with high-dose IL-2. Progression-free survival was significantly higher in comparison to treatment with high-dose IL-2 alone[93].

Even though these results encourage the use of peptides defined by minimal T cell epitopes, those short synthetic peptides may have important drawbacks. Because they do not require processing by antigen-presenting cells, they can associate with MHC class I complexes on the surface of all nucleated cells in the body[94]. However, only properly activated dendritic cells express the necessary co-stimulatory receptors to activate naïve T cells. For those reasons, exogenously loaded minimal peptides have repeatedly been shown to confer immunological tolerance rather than immunity[95,96] if they are displayed in the wrong immunological setting. The injection of minimal epitope peptides in IFA or Montanide without other immunostimulatory adjuvants might further tolerize peptide-specific T cells over a long period of time due to the depot effect of those emulsions[97]. However, addition of strong adjuvants like CpG-oligodeoxynucleotides has been shown to increase immunogenicity significantly[55]. Another study compared vaccination of a minimal HPV16-derived $CD8^+$ T cell epitope with a longer peptide containing the same epitope. Higher immunogenicity of the longer peptide was observed even in MHC class $II^{-/-}$ and $CD40^{-/-}$ mice, precluding that more robust CTL responses are due to induction of T cell help from additional helper epitopes in the long peptide. These studies proposed that longer peptides induce better CTL responses, presumably because they are preferentially endocytosed, processed and presented by professional antigen-presenting cells[98].

Because of the self-antigenic nature of most tumor peptides, it was soon recognized that concomitant induction of T cell help potentially improves the differentiation and function of vaccine-primed CD8 T cells. Studies in mice have shown that inclusion of helper T cell epitopes increased $CD8^+$ T cell responses, but most importantly, coupling of the helper peptide with the minimal $CD8^+$ T cell epitope led to even stronger responses[99]. Such results not only encouraged the inclusion of $CD4^+$ T cell epitopes but at the same time the use of longer peptides that encompass both $CD4^+$ and $CD8^+$ T cell epitopes (Figure 5.1b). The advantage of using long synthetic peptides is that they have the capacity to induce immune responses to subdominant epitopes, which are not induced when vaccinating with short peptides covering exact epitopes or with proteins[97]. However, studies have shown that T cells specific to subdominant epitopes also contribute in anti-tumor immune responses[82]. Therefore, the broadest variety of T cell specificities and the best anti-tumor immunity should be generated when vaccinating with a pool of long synthetic peptides covering the entire protein sequence. Several studies in clinical trials have confirmed those conclusions. Vaccination of melanoma patients with six previously described helper peptides from MAGE and melanocytic differentiation antigens encouraged the use of $CD4^+$ T cell epitopes, since a phase I/II trial has shown early evidence of clinical activity[100]. Several other studies using long peptides harboring both $CD4^+$ and $CD8^+$ T cell epitopes reported promising immunological and clinical results. We also refer to an excellent review on the advantages of such long peptides for vaccination[97].

Vaccination of ovarian carcinoma patients with a NY-ESO-1 14-mer peptide containing both $CD4^+$ and $CD8^+$ T cell epitopes and vaccination of both ovarian and colorectal carcinoma patients with a set of 10 overlapping peptides spanning amino acids 70–248 of the p53 peptide resulted in long-lasting T cell responses between 6 and 12 months after vaccination[101–103], in line with the paradigm that $CD4^+$ T cell help is important during $CD8^+$ T cell priming and memory formation[104,105]. Even though these trials did not report on a clinical benefit, other trials that induced comparable long-term circulating T cells could show a direct correlation with a survival benefit for the patients. For example, inoperable stage III non-small cell lung cancer patients were vaccinated after chemoradiotherapy with a 16 amino acid long peptide derived from telomerase reverse transcriptase, which binds several MHC class II molecules and contains nested putative HLA class I epitopes. In an early phase I/II clinical trial, 13/24 patients developed an immune response against the peptide, and median survival of those responders was 19 months versus 3.5 months in immune nonresponders. Two patients showed complete regression. All long-term survivors displayed durable T cell responses with a polyfunctional cytokine profile that was IFN-γ dominated. A subsequent phase II clinical trial confirmed those response rates[106]. Other encouraging results are reported from a phase IIB randomized, multicenter, open-label trial with stage IIIB or IV non-small cell lung cancer patients receiving a 25 amino acid long MUC-1-derived peptide-liposome vaccine. The median survival time was increased 4.2 months in comparison to non-vaccinated patients. A subgroup of those patients with stage IIIB locoregional disease benefitted most from the treatment, since their median survival time was 17.3 months longer[107]. The pivotal phase III clinical trial with this vaccine is underway[108].

Other studies have used long overlapping peptides that cover the entire target protein. This approach not only ensures maximal MHC class I and II epitope content but also provides the same advantage of recombinant proteins that can be administered to all patients independent of their MHC haplotype. This was explored in a peptide vaccination study in women with premalignant vulvar lesions using a 30-mer peptide pool covering the entire HPV16 E6 and E7 oncoproteins. Broad immune responses were observed in 19/20 patients, and excellent immune memory was confirmed during 2 years of follow-up. Vaccination-induced complete regression in half of the patients [109] and detailed analysis revealed that those regressions were mostly observed in patients with smaller lesions. These patients displayed stronger and broader T cell responses and lower induction of HPV16-specific Tregs. Interestingly, those correlations are only valid when the size of the lesion, but not the duration of disease, was considered[110].

These results indicate that intervention via peptide vaccination at early stages of disease leads to best clinical results. Once a tumor accumulates escape mechanisms and disease progresses, peptide vaccination alone might not be sufficient to break immune tolerance and reconstitute tumor control. Additional approaches for targeting and neutralizing an immune-hostile tumor microenvironment and depleting tumor-tolerating immune cells might be necessary to allow vaccine-induced T cells to re-establish immune surveillance. Combination strategies for immunotherapy are addressed in more detail in chapters 13 and 14.

5.3 Future Directions for Peptide Vaccination Approaches

5.3.1 Prime-Boost Vaccines

Peptide analogues have proven to have strong potential in the induction of more robust T cell responses. However, one of their drawbacks is that cross-reaction with the native peptide and therefore tumor recognition can be significantly reduced[24,111]. Since T cells induced upon vaccination with native peptides retain the ability to cross-react with their peptide analogue counterparts, a possibility would be to prime and boost with different peptides. During priming with the native peptide, a large pool of high-affinity TCRs would be selected, and boosting with the peptide analogue would drive the efficient expansion and differentiation of already primed, tumor-reactive T cells. Moreover, short synthetic peptides mimicking strong MHC class I-restricted $CD8^+$ T cell antigens could be used to prime responses that could then be subsequently boosted by immunization with the parental recombinant protein.

5.3.2 Dendritic Cell Targeting

Approaches to ensure loading of peptides exclusively on antigen-presenting cells might rely on the coupling to antibodies that target receptors selectively expressed by dendritic cells. Several members of the C-type lectin receptor family fulfil these criteria. For example, targeting of DC-SIGN via antibodies specific to its neck region led to clathrin-independent internalisation, routing into early endosomes and delayed lysosomal degradation. Cross-presentation of protein antigens conjugated to those antibodies was approximately three orders of magnitude higher[112]. Similar results were obtained via targeting of DEC205, a dendritic cell-restricted endocytic receptor. However, both methods require additional dendritic cell activation to circumvent tolerance induction. CD40 ligation of targeted dendritic cells induced prolonged T cell activation and immunity[113], showing that dendritic cell targeting is a promising tool for using short peptides in combination with helper peptides and strong adjuvants. Dendritic cell targeting by viral vectors is extensively addressed in chapter 6.

A different approach to ensure uptake of peptide antigens by professional antigen-presenting cells is their linkage to small particles, such as virus-like particles that represent another delivery system for vaccine antigens and can incorporate adjuvant properties. The adjuvant effect of virus-like particles is derived from the geometrical configuration of the particle, ensuring that the antigen is displayed in a highly ordered structure, thereby facilitating the efficient activation of B cells through cross-linking to the B cell receptor[114]. Great success with virus-like particles as delivery forms for vaccine antigen was achieved with the recently approved human papilloma virus vaccines, in which the recombinant HPV L1

capsid proteins themselves assemble into virus-like particles. However, virus-like particles not formed by the vaccine antigen are themselves antigenic and can confer immunity against themselves. However, studies suggested that induction of virus-like particle-specific CD4$^+$ T cells equally provide help to tumor-specific CD8$^+$ T cells during priming and thereby enhance CD8$^+$ memory formation[115]. In line with this concept, tumor-unrelated CD4$^+$ helper peptides have been shown to be as efficient as strong, tumor-related CD4$^+$ helper peptides in improving vaccine-induced immune responses in mice[116]. In randomized clinical trials, vaccination with a tetanus helper peptide or a mixture of six tumor-associated helper peptides concomitant to tumor-related CD8 peptides showed higher immunogenicity of the tetanus peptide combination[117]. Virus-like particles should also be considered as powerful antigenic carriers because vaccine antigens displayed on their surface are of similar immunogenicity than the virus-like particles themselves[118]. Virus-like particles are therefore capable of inducing immune responses against self-peptides even in the absence of common adjuvants[119–121]. Besides inducing potent antibody responses, recent data provides evidence that virus-like particles in conjugation with A-type CpG-oligodeoxynucleotides are capable of inducing robust Th1 biased CD4$^+$ T cell responses, as well as priming of central memory CD8$^+$ T cells[115,122].

5.3.3 *Induction of T Cell Help*

Vaccination with long peptides in the absence of T cell help provides evidence that longer peptides are more efficiently endocytosed and selectively presented by professional antigen-presenting cells, thereby precluding induction of tolerance[98]. By providing MHC class I and class II epitopes in the same peptide, antigen-presenting cells recruit CD4$^+$ and CD8$^+$ T cells at their surface. T cell help is then provided to CTLs via distinct pathways. First, interaction via CD40–CD40L ensures full activation of the dendritic cells which is necessary for optimal CD8$^+$ T cell priming[123]. Second, activated CD4$^+$ T cells release the chemokines CCL3 and CCL4, which play important roles in the recruitment of naïve CD8$^+$ T cells to the antigen priming site, and contribute to enhanced expression of IL-6Rα and IL-7Rα, thereby enhancing T cell survival and promoting memory formation[124,125]. As a consequence, the three cell interaction model in which an antigen-presenting cell interacts with CD4$^+$ and CD8$^+$ T cells at the same time becomes more probable. Third, in this way, CD4$^+$ T cells can further provide IL-2 to neighboring CD8$^+$ T cells, which further enhances their effector function. Besides their role in priming and memory formation of CD8$^+$ T cells[104,105], CD4$^+$ T helper cells were also recently shown to be implicated in CTL homing to the tissue of interest[126,127]. Importantly, this was even true for low-avidity CD8$^+$ T cells that are commonly found among tumor-specific CTLs[128]. The mechanism of action for this recruitment was dependent on IFN-γ secretion by activated Th1 cells entering the tissue, which in turn induced the production and secretion of various CTL-attracting chemokines, including CXCL9 and CXCL10, by resident epithelial cells. It is therefore highly

recommended to include strong tumor-specific $CD4^+$ T cell epitopes in future vaccination strategies in order to endow T cells with the capacity to home to the tumor where they need to execute their effector function. Long synthetic peptides have already shown to harbour such potential.

5.3.4 Peptide-Adjuvant Fusion Constructs

Another factor that is crucial for induction of potent T cell responses is the optimal formulation with adjuvants in order to trigger dendritic cell activation and display the low immunogenic tumor antigen with a strong danger signal. A clever innovation in the design of peptides for vaccination was the coupling of the peptide directly to the adjuvant to ensure activation of those antigen-presenting cells that process and present the vaccine peptide. Studies in mice have demonstrated that peptides coupled to TLR ligands, to CpG or bacterial lipopeptides, are not only efficiently taken up by professional antigen-presenting cells but that induction of CTL responses was comparable to live vaccines[129–131]. Combinations of such fusion constructs with peptides or proteins that contain additional T helper epitopes further supported the cytolytic activity of primed CTLs[132]. This is consistent with the fact that antigen-presenting cell activation via TLR triggering and CD40 ligation act synergistically[57]. Interestingly, the recently FDA-approved sipuleucel-T treatment of prostate cancer patients support the combination of such constructs. The vaccine consists of the tumor antigen prostatic acid phosphatase physically linked to GM-CSF and is administered after ex vivo loading onto autologous peripheral blood mononuclear cells[133].

5.3.5 Lymphodepletion and Regulatory T Cell Targeting

Even though the inclusion of $CD4^+$ T cell epitopes in peptide vaccines seems promising for many reasons, the concomitant induction of Treg responses during later stages of disease[110] emphasises the requirement of strategies to prevent priming of Tregs. Cyclophosphamide is a chemotherapeutic agent that is commonly used in combination with other drugs to treat lymphomas, leukaemias and some solid tumors by inhibiting tumor cell growth[134,135]. At high doses, it is linked to cytotoxicity of rapidly dividing cells and can thus result in immunosuppression. Low-dose continuous or metronomic administration of cyclophosphamide, in contrast, has immunostimulatory properties[136]. Some clinical observations might reflect this property. The use of a single dose of cyclophosphamide before MUC-1 vaccination resulted in successful clinical outcomes[107], and its use in combination with p53-SLP vaccination in recurrent ovarian cancer led to enhanced IFN-γ-producing p53-specific T cells in comparison to the same immunization without cyclophosphamide treatment[137]. The mechanisms behind the immune-enhancing effects of cyclophosphamide are not fully

understood, but it has been shown that treatment with repeated low doses of cyclophosphamide selectively depletes Tregs[138–140] and leads to improved maturation of dendritic cells and proliferation of effector T cells[141,142]. In a mouse model of therapeutic lentivector immunization against tumor self-antigens combined with cyclophosphamide treatment, it was shown that combination therapy led to improved tumor protection and to an increase in the presence of TILs with enhanced survival and function compared to the effect of vaccination alone. This outcome was similar to that achieved by combining immunization with antibody blockade of the PD-L1/PD-1 inhibitory pathway[143]. Cyclophosphamide used in combination with exosome-based dendritic cell vaccines was able to overcome suppression by $CD4^{+}CD25^{+}Foxp3^{+}$ Tregs, as well as enhancing the memory response induced by vaccination, leading to increased tumor protection in a therapeutic mouse model[144]. Additionally, repeated, low-dose cyclophosphamide therapy was shown to deplete $CD4^{+}CD25^{+}$ Tregs selectively and reduce their suppressive function in end-stage cancer patients, leading to recovery of function by effector T cells and NK cells[138].

These data support the role of cyclophosphamide in reducing tumor immunosuppression mediated by Tregs and thus contributing to the enhancement of the vaccination-induced antitumor effects of CTL. However, no differences in clinical response rates to secondary chemotherapy or in disease-specific survival were observed between immunized patients and historical controls when cyclophosphamide was used in combination with therapeutic cancer vaccination[117,145], and other strategies are therefore needed to improve the efficacy of cancer vaccines in the clinic.

5.4 Modulating the T Effector/Regulatory T Cell Ratio Through Vaccination with Molecularly Defined Adjuvants

Cancer vaccines can concomitantly induce the expansion of Tregs[146], as well as activating effector T cells. Indeed, as described earlier, the promise held by the inclusion of $CD4^{+}$ T cell epitopes and the fusion of adjuvants to peptide vaccines is dampened by the concomitant induction of Treg responses during later stages of disease[110]. This emphasizes the requirement for strategies to prevent priming or expansion of Tregs. One way to address this problem might be to take a more focused approach aimed at optimizing the use of molecularly defined adjuvants in the vaccine formulation. Dendritic cells matured with the yeast *Saccharomyces cerevisiae*, followed by coculture with naïve $CD4^{+}$ T cells, result in a reduction in the number and function of Tregs and enhance the ratio of antigen-specific effector to Tregs. The ensuing effector T cell population produces increased levels of Th1-associated cytokines and proliferates more vigorously in response to the tumor antigen CEA[147]. This supports the concept that given the right inflammatory stimulus or "danger signal", the balance of effector and regulatory cells can be regulated. More precise and well-defined adjuvants, including TLR ligands,

have been shown to enhance the effects of vaccination by stimulating murine dendritic cells to produce inflammatory cytokines and enhancing Th1 and CTL responses[148–150].

More important, clinical data in support of the role of TLR ligands in controlling the tumor antigen-specific T effector to Treg balance also exist from the study of advanced metastatic melanoma patients vaccinated with Melan-A peptide in combination with Montanide and CpG-oligodeoxynucleotides[151]. The proportion of $Foxp3^+$ Tregs among the total $CD4^+$ T cell pool was comparable to that of healthy donors and remained unchanged following vaccination. In contrast, analysis of the Melan-A25-36-specific $CD4^+$ T cells in these patients revealed that the proportion of $Foxp3^+$ cells within this population was extremely high before treatment (20–75%). However, following multiple rounds of vaccination, the percentage dropped to ~10 %. A concurrent rise in the frequency of $CD8^+$ effector T cells meant that the antigen-specific effector T cell to Treg ratio was dramatically increased. The proliferative potential and effector function of both the vaccination antigen-specific $CD8^+$ and $CD4^+$ T cells was also enhanced. Additionally, a study of the antigen specificity of effector and Tregs in colorectal carcinoma patients showed that Tregs were specific for only a small set of tumor antigens and that there was a degree of cross-over with the antigen specificity of the effector T cell population. This provided further evidence that Tregs suppress effector T cells in an antigen-specific manner. This was confirmed by the in vitro stimulation of patient T cells with autologous dendritic cells loaded with long synthetic peptides derived from tumor antigen. The outcome of Treg depletion in this system was that proliferation of tumor-specific memory T cells was only enhanced when the stimulating dendritic cells were loaded with peptides for which there was shared specificity between effector and Tregs[152].

These observations have prompted the suggestion that the balance of Tregs to effector T cells might not only dictate the success or failure of antitumor immunity[153,154] but that it might also be a useful predictive biomarker of the clinical efficacy of cancer vaccines. Experimental and clinical data have already shown a link between the natural effector to Treg ratio and disease outcome. In a chemically induced mouse tumor model, high proportions of proliferating $Foxp3^+$ Tregs found in certain tumors were shown to correlate with tumor progression, despite the presence of activated $CD8^+$ effector T cells. Nonprogressing tumors also developed in the same system contained equivalent numbers of $CD8^+$ T cells but were characterized by the presence of fewer Tregs, and consequently the antitumor immune response could be predicted to have a successful outcome[197]. In several different types of human cancer, increased numbers of $CD4^+Foxp3^+$ TILs have also been associated with poor prognosis[155–161]. Conversely, it was reported that patients with a higher tumor-infiltrating $CD8^+$ to $CD4^+Foxp3^+$ T cell ratio in tumors such as cervical cancer, ovarian cancer, cutaneous T cell lymphoma and lung cancer displayed an improved prognosis[162–166]. In fact, the balance between tumor-infiltrating $CD8^+$ and $CD4^+Foxp3^+$ T cells has been proposed as an independent prognostic factor in hepatic carcinoma[167,168], Hodgkin lymphoma[169] and cervical cancer[163].

Experimental data from mouse disease models also supports the importance of the antigen-specific effector to Treg ratio in controlling the functional outcome of immune responses. In a model of OVA-specific T cell-mediated skin-graft rejection, immunotherapy with anti-CD154 (CD40L) blocking antibodies induced the development of OVA-specific Foxp3$^+$ Tregs and delayed the activation of multi-cytokine-producing CD8$^+$ and CD4$^+$ effector T cells resulting in graft tolerance[170]. In a mouse model of multiple sclerosis (experimental allergic encephalomyelitis), immunization with myelin oligodendrocyte glycopeptide (MOG$_{35\text{-}55}$) induces central nervous system disease. This type of immunization has been shown to induce MOG-specific Foxp3$^+$ Tregs as well as the CD4$^+$ effector T cells that mediate disease. At the peak of the disease phase, MOG-specific effector cells greatly outnumbered Foxp3$^+$ Tregs and developed polyfunctional effector activity. However, after the peak of the response, effector cells were reduced in number and function, while Treg numbers remained stable. This resulted in a marked reduction in the effector to Treg ratio and was accompanied by increased IL-10 production and disease recovery[171].

Controlling the presence and/or function of Tregs has therefore become a key goal in the advancement of cancer immunotherapy strategies. It has been clearly demonstrated that the ratio of effector to Tregs is a strong prognostic indicator of cancer progression in many different types of cancer. Additional experimental evidence exists supporting a link between positive regulation of the effector to Treg balance, the enhancement of polyfunctional effector T cell responses and disease outcome following the immunotherapy of other diseases. Further research is necessary to confirm that this will also be a reliable biomarker to use for monitoring the clinical efficacy of new and existing cancer vaccines, targeted at controlling the balance between the tumor-specific effector and regulatory subsets, which has emerged as being a critical factor in the success of antitumor immunity. See chapter 9 for additional Treg details including strategies to control them therapeutically in cancer.

5.4.1 Manipulation of T Cell Memory

Besides the induction of neutralizing antibodies, a powerful vaccine has to evoke a strong T cell response. The latter is obtained by generating long-lived memory CD8$^+$ T cells with enhanced antitumor activity. Therefore efforts must be made to develop vaccines which raise the quantity as well as the functional quality of memory CD8$^+$ T cells[172]. For this purpose, the inhibition of mTOR by rapamycin seems to be a promising strategy. Rapamycin is a drug used to avoid transplant rejection and in cancer therapy, and was initially considered strictly immunosuppressive. Paradoxically, rapamycin has been shown to increase the quantity and functional qualities—like proliferative ability, protective power and longevity—of virus-specific memory CD8$^+$ T cells in mice and *rhesus* macaques. Rapamycin increased the number of memory precursor T cells in the expansion phase and

advanced memory CD8$^+$ T cell differentiation in the contraction phase during primary and secondary T cell responses[173].

These findings suggest the use of rapamycin as an innovative vaccine adjuvant. In contrast to currently used adjuvants, rapamycin acts by inhibition of the intracellular kinase mTOR in antigen-specific CD8$^+$ T cells. Synergistic effects might be achieved by the combination of rapamycin and adjuvants that stimulate dendritic cells and act through TLRs. A further advantage of rapamycin is its oral application form which would not interfere with vaccine formulations. Future issues regarding the use of rapamycin as a vaccine adjuvant will have to address the optimal dose to achieve maximum immunostimulatory and minimum immunosuppressive effects. Furthermore, efforts will have to be made to identify other signalling pathways in memory CD8$^+$ T cell differentiation that could function as additional pharmacological targets to improve vaccine efficacy[172,174].

5.4.2 Combinatorial Therapies Using Small Molecules and Antibodies

Each tumor accumulates oncogenic mutations during carcinogenesis, some of which can be specifically targeted with small molecules or blocking antibodies. Several compounds have been FDA approved recently, and combination with peptide vaccination seems promising to enhance the antitumor response. Sunitinib is a small molecule that targets multiple receptor tyrosine kinases, including those for platelet-derived growth factors and vascular endothelial growth factors that are implicated in tumor angiogenesis and proliferation. Indications include metastatic renal cell carcinoma and gastrointestinal stromal tumors. Recent studies suggest that sunitinib can decrease myeloid-derived suppressor cells and Tregs[175,176], thereby decreasing local suppressive mechanisms at the tumor site. Disruption of such tumor-driven dysfunctional immune responses might be necessary for paving the way for tumor-specific T cell therapies, since T cells develop exhausted phenotypes in such suppressive microenvironments[177].

The protein kinase B-RAF is one of the most frequently found mutated kinases in human cancers. About 50% of melanoma tumors harbour a V600E oncogenic mutation. Treatment with a specific inhibitor, PLX4032 (vemurafenib), improved rates of overall and progression-free survival of metastatic melanoma patients[178]. Treatment results in fast shrinkage of melanoma tumor masses in approximately half of the patients, but relapses occur frequently because of rapid establishment of tumor resistance. Therefore, combinations with other treatment approaches become necessary. Concomitant peptide vaccination would have the advantage of generating tumor-specific T cells that could infiltrate the tumor and eliminate residual tumor cells that cannot be targeted with the drug. Because inhibition of mutated B-RAF leads to rapid tumor destruction, major changes in the tumor

microenvironment are likely to occur, and local suppressive mechanisms would be disturbed. Massive tumor cell death possibly also leads to exposure of new tumor proteins and epitope spreading. Therefore, the reinforcement of T cell responses with vaccines during and after B-RAF treatment could have a great potential to re-establish tumor immune control.

Recent studies have shown that functional T cells are not only dependent on cell intrinsic, but also extrinsic factors. A most efficient anti-tumor immune response is likely achieved by targeting multiple factors in a deregulated tumor-microenvironment, as illustrated in Figure 5.2.

CTLA-4 is a receptor expressed on recently activated T cells that mediate inhibitory signals, and CTLA-4 in Tregs is important for their function. Mouse models have suggested that the enhancement of specific $CD8^+$ T cell responses upon anti-CTLA-4 blockade generates tumor protection in combination with vaccination[179]. Thus a major focus has been the design of anti-CTLA-4 blocking antibodies for human use. The humanized antibody ipilimumab has successfully passed clinical development where it showed improvement of overall survival in metastatic melanoma[180] and is now FDA approved as second line treatment of advanced metastatic melanoma. Treatment with anti-CTLA-4 antibody generates nonspecific activation of immune stimulatory mechanisms that modify the function of a large set of immune cells. It therefore also has potential to disrupt established tumor microenvironments, and peptide vaccination approaches might have enhanced potential in re-establishing protective immunity. A limited number of recent clinical studies have started to explore the effect of CTLA-4 blockade on the formation of effector and memory T cells upon vaccination[181–183]. The results, although encouraging, suggest the need to define the timing of CTLA-4 blockade carefully relative to immunization schedules and to monitor many parameters of the immune response.

PD-1 is another co-inhibitory receptor that is expressed upon T lymphocyte activation. Studies in mouse and human clinical settings of chronic viral infection have revealed its association with functionally exhausted T cells[184,185]. Significant evidence has accumulated that PD-1 blockade can reverse T cell dysfunction not only in established chronic viral infections such as in acquired immunodeficiency syndrome and chronic hepatitis due to hepatitis C virus infection but also in metastatic tumors[186,187]. A humanized blocking anti-PD-1 antibody has already been tested in phase I clinical trials in cancer patients, and encouraging results in terms of clinical responses in cancer patients as well as tolerability and safety have been reported[188]. Thus, the stage may be set for future studies of combination of cancer vaccines and PD-1 blockade. Preclinical studies certainly suggest the power of this combination[143,189,190]. Finally, other co-inhibitory receptors could also be therapeutically relevant targets for cancer immunotherapy and amenable to combinations with vaccination. These include Tim-3[191], Lag-3[192,193] and possibly BTLA[194–196]. Please see chapter 8 for many additional details on co-signalling blockade in cancer immunotherapy.

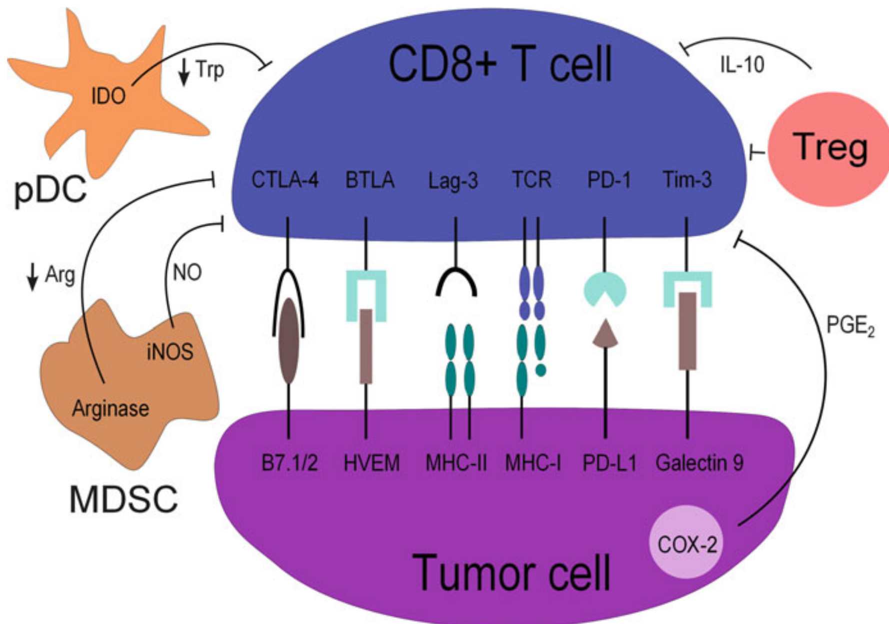

Fig. 5.2 T cell extrinsic and intrinsic immune regulatory factors in the tumor microenvironment. Regulatory mechanisms present in the tumor microenvironment oppose the activity of tumor-specific T cells in anti-cancer immune responses. Several regulatory cell types are present in the tumor microenvironment. One of these is the regulatory T cell (Treg) population, which can suppress by direct contact with effector T cells or through IL-10 production. Myeloid-derived suppressor cells (MDSC) are also enriched at the tumor site. They express the inducible nitric oxide synthase (iNOS) allowing them to produce high amounts of nitric oxide (NO), which inhibits T cell migration as well as T cell receptor (TCR) and cytokine signalling. Degradation of essential amino acids is another mechanism of tumor suppression employed by MDSCs as well as plasmacytoid dendritic cells (pDC) at the tumor site. MDSCs produce arginase, which degrades arginine (↓Arg). pDC can be induced by the suppressive tumor environment to produce IDO, which degrades tryptophan (↓Trp). This leads to an impairment of T cell proliferation and survival through metabolic starvation. In addition to the immune regulatory populations present in the tumor microenvironment, tumor cells themselves contribute to immunosuppression by producing cyclooxygenase-2 (COX-2) and its enzymatic product prostaglandin-E_2 (PGE_2), which inhibits antigen presenting cell maturation and T cell activation, and promotes angiogenesis and metastasis. Tumor cells also directly inhibit T cell activation and function through the expression of costimulatory/inhibitory molecules, which are recognized by corresponding inhibitory receptors present on the surface of highly activated effector T cells. Such inhibitory ligand-receptor pairs include B7.1/2-CTLA-4, PD-L1-PD-1, HVEM-BTLA, Galectin-9-Tim-3 and MHC-II-Lag-3. These regulatory cell types and inhibitory molecules are promising therapeutic targets for the treatment of cancer. Therapies targeting some of these factors are already approved for clinical use, while many others are in early or pre-clinical development.

5.5 Conclusion

The immune system actively participates in sculpting tumor immunogenicity during cancerogenesis. Once a tumor has been established, it progresses in the presence of an immune system that has largely failed to control tumor growth. Since

clinically detectable tumors have already reached considerable sizes, immune tolerance needs to be overcome in order to reinvigorate immune surveillance. Peptide/protein therapeutic vaccination approaches have undergone considerable improvement through numerous early phase clinical trials in patients with various tumor types. Minimal epitopes still remain the most powerful peptides to induce large numbers of $CD8^+$ T cells, but approaches are needed to ensure their presentation solely on professional antigen-presenting cells. These in turn are only able to mobilize antitumor immune responses when they have been properly activated, and the combination of the most potent adjuvants and strong $CD4^+$ T cell epitopes in the vaccine formulation is crucial in this process. T cell help is also implicated in the formation of CTL memory, which is important for establishing long-lasting tumor control that has been shown to correlate with increased patient survival. Tumor-specific $CD8^+$ T cells further need to infiltrate into the tumor to execute their destructive mission. $CD4^+$ T cells can help $CD8^+$ T cells home to the tumor, which underscores the importance of considering strong MHC class II epitopes (that promote $CD4^+$ T cell help) during peptide/protein vaccine design. However, once CTLs enter into tumor tissue, they are confronted with an immune-hostile tumor suppressive microenvironment, which impedes their proper function and renders them hypofunctional. Therefore, the future of peptide vaccinations lie in their combination with other approaches. In this regard, the massive destruction of tumor masses with drugs specifically targeting tumor-specific mutations has the potential to disrupt established microenvironments. At the same time, the targeting of Tregs and myeloid-derived suppressor cells using depleting agents such as cyclophosphamide and sunitinib holds great promise. On the other hand, treatments that enhance immune functions such as with ipilimumab have already proven successful. Combination therapies using improved peptide/protein vaccination protocols in parallel with such immunomodulators represent promising strategies to induce protective immunity and provide new avenues for the treatment of cancer.

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Chapter 6
Antigen Targeting to Dendritic Cells for Cancer Immunotherapy

Dinja Oosterhoff, David T. Curiel, and Tanja D. de Gruijl

Abstract Dendritic cells (DC) are antigen presenting cells that play a crucial role in initiating anti-tumor immunity. DC capture antigens, process them, and migrate to the draining lymph nodes where they can induce an antigen-specific T cell response. A promising strategy to induce a potent, specific, and lasting anti-tumor response is to target tumor antigens to DC in vivo. This represents a clinically generally applicable and cost-effective approach to DC-based vaccination against cancer. Here, an overview is provided of the different delivery vehicles (e.g., viruses, proteins, liposomes, and nanobodies) that are currently being explored for the development of therapeutic cancer vaccines and considerations for their successful application as well as future developments.

6.1 Introduction: The Case for DC-Targeted Vaccines

Dendritic cells (DC) are generally regarded as the most powerful antigen-presenting cells (APC) with a singular ability to prime naive T cells and thus initiate adaptive immunity. They form a crucial link between the innate and the adaptive arms of the immune system and are central regulators in numerous immune processes. They stem from a common $CD34^{+}$ bone marrow (BM)-derived precursor and can differentiate into various subsets, which can be myeloid (conventional DC) or more lymphoid in nature (plasmacytoid DC). From the blood, DC precursors home to peripheral tissues where they develop into immature DC.

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Immature DC patrol all tissues of the body and carry specialized receptors to bind and detect pathogen- or danger-associated molecular patterns (PAMP and DAMP, respectively). Upon their activation through these infectious and/or pro-inflammatory stimuli, they reach end-stage maturation, at which point they acquire the ability to migrate to secondary lymphoid organs and activate (naïve) T cells in an antigen-specific manner, thus starting an adaptive immune response[1, 2].

Numerous clinical trials have been carried out and are currently underway to study the efficacy of DC vaccines. A common strategy in the DC-based immunotherapy of cancer is the ex vivo generation of autologous DC from blood-derived DC precursors, which are then loaded with proteins or peptides that carry known T cell epitopes from tumor-associated antigens (TAA). Such DC vaccines are subsequently readministered to the patient. Alternatively, TAA-encoding genes can be transferred to DC. Genetic modification of DC for immunotherapy has distinct advantages. In contrast to the use of proteins, a genetic TAA vaccine provides a long-lived and continuous source of antigen, facilitating durable presentation of TAA-derived epitopes to both cytotoxic T lymphocytes (CTL) and helper T cells (Th). Endogenous TAA expression resulting from gene transfer ensures access to the MHC class I processing pathway, which is essential for subsequent activation of specific CTL, the proposed main effector cells of anti-tumor immunity. Although vaccination with ex vivo generated autologous DC has led to some clinical successes, its wide-scale implementation is hindered by limitations with respect to logistics, costs, and standardization. Indeed, there is a general consensus that this methodology requires optimization to improve therapeutic efficacy and alternative tumor vaccination approaches are actively being pursued[3].

An ever expanding knowledge of DC biology has led to a new generation of genetically modified vaccines that can specifically target DC in vivo. By simultaneously ensuring proper DC activation these generally applicable DC-targeted vaccines may ultimately render the ex vivo generation and loading of DC redundant. Indeed, vaccines based on the targeting and triggering of tissue-resident DC can be designed to exploit the physiological processes already in place to facilitate DC activation, migration, lymph node homing, and subsequent T cell activation. Additionally, the presence of different DC subsets in peripheral tissues allows the targeting of specific DC subsets that have been demonstrated to hold potent immunostimulatory capacities. For example, for anti-tumor immunization it may prove beneficial to target Langerhans cells specifically, i.e., the DC subset residing in the epidermis and other epithelial surfaces, as these have been implicated in the selective generation of cell-mediated immunity[1, 3].

6.2 DC Targeting Motifs

In mouse studies, in vivo immunotargeting of protein antigens to DC-restricted markers, such as DEC-205, was shown to induce strong immune responses[4, 5]. However, for effective CTL-mediated anti-tumor immunity, DC targeting alone

may not be enough; additional activation is required. For instance, it has been shown by Steinman and colleagues that targeting of model tumor-, or HIV-derived antigens to the DEC-205 receptor (or other DC-associated C-type lectin receptors [CLR], like Langerin and Clec9A) on DC led to specific T cell unresponsiveness within 7 days after immunization[6, 7]. This unresponsiveness was only overcome after the co-injection of a CD40 agonistic antibody or activating ligands of toll-like receptors (TLR), like CpG oligodeoxynucleotides. Therefore, in selecting DC targeting motifs two important factors should be considered: selectivity and activation.

The choice of molecules to target for DC-specific gene transfer is closely related to the subset, the maturation state, and the anatomical location of the DC in question. The most attractive targets should (a) be only expressed on DC, (b) be rapidly internalized upon binding, (c) route internalized antigens into MHC class I and II processing pathways, and (d) induce DC maturation and migration upon binding, to allow for optimal CTL and other immune cell activation. DC express many different antigen-capture and PAMP or DAMP-binding molecules at their surface, collectively referred to as pattern recognition receptors (PRR). PRR are by definition attractive targets, because it is their natural function to internalize antigens and mediate their routing to antigen processing pathways in order to facilitate generation of a T cell response (reviewed in reference 8). PRR include CLR, TLR, scavenger receptors (SR) and NOD-like receptors. Upon infection and/or tissue damage, DC bind PAMPs and/or DAMPs, leading to endocytosis, processing, and presentation of associated antigens. Their ability to capture and process antigens for subsequent T cell activation, coupled to the capacity of some to also induce DC maturation, make PRR attractive candidate targeting motifs for in vivo DC vaccination. In addition, their differential expression on DC subsets may allow for targeting of specific subsets with specialized functions: for example, Langerin for Langerhans cells (CTL activation); MR, TLR2, and DC-SIGN for dermal or interstitial DC (B- and T cell activation); or CD141 and Clec9A for enhanced cross-priming in specific DC subsets[3]. Upon binding of their ligand(s), some PRR (e.g., TLR) can activate DC, whereas others do not (e.g., most CLR), necessitating the incorporation of DC-activating signals in the vaccine formulation. Alternatively, DC-activating receptors that are (relatively) over-expressed on DC might be directly targeted to achieve simultaneous DC targeting and activation. Members of the family of TNF receptors are attractive candidates in this respect. For instance, we found that CD40-targeted adenovirus-mediated TAA gene transfer resulted in selective DC transduction in human skin explants and skin-draining lymph nodes and simultaneously induced their activation, leading to the high-efficiency priming and activation of tumor-reactive CTL[9, 10].

6.3 Targeting DC In Vivo: Delivery Vehicles

DC-targeted vaccines generally consist of antigenic proteins or genetic material encoding antigenic sequences (Fig. 6.1). Immunogenic DC targeting of either modality has to adhere to a separate and specific set of requirements. For protein

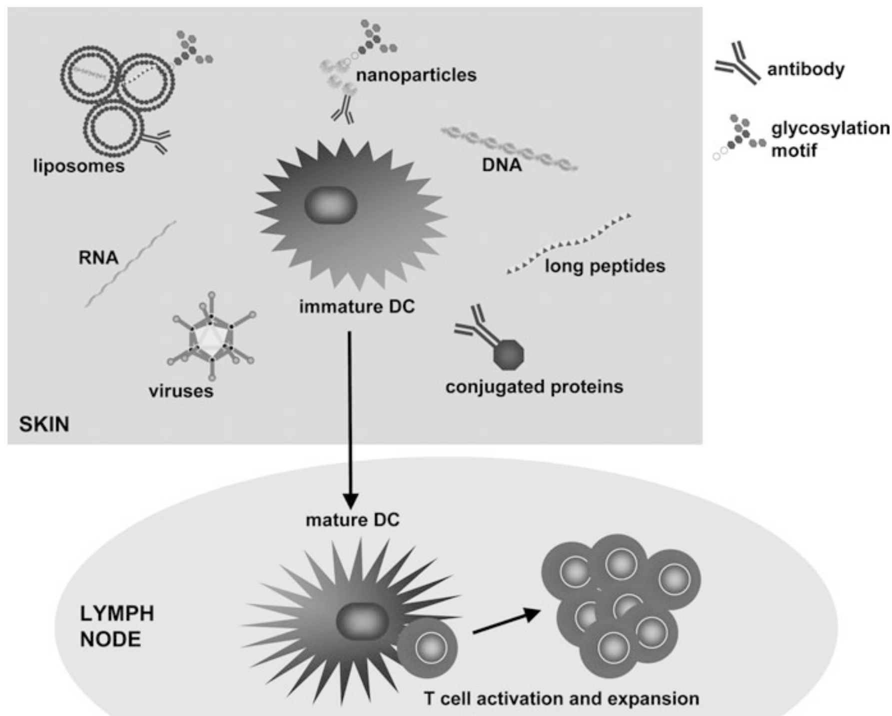

Fig. 6.1 Representation of the different mechanisms to target dendritic cells (DC). Tumor associated antigens can be targeted to DC residing in the skin via several mechanisms. The antigen itself can be coupled to an antibody specific for DC. Another option is to inject DNA or RNA encoding the antigen of choice directly into the skin or incorporate the DNA/RNA in a viral vector, liposome, or a nanobody manipulated in such a way that it will only bind to DC (such as through specific glycosylation motifs or antibodies). A last option is to use synthetic long peptides that will be taken up and processed by the DC. Upon capture, the DC will start to mature and migrate towards the draining lymph nodes where the mature DC can induce an antigen specific T cell response against antigen-derived epitopes.

targeting it is essential that upon binding to the DC surface motif, the protein is efficiently endocytosed and routed to MHC processing pathways for subsequent presentation to T cells. In contrast, for viral transduction, binding to a DC-specific docking molecule per se might be sufficient, as most viruses have co-receptors (e.g., integrins) and other mechanisms in place for the induction of subsequent uptake and release from endosomes into the cytoplasm. Various vehicles are now available for targeted in vivo gene and/or protein transfer to DC, both viral and nonviral (see Fig. 6.1). An overview is given below.

6.4 Viruses

There are some major advantages to the use of viral vectors for gene delivery to DC:

(1) Many viruses exhibit a natural tropism for DC (e.g., lentiviruses) that might be utilized for DC-targeted vaccination.
(2) Viruses have a natural ability to infect target cells, e.g., to be efficiently endocytosed by DC.
(3) Viruses have developed mechanisms to transfer their genetic cargo efficiently to the host cytoplasm and/or nucleus to take over the host replication and/or transcription machinery and thus ensure high-level expression of the transgenes they carry.

These characteristics make viruses extremely attractive vaccine vehicles, despite regulatory restrictions that complicate their clinical implementation and concerns about preexisting or induced neutralizing antibody responses. Nonviral vehicles often need to be chemically altered to achieve the above listed advantageous traits for DC-targeted vaccination that viruses often possess naturally.

6.5 DNA Viruses

6.5.1 Adenovirus

One of the most commonly used gene transfer vectors for DC is the adenovirus serotype 5 (Ad5). Advantages of adenoviruses over other delivery vehicles, such as retroviral vectors, are that Ad vectors can efficiently infect both dividing and nondividing cells, that they can be produced at high titers, and that they are relatively safe, since they do not integrate into the host cell genome. Importantly, the perceived unsuitability of Ad5 vectors as vaccine vehicles, due to preexistent or rapidly induced neutralizing antibody responses that would prohibit their use in prime/boost set-ups, can now be overcome by specific ablation of antibody binding sites in the hexon protein of the Ad capsid[11].

In 1997, Wan et al. described the ex vivo transduction of DC with a replication deficient adenoviral vector encoding the polyoma middle T antigen[12]. A single injection of DC transduced with the Ad vector expressing polyoma middle T provided complete and specific protection against tumor cell challenge in 100% of vaccinated animals. A comparable study was performed by Song et al. with a replication deficient adenovirus expressing the reporter gene beta-galactosidase[13]. Using a murine metastatic lung tumor model with syngeneic colon carcinoma cells expressing beta-galactosidase, it was shown that immunization of mice with a genetically modified DC line or bone marrow-derived DC confers potent protection against a lethal tumor challenge, as well as suppression of pre-established tumors, resulting in a significant survival advantage. Since then, many similar

tumor vaccination studies with Ad vectors encoding a myriad of TAA for various tumor types have been performed[3–23]. All these studies demonstrated that adenoviral transduction of DC resulted in high expression levels of the TAA of interest and efficient generation of immune responses directed against the tumor. A more general approach to treat different types of cancer is to transduce DC with Ad vectors encoding wild type p53. The tumor suppressor protein p53 is an attractive candidate for DC-based immunotherapy, because this protein is found abundantly in 50% of human malignancies but not in normal tissues. Several reports demonstrated that treatment with Ad-p53-transduced DC generated CTL directed to p53 and significantly slowed the growth of established tumors[14, 15]. Thus, transducing DC with wild-type p53 may be a promising new tool for the immunotherapy of cancer.

In all the studies listed above, DC were ex vivo transduced with Ad vectors. Although more attractive, direct in vivo administration of Ad-based vaccines to patients is complicated by the fact that DC are relatively resistant to Ad infection. The infection of host cells by Ad5 is a two-step process. The first step is a high-affinity interaction of the knob domain of the Ad fiber with the cell surface receptor coxsackie and adenovirus receptor (CAR)[16, 17]. Subsequent internalization, via receptor-mediated endocytosis, involves interactions between the Arg-Gly-Asp (RGD) sequences of the adenovirus penton base proteins with cellular $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrin receptors. Unfortunately, DC lack surface CAR expression, whereas CAR is abundantly expressed on many other cell types. In vitro the resistance to Ad infection of DC can be overcome by the use of high virus titers (at multiplicities of infection [MOI] exceeding 1,000). In vivo, however, this would lead to preferential bystander (non-DC) infection and unwanted cytopathic side effects. A logical approach to circumvent inefficient CAR-mediated Ad5 transduction is redirecting Ad5 entry (targeting) via alternative cell surface molecules abundantly expressed on DC. Several strategies have been explored in this regard (see Fig. 6.2). First of all, it proved possible to replace the tropism-determining fiber knob domain of Ad5 with that of a different Ad serotype. Replacement of the Ad5 fiber knob with the Ad35 fiber knob resulted in a dramatic increase in gene transfer efficiency to DC and their high-efficiency in situ transduction in human skin explants[18, 19]. Similarly, it was recently demonstrated by Stone et al. that the use of the Ad11 led to an increased transduction efficiency of human immature DC as compared to Ad5[20]. We have also shown that replacement of the Ad5 fiber knob with that of Ad3 resulted in increased transduction efficiencies of human DC and that this Ad5/3 was more specific for mature $CD1a^{+}CD83^{+}$ DC than Ad5/35, selectively targeting DC in the context of skin and melanoma-draining lymph nodes through binding to CD80/CD86[21]. Obviously, the utility of pseudotyping is limited by the natural diversity of Ad receptor recognition. More precise targeting of DC specific surface molecules requires synthetic design of targeted adenoviruses. For this, single- and two-component systems are being explored.

The design of single-component targeted Ad vectors by incorporating targeting ligands into adenovirus capsid proteins has been widely explored in the context of tumor targeting. For DC targeting, the RGD sequence has been incorporated in the

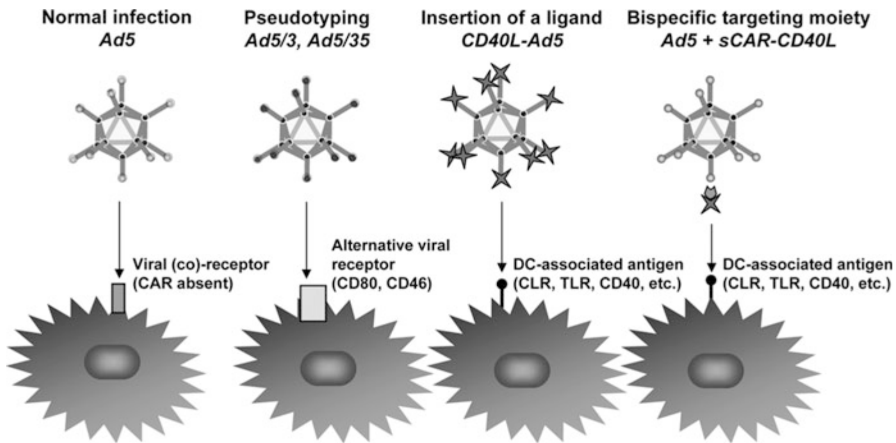

Fig. 6.2 Schematic representation of different approaches to target adenoviruses to DC. During native infection, adenovirus serotype 5 (Ad5) enters the cell following high-affinity binding to the cellular receptor CAR, which is not expressed by DC. Replacing the tropism-determining fiber knob domain of Ad5 with that of a different adenovirus serotype results in a virus with a modified tropism, potentially leading to improved transduction of DC. Examples are Ad5/3 and Ad5/35 that can bind to CD80 and CD46 on the DC. Targeting Ad5 to DC has also been established by inserting peptide motifs, like the binding domain of CD40L, in the virus capsid that can bind to receptors on DC. Furthermore, bispecific targeting moieties have been used to target Ad5 to DC. These molecules can bind on one side to the adenoviral fiber and on the other side to a receptor on the DC. An example is the fusion protein sCAR-CD40L which can bind to the knob domain of Ad5 (thereby neutralizing its natural receptor binding) and to DC expressing CD40.

fiber knob[22–24]. RGD targeting greatly enhanced DC transduction efficiency. This modification, however, does not abrogate binding to CAR and thus expands rather than targets Ad entry. However, it should be possible to combine such modifications with capsid protein mutations known to abolish native tropism[25]. Belousova et al. constructed a chimeric Ad containing the CAR-binding mutated wild-type fiber and a bacteriophage T4 fibritin fiber in which CD40L was incorporated[26]. Intradermal injection of this vector in human skin explants resulted in targeted, enhanced gene transfer to migrating DC, as well as in their phenotypic maturation[27]. Production of a virus containing only the CD40 targeted fibritin fiber unfortunately proved suboptimal, and thereby unsuitable for clinical application.

Complex binding ligands including antibodies have been successfully employed in two-component targeting strategies, where they were bound to the Ad fiber indirectly via a second protein moiety. We and others have demonstrated that using this approach to target Ad5 to CD40 expressing cells, the transduction efficiency increased to 95% at MOI 100[10, 28–30]. Indeed, immune conjugate-mediated targeting of Ad5 vectors to CD40 resulted in the selective and enhanced transduction of DC in human skin explants and in lymph node suspensions and facilitated the efficient priming of high-avidity melanoma-reactive CTL[9, 10]. Moreover, in vivo delivery of an Ad5 vector carrying tumor antigens and retargeted to CD40 through a CD40L-sCAR adapter protein resulted in efficient DC transduction

in lymph nodes and resulted in superior tumor protection in the B16 melanoma model[31]. Indeed, we envisage the clinical use of such a recombinant CD40 adapter protein (consisting of the TNF-like domain of CD40L fused to soluble CAR), which represents a highly defined product that is clinically applicable with Ad5 as a highly flexible two-component DC-targeted Ad vector configuration, ultimately allowing vaccination with different TAA-encoding Ad vectors simultaneously, depending on the TAA expression profiles of the targeted primary or metastatic tumors.

6.5.2 Adeno-Associated Virus

Adeno-associated virus (AAV) are small, nonpathogenic parvoviruses that are dependent on larger helper viruses, such as adenoviruses, for their replication. AAV has established its position as one of the most popular gene delivery systems. This is mainly because of the long-term and efficient transgene expression in various cell types in many tissues including liver, muscle, retina, and the central nervous system[32]. However, there are some disadvantages associated with the application of AAV. The packaging capacity is relatively restricted and the large-scale production inefficient. Furthermore, the integration into the host genome is random, which can lead to unexpected activation or inhibition of endogenous gene expression (a major obstacle to in vivo clinical application). Different AAV serotypes have shown remarkably different expression patterns because of differences in cell entry and intracellular activities. For example, Ponnazhagan et al. demonstrated that at an MOI of 100 of AAV serotype 2, the efficiency of transduction among DC cultures derived from different normal blood donors, varied between 2 and 55%[33]. Nevertheless, transduction of DC with an AAV containing the cDNA encoding the HPV-16 E7 antigen generated CTL that showed MHC class I-restricted killing of cervical cancer cells[34]. Flow cytometric analysis of the DC populations revealed that AAV/E6 vector-pulsed DC had higher levels of CD80 and lower levels of CD86 than protein-pulsed DC[35]. Importantly, transducing DC with AAV encoding self-antigens resulted in the generation of functional CTL, thus suggesting that AAV-loading of DC is a good approach for generating CTL against TAA with low immunogenicity[36, 37].

6.5.3 Vaccinia Virus

Vaccinia virus is a double-stranded DNA virus of which the entire life cycle takes place within the cytoplasm of host cells. It has a wide host range and is capable of infecting almost all human cell types with high efficiency. This represents a clear disadvantage for DC targeting. An advantage of Vaccinia virus is its capacity for efficient infection and gene expression. A number of viral promoters can be chosen to control the timing and the level of transgene expression. Furthermore, the

Vaccinia virus genome can accommodate at least 25 kb of foreign DNA sequence[38] and its replication occurs exclusively in the cytoplasm, eliminating the possibility of chromosomal integration. An important potential disadvantage for clinical application of Vaccinia virus-based vaccines may be preexistent immunity in older patients vaccinated for smallpox. This may limit vaccination efficacy.

In 1998, Di Nicola et al. described that mature monocyte-derived DC were transducible with Vaccinia virus[39]. Since then, various studies have been performed demonstrating Vaccinia virus-mediated transduction of genes encoding the TAA EBNA-3A[40], gp100[41], MUC1[42, 43], CEA[43], or HPV16-E7. In general, all these studies showed the induction of antigen-specific Th and CTL responses, resulting in in vivo tumor rejection. However, it has been demonstrated that Vaccinia virus transduction hampers proper DC maturation[44, 45] making it necessary to induce DC maturation prior to transduction with this virus. Furthermore, transduction with Vaccinia virus inhibited expression of HLA-DR and reduced the secretion of cytokines important for DC migration, like RANTES, MIP-1α, and TNF-α[46].

Two phase I clinical trials with Vaccinia virus-based melanoma vaccines have been performed[47, 48]. In the first study, 6 patients were injected intravenously and subcutaneously with DC transduced in vitro with a modified Vaccinia virus encoding the human tyrosinase gene[47]. Treatment was well tolerated, except for low-grade fever (in 3/6 patients), mild erythema at the injection site (in 5/6 patients), and vitiligo (in 2/6 patients). A partial response, involving shrinkage of a subcutaneous nodule, later surgically removed, was observed in 1 patient, who then remained disease-free (>850 days). In 4 of 5 patients, significant and often long-lasting increases in frequency of T cells directed to tyrosinase were documented. In another study a comparable vaccine was directly injected in 20 patients three times at 4-week intervals (5×10^8 IU/injection)[48]. This did not elicit a measurable immune response to its transgene product in patients with stage II melanoma after repeated combined intradermal and subcutaneous vaccination, probably because DC maturation was hampered.

Another clinically tested Vaccinia virus-based vaccine is PROSTVAC-VF (Tricom). It consists of two vectors, both encoding the prostate tumor antigen prostate specific antigen (PSA) and three co-stimulatory molecules: ICAM-1, LFA-3, and B7.1 (CD80). A Vaccinia virus is used for the priming vaccination, followed by boost vaccinations with fowlpox vectors. The viruses are subcutaneously injected together with GM-CSF. In a randomized controlled phase II trial of 125 patients with castration resistant prostate cancer it was demonstrated that patients receiving PROSTVAC-VF had a longer median overall survival (25.1 months versus 16.6 months for patients receiving an empty control vector)[49]. A large phase III trial is now planned to confirm these promising results.

6.5.4 Herpes Simplex Virus

Herpes simplex virus (HSV) is a large DNA virus of which type I can infect DC with intermediate to high efficiency. DC infected with replication deficient HSV

fail to become activated, downregulate a number of surface markers, and fail to produce a number of cytokines in response to activation stimuli, such that their T cell-activating capabilities are minimal[50, 51]. To overcome this immune problem, the viral gene encoding virion host shut-off protein has been deleted[52]. This protein destabilizes mRNA in infected cells so that host protein synthesis is reduced in favor of translation from more rapidly produced viral mRNA. The resulting virus transduced DC as efficiently as the parental virus, but induced both expression of CD86 and an enhanced specific T cell-proliferative response. Transduction of DC with HSV–OVA (ovalbumin) or HSV–PSA and co-culture with CTL hybridomas resulted in specific activation of the CTL, indicating that transduced DC express these transgenes and process the tumor antigens for MHC-I mediated presentation to CTL. Mice immunized with HSV–PSA-transduced DC generated a specific CTL response that could be detected in vitro by a classic chromium release assay and these mice were protected from challenge with tumors that expressed PSA[53]. Thus far, HSV vectors have not been clinically used for in vivo vaccination or DC targeting.

6.6 RNA Viruses

6.6.1 Retrovirus

The idea of using retroviruses as gene delivery tools was introduced by Mann et al.[54]. The retrovirus family consists of single-stranded RNA viruses that measure 80–120 nm in diameter. These single-stranded RNA viruses replicate through a double-stranded DNA intermediate, which is integrated in the host genome. The most commonly used retroviral vectors are based on Moloney murine leukemia virus, in which the *gag*, *pol*, and *env* genes are replaced with an expression cassette. The major advantage of retroviral vectors is the lack of immunogenicity due to the removal of the genes encoding viral proteins. However, limitations of this vector are the instability of the viral particle, low viral titers, and the inability to transduce nondividing cells. DC residing in tissues and secondary lymphoid organs have lost their proliferative capacity, rendering retroviruses useless in terms of in vivo DC targeting.

6.6.2 Lentivirus

In contrast to oncoretroviral vectors, lentiviral vectors are capable of transducing nondividing cells, such as DC, at high transduction efficiencies. Importantly, like oncoretroviral vectors, lentiviral vectors do not encode viral proteins, thereby minimizing the potential for interfering with the function of the transduced DC[55].

Third-generation lentiviral vectors with enhanced safety profiles have been developed and used to transduce murine and human DC efficiently. These improved vectors contain a chimeric Rous sarcoma virus/HIV 5′ long terminal repeat (5′LTR) enhancer and promoter to initiate the transcription of genomic viral RNA[56]. The stronger chimeric promoter does not require HIV Tat protein, a transactivator of the transcription of HIV genomic RNA, to generate vector transcripts. In addition, the vectors have been made self-inactivating by deleting the majority of the U3 region in 3′LTR so that viral RNA cannot be produced in target cells[57]. These additional safety modifications further prevent the generation of replication-competent recombinants and should feasibilize clinical implementation.

It has been shown that immature DC are efficiently transduced with increasing doses of lentivirus without affecting cell viability. Transduction at low MOI did not result in phenotypical or functional maturation. Higher doses of lentivirus, however, resulted in upregulation of adhesion, costimulatory, and HLA molecules, as well as in increased allostimulatory capacity and secretion of interleukin (IL)-6, IL-8, and tumor necrosis factor-alpha[57]. Li et al. described that a single injection of murine bone marrow-derived DC transduced with a lentiviral vector encoding a truncated form of Neu protein stimulated the induction of $CD4^+$ and $CD8^+$ T cells in vivo and suppressed the growth of Her2/Neu overexpressing tumors[58].

Recently, two papers were published describing the construction of lentiviral vectors in which expression of the transgene was targeted to DC. Lopes et al. used the mouse dectin-2 promoter to target expression of GFP to dectin-2 positive cells. This lentivector effected transgene expression in mouse bone marrow-derived DC and in human skin-derived Langerhans cells and dermal DC. In mice, transgene expression was detected in splenic dectin-2^+ cells after intravenous injection and in $CD11c^+$ DC in the draining lymph node after subcutaneous injection. A dectin-2 targeted lentivector encoding the human cancer antigen NY-ESO-1 primed an NY-ESO-1-specific $CD8^+$ T cell response in HLA-A2 transgenic mice and stimulated a $CD4^+$ T cell response[59]. Furthermore, a transcriptionally DC-targeted vector was constructed using the DC-STAMP promoter region to induce tolerance by transducing hematopoietic stem cells. This should result in tolerance because the DC that generated from these stem cells are not activated by the vaccine and thus remain in a steady-state immature condition. This vector induced long-term and cell-selective transgene expression in vivo. As expected, these transcriptionally targeted DC induced functional, antigen-specific $CD4^+$ and $CD8^+$ T cell tolerance in vivo, which could not be broken by subsequent immunization[60].

Recently, a DC-SIGN targeted lentiviral vector was constructed by incorporation of an engineered glycoprotein derived from Sindbis virus. This targeted lentivector transduced DC in vitro with high specificity. Direct subcutaneous administration of the targeted lentivector in DC-SIGN transgenic mice induced a strong antigen-specific T cell and antibody response[61].

Thus, there is a growing body of evidence to show that it is indeed possible to use lentiviral vectors for in vivo DC targeting applications.

6.7 Nonviral Gene Vehicles

6.7.1 Naked DNA and RNA

6.7.1.1 DNA

An elegant approach to circumvent (mostly safety-related) disadvantages associated with viral vectors is to transfect DC directly with plasmid DNA encoding full-length TAA. Advantages of DNA transfection include the easy construction and high stability of plasmid DNA and the possibility to include sequences that lead to better antigen presentation or DC activation. Furthermore, DNA vaccines are relatively safe, because there is no risk for recombination with wild-type viruses and the risk for insertional mutagenesis is low. Finally, it has been demonstrated that cutaneously applied plasmids can remain present in the skin for up to 5 months[62]. DNA-based vaccines thus direct antigen expression for extended periods, supporting persistent anti-tumor immune responses that could theoretically protect a patient from relapse. Major hurdles to the use of DNA as immunotherapeutic tools are the low efficiency with which DC are transfected and the general weakness of elicited immune responses. Possible ways of administration include the modification of target cells with the DNA ex vivo and the direct delivery of the DNA plasmid into the patient, for example, with the gene gun method, by tattooing into skin, or simply by intradermal or intramuscular injection. The gene gun methodology entails delivery of the DNA following its precipitation onto gold microparticles that are delivered to the skin under pressure by a ballistic delivery device[63]. This process does not induce traumatic injury and requires much less DNA to achieve comparable humoral immune responses as compared to intramuscular administration[64]. More recently, Bins et al. pioneered a tattoo approach whereby antigen-encoding DNA can be delivered to the epidermis[65]. In mice and primates this was shown to induce immunity to tumor and HIV antigens, respectively[66]. The resulting trauma to the epidermis also ensured DC activation and migration. The exact mechanism for induction of the immune response is still not entirely clear, but appears to involve processing of the antigen through both endogenous and exogenous pathways, leading to presentation of the antigen in the context of both MHC class I and II. The disadvantage of direct administration of DNA to patients is that any cell encountering the plasmid could be transfected with it. It was demonstrated by Raz et al. that after intradermal injection of plasmid DNA, cells resembling macrophages and DC, but also keratinocytes and fibroblasts, were transfected[62]. Other studies, however, demonstrated direct transfection only of skin DC following gene gun administration[67, 68]. In contrast to this, Corr et al. published that the elicited immune response was the result of expression of the antigen by non-lymphoid tissues and transfer to APC[69]. After DNA vaccination DC appear to acquire antigen both by direct transfection and by cross priming. A study by Condon et al. furthermore revealed that gene gun immunization resulted in the migration of transfected skin-derived DC to the draining lymph nodes[67]. Recently, a way to target the expression of the gene to DC was described by Ni et al. who used a DC specific

promoter based on a short sequence of the CD11c promoter to target expression of lacZ or EpCAM to DC in mice[70].

A number of features can influence the nature and the potency of the DNA-elicited immune response. The composition of the DNA is a first important consideration for plasmid vaccines. For instance, hypomethylated CpG dinucleotide sequences that are relatively underrepresented in eukaryotic DNA serve as a PAMP and in man bind to TLR9 in plasmacytoid DC, increasing the immunogenicity of DNA vaccines[71]. Secondly, to increase the level of transgene expression, a strong promoter, like the CMV promoter, is required. Finally, the antigenicity of the encoded protein is of considerable importance in generating an effective immune response. Although DNA vaccines have shown promise in eliciting effective CTL responses to neoantigens, the fact that most TAA are self-antigens and thus weakly immunogenic requires DNA vaccines to be very potent to be clinically useful. Many studies have therefore focused on enhancing the immune response that is raised by DNA vaccines. Different approaches have tried to improve the delivery of the vaccine[72], modification of the encoded antigen to increase its immunogenicity and DC targeting potential, for example, by fusion of the antigen to CD40L[73] or FLT3L[74], or modification of the microenvironment by addition of (DNA encoding) cytokines or chemokines[75].

Only a few clinical studies with plasmid DNA in cancer patients have been published. In general, DNA vaccines were well tolerated, but had mixed results in raising cellular immunity. Tagawa et al. conducted a phase I trial in patients with stage IV melanoma[76]. Patients received intranodal injections of a DNA vaccine encoding tyrosinase epitopes. The vaccine was tolerated well, with only five patients demonstrating grade I–II toxicity. Immune responses by peptide-tetramer assay to tyrosinase were detected in 11 of 26 patients. However, no clinical responses were seen. In a study by Rosenberg et al., 22 patients with metastatic melanoma were injected intradermally or intramuscularly with plasmid DNA encoding gp100 melanoma–melanocyte differentiation antigen[77]. One patient exhibited a partial response of several subcentimeter cutaneous nodules, whereas all other patients had progressive disease. Of 13 patients with cells available before and after immunization, none exhibited evidence of the development of anti-gp100 T cell responses. A significant clinical or immunological response to plasmid DNA encoding the gp100 tumor antigen was thus not demonstrated. Recently, Miller et al. published the results of a phase I trial in which 6 patients with hormone-refractory prostate cancer were monitored for their ability to mount PSA-specific cellular responses with recombinant GM-CSF and IL-12 as immunoadjuvants after receiving a PSA DNA vaccine[78]. After vaccination, T cells recognized both PSA peptides and the naturally processed PSA protein. Several trials of DNA vaccines against human papillomavirus (HPV) related malignancies have been performed. HPV-related malignancies have the advantage that foreign HPV antigens are expressed and serve as TAA, rather than self antigens. Plasmid DNA encoding HLA-A2 epitopes from HPV16 E7 protein was incorporated in polymer microparticles and delivered intramuscularly. In a trial for anal dysplasia, increased T cell responses were reported in 10 of 12 patients[79]. In another study, the same

plasmid was delivered to women with cervical intraepithelial neoplasia and most patients mounted a detectable immune response to HPV16-E7. More importantly, in 33% of the participating women, complete histological responses were documented.

Although definitive clinical evidence of the efficacy of DNA vaccines remains to be demonstrated, DNA vaccines do have several advantages and have proven to be safe in clinical applications. Further clinical investigations to improve their efficacy are therefore warranted.

6.7.1.2 RNA

Transfection of DC with specific or whole cell lysate-derived ribonucleic acid (RNA) has been demonstrated to be very effective in inducing potent TAA-specific cytotoxic T-lymphocytes[80, 81]. Using whole cell lysates has the advantage that it does not require the definition of specific TAA and that it therefore might have broad clinical applicability. On the other hand, a potential drawback is the increased risk of inducing autoimmunity. The first preclinical data using RNA-loaded DC in vivo were presented by Boczkowski et al.[80]. This study showed that DC pulsed with mRNA from ovalbumin-expressing tumor cells were as effective in inducing CTL responses as DC pulsed with ovalbumin peptide. Since then, the effectiveness of this approach has been demonstrated in many in vitro studies using RNA coding for different TAA, like PSA[82, 83], the human papillomavirus proteins E6 and E7[84], human telomerase reverse transcriptase (hTERT)[85] and human immunodeficiency virus capsid proteins[86]. Recently, Grunebach et al. demonstrated that transfection of monocyte derived DC with the RNA encoding Her-2/Neu and 4-IBBL resulted in an increased specific lysis of target cells by induced CTL lines[87] compared to untransfected monocyte derived DC. More importantly, vaccination with mRNA loaded DC has been shown to induce protective and therapeutic anti-tumor responses in mice[88, 89]. Several RNA delivery strategies have been explored, like electroporation[90], lipofection[85], or transfer through receptor-mediated endocytosis. Strobel et al. demonstrated that the use of liposomes was more effective than electroporation[90] whereas Van Tendeloo et al. found that electroporation was more potent compared to lipofection or CD71 based endocytosis[91].

RNA transfection thus represents a promising approach to engineer DC to present the whole and unique antigenic spectrum of a patient's tumor and therefore several clinical trials have been performed to assess the efficacy of this approach in patients. The first vaccination study using RNA-transfected DC was a phase I trial designed to evaluate the safety, feasibility, and efficacy to induce T cell responses in patients with metastatic prostate cancer[92]. Immature monocyte-derived DC were transfected with in vitro transcribed PSA RNA. Increasing doses ($1–5 \times 10^7$) of the modified DC were administered intravenously every 2 weeks and additionally 1×10^7 cells were administered intradermally at each vaccination. No major toxicity was observed and in general vaccination was well tolerated. After vaccination, all analyzed patients had PSA-reactive, IFN-γ secreting T cells, whereas in the pre-therapy samples no IFN-γ secreting T cells were detected. Furthermore, in a

chromium release assay it was demonstrated that after vaccination there was a significant increase in PSA-specific killing of target cells[90]. In a phase II trial for the same disease, hTERT mRNA-transfected DC were administered to 20 patients[93]. Eleven of the patients received DC transfected with hTERT-encoding mRNA, whereas 9 patients received DC transfected with the mRNA encoding a chimeric lysosome-associated membrane (LAMP)-hTERT fusion protein to direct hTERT antigen processing into the class II pathway[94]. It was demonstrated that patients receiving the fusion protein mRNA construct exhibited more pronounced delayed type hypersensitivity reactions, enhanced $CD4^+$ T cell responses, increased antigen-specific proliferative responses and improved CTL-mediated lytic activity when compared with immunization with the unmodified hTERT construct.

Several subsequent trials demonstrated safety, feasibility, immunogenicity, and moderate clinical efficacy of DC vaccines pulsed in vitro with TAA RNA[95–97]. It is, however, also possible to inject mRNA directly into skin. Intradermal application of naked mRNA in mice resulted in protein expression and the development of an immune response[98]. The same approach was used to vaccinate 15 melanoma patients[99]. For each patient a growing metastasis was removed and copy mRNA was produced. Autologous preparations were applied intradermally in combination with GM-CSF as adjuvant. This treatment proved to be feasible and safe. Furthermore, an increase in anti-tumor humoral immune responses was seen in some patients. However, a demonstration of clinical efficacy of direct injection of mRNA for anti-tumor immunotherapy was not shown in this study and must be evaluated in subsequent trials. Further strategies to stabilize naked RNA for in vivo applications should prove instrumental in this regard.

6.8 Nanoparticles and Liposomes

Nanotechnology is a relatively new focus of anti-cancer research and is used as a general term for the manufacture, manipulation and application of structures in the nanometer range. The ultimate goal of nanomedicine is to create medically useful nanodevices that can function inside the body. It is envisioned that nanodevices will be hybrids of biological molecules and synthetic polymers that can enter the cell and can interact with the DNA and proteins. In this regard, it should be possible to incorporate TAA into nanodevices and target them specifically to DC.

One of the first studies using this technology for immunotherapy described the construction of 100 nm cationic nanoparticles from warm oil-in-water micro-emulsion precursors. Plasmid DNA was coated on the surface of these cationic nanoparticles and the DC-targeting ligand mannan was incorporated in or deposited on the particles[100]. This approach significantly increased both IgG titer and Th1 cytokines upon immunization as compared to naked DNA transfection.

Fifis et al. coupled ovalbumin to smaller solid-core nanobeads of 40–50 nm, which, upon in vivo delivery, allowed them to localize to DC in the draining lymph nodes[101]. This resulted in the induction of two- to tenfold stronger immune

responses as compared to larger bead sizes, indicating that the size of the nanodevice is important. A single dose of these beads protected the mice from tumors in two different models. Since then, many more studies were performed in mice, although it is difficult to compare all these, because all the nanoparticles used have different compositions and sizes and were tested in different mouse models.

In human skin, particles with a size of 40 nm were efficiently taken up by epidermal Langerhans cells after transcutaneous vaccination, whereas larger particles were only taken up by Langerhans cells around hair follicles[102]. The same group recently published a paper in which influenza protein-based nanoparticles were transcutaneously injected and compared to intramuscular vaccination in humans. In a study on 11 healthy volunteers, it was found that transcutaneous vaccination induced both effector $CD4^+$ and $CD8^+$ T cell responses, whereas intramuscular injection induced effector $CD4^+$ T cells in the absence of $CD8^+$ T cells[103]. An interesting paper by Prasad et al. was recently published that described the construction of nanoparticles containing whole tumor lysates from human solid tumors. Compared to conventional tumor lysates the nanoparticles containing tumor lysates were more efficient in inducing IFN-γ production in vitro while reducing the production of potentially immunosuppressive IL-10, although the amount of administered lysate was five times lower in the nanoparticle containing lysate as compared to conventional tumor lysate[104]. An explanation for this could be that nanoparticles can function as a Th1 adjuvant, because the particles can bind and activate TLR-2[105], thereby inducing DC maturation.

The surface of nanoparticles can be modified to improve stability, but also to conjugate ligands to target the particles specifically to target cells (see Fig. 6.1). Ghotbi et al. described the construction of nanoparticles into which mannan was incorporated. These targeted nanoparticles were more efficiently taken up by murine bone marrow-derived DC than untargeted particles[106]. Cruz et al. recently reported on antibody-modified nanoparticles targeting DC through DC-SIGN and DEC-205[95, 107], resulting in T cell activation. Clearly such particles are attractive candidates for clinical translation as DC-targeted nanoparticle-based vaccines.

Liposomes can be regarded as a subtype of nanoparticles. Liposomes, bilayered phospholipid spheres of $\geq$100 nm, are excellent carriers of drugs or antigens that are currently used in a number of immunotherapeutic applications. They can accommodate almost any molecule of interest, whether it be peptides, proteins, or DNA, for the purpose of targeting, sustained release, and protection from degradation. The components of the lipids may vary, with neutral liposomes containing neutral lipids, and cationic and anionic liposomes containing lipids that are either positively or negatively charged. Depending on their lipid composition, liposomes can exhibit potent adjuvant-like properties[96]. Like nanoparticles, many liposomes with different composition and sizes have been tested for their capacity to target DC. The delivery of antigen-containing liposomes to DC can be facilitated by introduction of agents into the bilayer that bind selectively to molecular structures on the surface of the DC, such as antibodies, nanobodies or glycosylated motifs that can bind to specific CLR (see Fig. 6.1).

Targeting liposomes containing antigen or DNA to DC enhanced their capacity to induce humoral and CTL responses in vivo[97, 108, 109]. Moreover, specialized liposomes can be designed to enhance delivery of their payload to the cytosol, such as by using bilayer compositions which are pH-sensitive (pH-sensitive liposomes). This should facilitate subsequent processing for MHC-I mediated activation of specific CTL. Studies in mice have shown that the glycan modification of liposomes for APC targeting is a promising approach for the treatment of cancer. These glycoliposomes can also incorporate TLR-L motifs as well as DNA, since cationic lipids will spontaneously complex with DNA to form lamellar structures, so-called lipoplexes. A recent paper described the construction of antigen-containing liposomes that were engrafted with peptides from the TLR-5-ligand flagellin[110]. These DC targeted liposomes were efficiently taken up by murine bone marrow-derived DC and induced their maturation. Vaccination of mice with ovalbumin containing TLR-5 targeted liposomes increased the number of antigen specific $CD8^+$ T cells, indicating that this is a promising approach to target liposomes to DC.

A novel subtype of nanoparticles are nano-engineered exosomes. Exosomes are small vesicles released by tumor cells and/or DC. Exosomes released by DC express high levels of costimulatory molecules including MHC class I and II, and because of this observation DC exosomes (dexosomes) are considered to represent an alternative pathway of antigen delivery and presentation. DC-derived exosomes can modulate immune responses by activating T cells. Exosomes can be engineered ex vivo and are an interesting new field in immunotherapy[111]. Artificial exosomes can be 30–100 nm in diameter and were developed by coating liposomes with peptide-MHC class I complexes. These artificial exosomes could activate and expand functional antigen specific T cells[111].

6.9 Proteins, Antibodies, and Nanobodies

Antigenic proteins can be targeted to DC by coupling them to DC-specific antibodies. After uptake of the antigen-antibody complex by DC, the antigen will be presented and antigen-specific T cell responses can be raised. This has been successfully demonstrated using antigen targeted to DEC-205 and Langerin in murine models[4, 5, 112, 113]. This approach also worked using DEC-205 or CD11c specific scFv antibodies coupled to gp100 and Her2-Neu, respectively[114, 115]. Of note, antigen-antibody complexes were recently reported to be stored in specialized subcellular compartments in DC, ensuring prolonged presentation to, and activation of CTL[116]. Although these studies demonstrated that antigen targeting to DC induces specific immune responses, translation of these results to humans is difficult, because of different target expression patterns on DC in humans and mice, as is the case, for example, with many CLR. Kretz-Rommel et al. therefore used a mouse model with a humanized immune system to study DC-SIGN targeting. Targeting antigens to DC-SIGN in this model induced antigen-specific T cell responses[117]. Recently, Flacher et al. published a paper describing targeting of DC using

antibodies in human skin[118]. Antibodies directed to DEC-205 or Langerin were injected intradermally and interestingly, efficient targeting of LC was observed, indicating that the antibodies can diffuse from the dermis towards the epidermis. The LC thus targeted through Langerin were not capable of priming T cell responses, however[118].

Nanobodies are single-domain antigen-binding fragments of camelid (from camels or llamas)-specific heavy chain-only antibodies. These nanobodies bind antigen without requiring domain pairing and have a therapeutic advantage over classic antibody fragments because of their smaller size, robustness, and preference to target unique epitopes. Nanobodies have been successfully used to target toxic enzymes or to block specific molecular interactions. Cortez-Retamozo et al. used a nanobody directed to CEA to target the prodrug converting enzyme β-lactamase specifically to tumor cells, resulting in tumor specific toxicity after injection of the prodrug[119]. Another approach described the construction of nanobodies directed to EGFR to block the binding of the growth factor EGF specifically to its receptor[120]. Currently, two nanobodies for the treatment of thrombosis are being tested in clinical trials. Thus far, no literature is available describing the use of nanobodies to activate the immune system. In theory, however, coupling of TAA to a DC-specific nanobody should result in effective targeting. Indeed, a recent paper by de Groeve et al. does describe the construction of a DC-specific nanobody[121].

Finally, synthetic long peptides should be mentioned as a DC-targeting strategy. Preclinical studies showed increased vaccination efficacy of synthetic long peptides over short peptides which was attributable to selective uptake by DC and prolonged antigen presentation to $CD4^+$ T cells and CTL[122]. Several clinical trials with synthetic long peptides tumor vaccines emulsified in Montanide have since been carried out. In a phase II trial in which women with stage III vulval intraepithelial neoplasia received 3–4 vaccines of an HPV16 E6/E7 SLP vaccine in incomplete Freund's adjuvant, clinical responses were observed in 15/19 patients, with complete regressions in 9 patients[123]. Responses were associated with the strength and breadth of induced $CD4^+$ and $CD8^+$ IFN-γ effector T cell responses. A next generation of synthetic long peptides vaccines is now under development, in which they are coupled to TLR ligands to ensure simultaneous DC targeting and activation[124].

6.10 Conclusions and Future Developments

A wide variety of vehicles and targeting motifs are now identified and available for use in the design of DC-targeted vaccines. Although some targets are more DC-restricted than others, their selection is mostly predicated by the DC subset to be targeted and, importantly, the vaccine delivery route: some targets may be relatively specific for DC in one tissue microenvironment, but not necessarily in another. Further translational studies are urgently needed to explore the best (combination of) DC targeting motifs and the preferred DC subset(s) to be targeted, as well as the most optimal route of in vivo delivery to achieve efficacious

DC-mediated immunization. When potentially immunosuppressive B cells, macrophages or other bystander APC are co-targeted as a collateral consequence of poor DC selectivity, this could lead to T cell tolerance rather than anti-tumor immunity. Also, care must be taken when selecting a DC-targeted vaccine formulation in terms of modulatory effects on the DC activation state and functionality. For tumor vaccination in particular it is of vital importance to ensure optimal DC activation upon vaccine delivery with preferential Th1 skewing and CTL activation, and to increase or decrease other arms of immunity as their relative importance in specific cancers becomes better known. In cancer patients where immunosuppressive conditions often prevail this may sometimes be a tall order, but it is nevertheless of the essence. Although DC-targeted vaccine approaches receive considerable attention and are becoming a major focus of attention in the tumor immunology field, clinical translation is seriously lagging with very few vaccines set to be tested in patients within the foreseeable future. Nevertheless, there is a general consensus in the field that in vivo DC targeting vaccines are a most promising way forward. Newly identified promising targeting motifs and advances in the fields of virology and nanotechnology should prove instrumental in developing effective new agents.

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Chapter 7
Cytokines in the Treatment of Cancer

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Abstract Cytokines are molecular mediators of intercellular signaling that function to regulate homeostasis of the immune system. There are five families of cytokines classified by their receptor usage. The effects of individual cytokines on immunity depend on several factors, including the local cytokine concentration, the pattern of cytokine receptor expression, and the integration of multiple signaling pathways in responding immune cells. Cytokines have shown therapeutic potential for the initiation and potentiation of antitumor immunity. Interferon-α and interleukin-2 (IL-2) have been approved as single agents for the treatment of metastatic melanoma and renal cell carcinoma, and several other cytokines have shown promise in preclinical tumor models. New strategies for improving the therapeutic benefit of cytokines are in development and include cytokine-antibody fusion molecules, delivery in recombinant viral vectors, expression by irradiated whole tumor cells, PEGylation, DNA vaccination, and ex vivo exposure to immune effector cells.

7.1 Introduction

Cytokines are molecular mediators of intercellular communication that signal through a series of shared receptors to regulate the function and homeostasis of the immune system. Using the immune system to prevent cancer and eradicate established tumors are major goals of tumor immunotherapy. The generation of potent, specific, and durable antitumor immunity requires a variety of cytokines that regulate important functions related to the balance between tumor recognition and

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rejection by antigen-specific effector cells on the one hand, and suppressive mechanisms that allow tumors to escape immunologic detection on the other. The significance of cytokines in tumor immunosurveillance has been demonstrated in mice genetically deficient in Type I or II interferon (IFN) receptors or elements of downstream IFN receptor signal transduction. These mice exhibit a higher frequency of spontaneous cancers compared to control mice[1–4]. The past decade has seen a growing understanding of the molecular and cellular basis of tumor immunosurveillance and antitumor immunity, with many tumor-specific and tumor-associated antigens being defined and a major focus on the importance of T cells in mediating tumor recognition and rejection. Since cytokines play a major role in regulating antigen presentation and all aspects of T cell activity, they have been widely investigated for their potential to enhance tumor immunotherapy.

The most extensively studied cytokines for cancer treatment are members of the IFN and interleukin families. Two cytokines (interferon-α and interleukin-2) have been approved as single agents for the treatment of metastatic renal cell carcinoma and melanoma. Interleukin-2 (IL-2), as well as other members of the IL-2-related family of T cell growth factors (e.g., IL-4, IL-7, IL-9, IL-15, and IL-21), utilize a common receptor signaling system that results in the activation and expansion of $CD4^+$ and $CD8^+$ T cells. More recently, the regulatory nature of IL-2 has been demonstrated by experiments showing similar activation kinetics for regulatory $CD4^+FoxP3^+$ T cells (Tregs) in response to IL-2-mediated signaling resulting in suppression of antigen-specific effector T cell function. The potential dominance of this regulatory function for IL-2 has been demonstrated in mice genetically lacking IL-2 or functional IL-2 receptor, which exhibit a phenotype characterized by loss of self-tolerance and development of autoimmunity[5]. This counter-regulatory role of IL-2 suggests its dual role in the homeostasis of T cells and their functions and has implications for the role of IL-2 treatment of cancer patients. These observations have also resulted in new cytokine-based therapeutic strategies designed to stimulate effector T cells while blocking suppressive/regulatory T cells but with the need to avoid dangerous autoimmune consequences.

The goal of this chapter is to review in brief the major cytokines involved in cancer treatment and discuss their basic biology and clinical applications. The chapter also describes new cytokines in preclinical development and discuss several novel approaches for cytokines in the treatment of cancer and potential new directions for future investigation using cytokines or combinations of biological agents for cancer therapy. An effort has been made to include cytokines that have already advanced into clinical use or have a strong preclinical basis for demonstrating therapeutic benefit in cancer patients.

7.2 Classification of Cytokines and Cytokine Receptors

Cytokines are secreted proteins that have pleiotropic effects on adaptive immunity, regulation of innate immunity, and hematopoiesis (Table 7.1). In the immune system, cytokines function in cascades and with some degree of redundancy.

Table 7.1 General features of cytokines

Cytokine	Primary cell source	Primary target cell	Biologic activity
GM-CSF	T cells Macrophages Endothelial cells Fibroblasts Mast cells	Bone marrow progenitor cells DC Macrophages	Promotes antigen presentation T cell homeostasis Hematopoetic cell growth factor
IL-1	Monocytes Macrophages Fibroblasts Epithelial Cells Endothelial Cells Astrocytes	T cells B cells Endothelial cells Hypothalamus Liver	Co-stimulation Cell activation Inflammation Fever Acute phase reactant
IL-2	T cells NK cells	T cells NK cells B cells Monocytes	Cell growth/activation
IL-3	T cells	Bone marrow progenitor cells	Cell growth and differentiation
IL-4	T cells	T cells B cells	Th2 differentiation Cell growth/activation IgE isotype switching
IL-5	T cells	B cells Eosinophils	Cell growth/activation
IL-6	T cells Macrophages Fibroblasts	T cells B cells Liver	Co-stimulation Cell growth/activation Acute phase reactant
IL-8	Macrophages Epithelial Cells Platelets	Neutrophils	Activation Chemotaxis
IL-10	Th2 cells	Macrophages T cells	Inhibits antigen-presenting cells Inhibits cytokine production
IL-12	Macrophages NK cells	T cells	Th1 differentiation
IL-15	Monocytes	T cells NK cells	Cell growth/activation NK cell development Blocks apoptosis
IL-18	Macrophages	T cells NK cells B cells	Cell growth/activation Inflammation
IL-21	CD4+ T cells NKT cells	NK cells T cells B cells	Cell growth/activation Control of allergic responses and viral infections
IL-23	Antigen-presenting cells	T cells NK cells DC	Chronic inflammation Promotes Th17 cells

(continued)

Table 7.1 (continued)

Cytokine	Primary cell source	Primary target cell	Biologic activity
IFN-α	Plasmacytoid DC NK cells Macrophages Fibroblasts Endothelial cells Osteoblasts	Macrophages NK cells T cells	Antiviral Enhances MHC expression Activates and promotes survival of T cells Activates NK cells, macrophages
IFN-γ	T cells NK cells	Monocytes Macrophages Endothelial Cells Tissue cells	Cell growth/activation Enhances MHC expression
TGF-β	T cells Macrophages	T cells	Inhibits cell growth/ activation
TNF-α	Macrophages T cells	T cells B cells Endothelial cells Hypothalamus Liver	Co-stimulation Cell activation Inflammation Fever Acute phase reactant

DC dendritic cell; *GM-CSF* granulocyte-macrophage colony stimulating factor; *IL* interleukin; *IFN* interferon; *TNF* tumor necrosis factor; *TGF* transforming growth factor

IFNs were the first cytokines identified and named for their ability to "interfere" with viral replication. Subsequent cytokines were referred to as interleukins because they were produced by and acted on leukocytes. The cytokines generally utilize a series of common and shared receptors, which have proven useful for a more functional classification of cytokines (Fig. 7.1). To date, there are seven cytokine receptor families (see Table 7.2): receptors for Type I cytokines, Type II cytokines, immunoglobulin superfamily members, tumor necrosis factors (TNF), transforming growth factor β (TGF-β), receptors coupled with cell membrane-based G-proteins, and the recently described IL-17 receptors. This chapter focuses on cytokines that signal through the Type I and II cytokine receptor families, as these have the most immediate clinical potential.

Type I cytokine receptors are expressed as transmembrane proteins on the surface of effector cells and respond to cytokines containing four α-helical strands. These receptors share a common amino acid motif (WSXWS) located in the extracellular portion of the receptor. The Type I receptors assemble as heteromeric chains made up of subunits that contribute to cytokine binding or signal transduction. The Type I cytokine receptors, which include receptors for IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21, share a common signaling subunit, the common γ chain (γ_c), that complexes with a cytokine-specific moiety to initiate intracellular signals

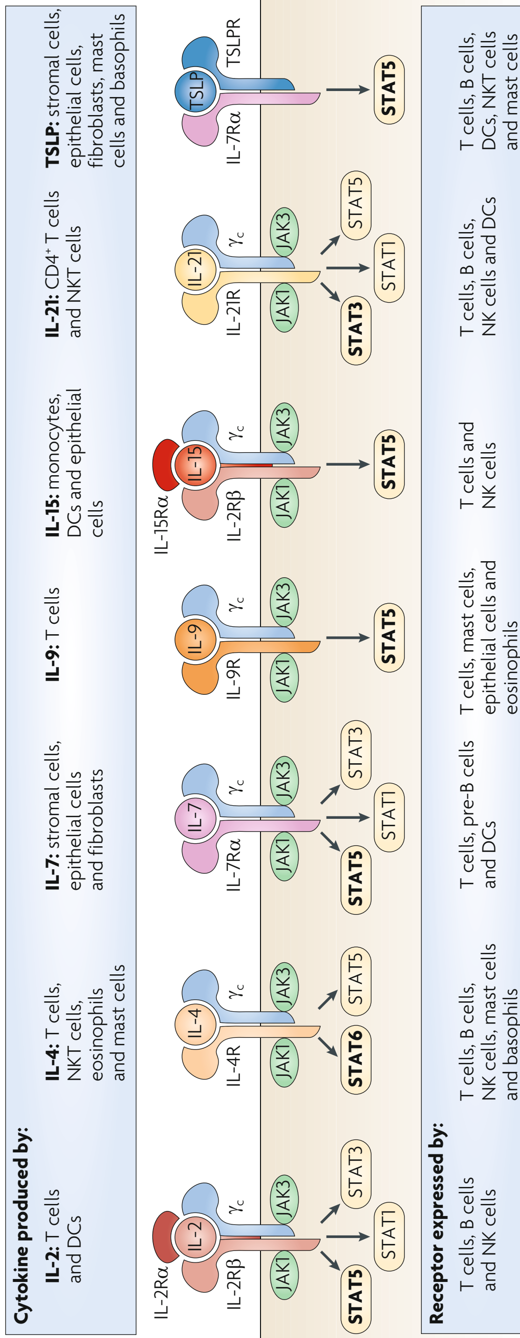

Fig. 7.1 Structural diagram of the major cytokine receptor families. Note that related receptors share common signaling chains and biologic activity is related to the presence of cytokine-binding chains, spatial orientation of the receptor complex, and the temporal and cellular pattern of receptor expression.

Table 7.2 Classification of cytokine receptor families

Receptor family	Ligands	Structure/function
Type I cytokine receptors	IL-2	Composed of multimeric chains
	IL-3	Responds to four α-helical cytokines
	Il-4	Contains WSXWS amino acid motif
	IL-5	Signals through JAK-STAT pathway using common signaling chain
	IL-6	
	IL-7	Contains cytokine-binding chains
	IL-9	
	IL-11	
	IL-12	
	IL-13	
	IL-15	
	IL-21	
	IL-23	
	IL-27	
	Erythropoietin	
	GM-CSF	
	G-CSF	
	Growth hormone	
	Prolactin	
	Oncostatin M	
	Leukemia inhibitory factor	
Type II cytokine receptors	Interferon-α/β	Immunoglobulin-like domains
	Interferon-γ	Uses heterodimer and multimeric chains
	IL-10	No WSXWS motif
	IL-20	Signals through JAK-STAT
	IL-22	
	IL-28	
Immunoglobulin superfamily receptors	IL-1	Shares homology with immunoglobulin structures
	CSF1	
	c-kit	
	IL-18	
IL-17 receptor	IL-17A	Contain four highly conserved cysteine residues
	IL-17B	
	IL-17C	
	IL-17D	
	IL-17E	
	IL-17F	
G protein-coupled receptors (GPCR)	IL-8	Contains a seven transmembrane helix
	CC chemokines	Functions to mediate cell activation and migration
	CXC chemokine	

(continued)

Table 7.2 (continued)

Receptor family	Ligands	Structure/function
TGF-β receptors 1/2	TGF-β	Contains serine/threonine kinase activity Signaling modulated by co-receptors Cytokine binding is sequential and cooperative Signals through Smad 2 and 3, and other pathways (e.g., MAPK) Regulates cell proliferation, differentiation, apoptosis, development, and tissue homeostasis
Tumor necrosis factor receptors (TNFR)	CD27 CD30 CD40 CD120 Lymphotoxin-β	Contains cysteine-rich extracellular domains Functions as co-stimulatory and co-inhibitory receptors

CD cluster of differentiation; *c-kit* mast/stem cell growth factor receptor; *CSF* colony-stimulating factor; *G-CSF* granulocyte-colony stimulating factor; *GM-CSF* granulocyte-macrophage colony stimulating factor; *IL* interleukin; *JAK* janus kinase; *STAT* signal transducer and activator of transcription; *TGF* transforming growth factor

through the coordinated activity of Janus kinases (JAK) 1 and 3, and signal transducers and activators of transcription (STAT) molecules (Fig. 7.1)[5]. Additional Type I cytokine receptor subgroups include the granulocyte/macrophage colony-stimulating factor (GM-CSF) and IL-6 receptor families, which share a common gp130 receptor subunit that mediates complex multi-pathway signal transduction in its target cells[6–9]. The gp130 signal transduction component is utilized by several receptor complexes, including IL-6, IL-11, leukemia inhibitory factor (LIF), oncostatin M, cardiotrophin-1, and ciliary neurotrophic factor, that have redundant and pleiotropic effects on the immune, hematopoietic, and nervous systems[10]. Likewise, IL-3, IL-5, and GM-CSF are also recognized by receptors in a separate GM-CSF receptor subfamily that shares a common β chain that complexes with the cytokine-specific α chain[11].

The effects of IFN-α, IFN-β, IFN-γ, and IL-10 are mediated by Type II cytokine receptors, which are composed of a signaling chain and a ligand-binding chain. The sequences of the Type II cytokine receptors resemble tandem Ig-like domains and the intracellular segments are typically associated with a tyrosine kinase of the JAK family[12]. The immunoglobulin superfamily receptors contain extracellular immunoglobulin domains and include the receptors for IL-1, IL-18, stem cell factor, and monocyte CSF[13].

Cytokines are responsible for the induction of active immune responses against tumors as well as the negative regulation of immune responses in maintaining homeostasis and self-tolerance. Self-tolerance is mediated by two major classes of $CD4^{+}FoxP3^{+}$ Tregs (see chapter 9 for details). Understanding how cytokines regulate the generation and maintenance of Tregs—and how to break this component of tolerance to achieve and maintain successful antitumor immunity—is an important area of current investigation[14]. $CD4^{+}FOXP3^{+}$ Tregs can arise in the periphery in a tolerogenic environment and mediate immune suppression.

In contrast, a naturally occurring $CD4^{+}FOXP3^{+}$ T cell population (nTreg) mediates immune suppression in a contact-dependent, cytokine-independent manner[15]. Throughout the process cytokines regulate the number and functionality of these cells as well as the effector cells that fight pathogens and tumors. It is this delicate balance between effector cells and Tregs that is critical for influencing the rejection or progression of tumors. IL-2, TGF-β, and IL-10 (and probably other cytokines) have been shown to modulate the generation of Tregs and may be involved in the fine balance between effector and regulatory populations[16, 17].

7.3 Structural Physiology and Functional Role of Cytokines

7.3.1 General Features of Cytokine Structure and Function

Cytokines play critical roles in the development of the immune system, host defense, and tumor immunobiology. Thus, understanding the biological activities and mechanism of action of these elements is central to developing cytokine-based immunotherapy in cancer treatment. Cytokines directly stimulate immune effector cells and stroma cells at the tumor site and enhance tumor cell recognition by cytotoxic effector T cells. When they become impaired at the tumor site, effector cells can then present an important obstacle to both spontaneous and deliberately induced T cell immunity against cancer. Numerous animal tumor model studies have demonstrated that cytokines have broad antitumor activity and this has been translated into a number of cytokine-based approaches for cancer therapy. To date, two cytokines have achieved FDA approval for cancer treatment: high-dose, bolus IL-2 for metastatic melanoma and renal cell carcinoma and IFN-α for the adjuvant therapy of Stage III melanoma (IFN-α is also used with bevacizumab for renal carcinoma and was formerly used for several hematologic malignancies until it was supplanted by agents with superior therapeutic indices, as detailed below). Other cytokines, including GM-CSF, IL-7, IL-12, IL-15, IL-18, and IL-21, have entered into clinical trials for patients with advanced cancers of various types. There is also preclinical data supporting the neutralization of suppressive or inflammatory cytokines, such as IL-6, IL-10, and TGF-β, in promoting antitumor immunity.

7.3.2 Interferons

The IFNs can be classified by their ability to bind to specific receptors termed Type I and Type II IFN receptors, which are a subset of the Type II cytokine receptors[18]. IFN-α and IFN-β are predominantly involved in cellular immune responses against viral infections[19, 20]. The IFN-α and IFN-β family actually represent over 20 distinct molecules that are classified according to their ability to activate Type I IFN receptors

(and are collectively referred to as Type I IFNs)[18–21]. The Type I IFNs all share the same receptor complex (INF-αR1 and INF-αR2), whereas Type II IFN-γ binds to a distinct receptor complex (IFNγR1 and IFNγR2)[22]. IFN-γ is the only Type II IFN and is also important in cell-mediated immunity and activates the Type II IFN receptor[23]. While the therapeutic potential for IFN-γ has been limited (see below), its secretion or the expression of its gene in effector lymphocytes is used commonly as a readout in laboratory assays for antigen-specific effector cell function.

Although numerous murine studies originally suggested an important role for IFN-γ in tumor immunity, the Type I IFNs have emerged as the most clinically useful IFNs for the treatment of cancer. These IFNs induce expression of major histocompatibility complex (MHC) class I molecules on tumor cells and mediate the maturation of a subset of dendritic cells (DCs)[24–27]. Type I IFNs can also activate cytotoxic T lymphocytes (CTLs), natural killer (NK) cells, and macrophages[28, 29]. In addition to their immunologic effects, the Type I IFNs can also exert a cytostatic effect on tumor cells and may also promote tumor cell apoptosis[30]. When administered at lower doses, they also have anti-angiogenic effects on tumor neovasculature[31]. Mice with targeted deletion of the Type I IFN receptor have a higher rate of carcinogen-induced cancer and increased tumor growth in transplantable tumor models supporting the hypothesis that the Type I IFNs are important in tumor immunosurveillance[32, 33].

IFN-α comprises a group of at least 12 distinct proteins. Recombinant IFNα-2a, IFNα-2b, and IFNα-2c differ by one or two amino acids and are the isoforms most commonly used in the clinic[18]. Since IFN-α and IFN-β signal through the same receptor, they would be expected to have similar biologic effects and have overlapping indications. This prediction has not, however, been confirmed clinically and the mechanism of antitumor activity in vivo is not completely defined for this group of IFNs. IFNs activate the JAK-STAT signaling pathway. IFN-γ phosphorylates JAK1 and JAK2 proteins which produces a recruitment site for STAT1 while Type I IFNs (IFNα or IFNβ) stimulate the activity of JAK1 and TYK2 proteins, leading to STAT2 tyrosine phosphorylation, and induce IL-4 secretion and subsequent activation of B cells[34]. IFN-α also induces direct apoptosis of tumor cells in a caspase-dependent manner, which may contribute to the well-known properties of Type 1 and 2 IFNs to enhance tumor cell antigen expression as well as co-stimulatory and co-inhibitory receptors that are essential to the type of immune reaction resulting between tumor and effector cells[35]. At low doses, IFN-α also acts as an anti-angiogenic agent[36]. While the specific mechanisms of IFN-mediated tumor rejection in animal models have not been fully elucidated, IFN-α has been the most widely investigated cytokine for human cancer treatment and may prove to be a valuable component of combinatorial strategies for immunotherapy of solid tumors.

IFN-γ is secreted by NK cells, NKT cells, $CD4^+$ T cells, $CD8^+$ T cells, antigen-presenting cells (APCs) and B cells[37–39]. IFN-γ activates macrophages and induces the expression of MHC class I, MHC class II, and co-stimulatory molecules on APCs[40–42]. Additionally, IFN-γ induces changes in the proteasome leading to enhanced antigen presentation[43, 44]. IFN-γ also promotes Th1 differentiation of

CD4$^+$ T cells and blocks IL-4 dependent isotype switching in B cells[40, 45]. Mice with targeted deletion of IFN-γ or the Type II IFN receptor have an increased risk of spontaneous and chemically induced tumors compared to controls[1, 3, 33, 44]. IFN-γ is cytotoxic to some malignant cells and has modest anti-angiogenic activity[46–49]. While the antitumor effects of IFN-γ in murine models suggested it would be effective against a wide spectrum of tumors, IFN-γ has demonstrated very limited clinical utility in cancer therapy, in part because of a narrow therapeutic index. IFN-γ may be an important regulator of antitumor activity mediated by other cytokines, in particular IL-12 and probably IL-2[50, 51].

7.3.3 Interleukin-2 Family

The biological effects of IL-2, a 15.5 kDa variably glycosylated protein comprises four antiparallel α-helices, are mediated by the IL-2 receptor, a trimeric complex composed of an α (CD25), β (CD122), and γ (CD132) chain. The β and γ chain are involved in signaling, while the ligand-specific α chain is only involved in cytokine binding. These subunits form a high (αβγ), intermediate (βγ), or low (α) affinity receptor depending on which of the chains are in the cell surface complex[52, 53]. Although the β and γ chains are expressed on T cells, B cells, and NK cells[54], the α chain is inducible and is expressed only by T cells but is present on several phenotypically and functionally distinct classes of T lymphocytes. The predominant cellular source of IL-2 is the CD4$^+$ T cell, predominantly the Th1 subset, and the major physiologic role of IL-2 is to promote the activation and proliferation of T and NK cells in an autocrine and paracrine manner[55]. In contrast to T cells, NK cells express the intermediate affinity IL-2 receptor (no α subunit). Exposure of NK cells to IL-2 results in proliferation, enhanced cytolytic activity, and secretion of other cytokines. B cells also express intermediate affinity IL-2 receptors and can secrete IL-2 in cooperation with other cytokines, resulting in B cell proliferation and differentiation[54].

IL-2 also plays a critical role in suppressing T cell responses. A subpopulation of CD4$^+$ T cells, characterized by high levels of CD25 and the forkhead/winged helix transcription factor FoxP3, function to suppress self-reactive T cells[56]. These Tregs maintain tolerance and prevent autoimmunity after activation of effector T cell responses. This is supported by data demonstrating that depletion of CD4$^+$FoxP3$^+$ Tregs breaks tolerance to self-antigens and can lead to increased autoimmunity[57]. Furthermore, in murine models, depletion of CD4$^+$FoxP3$^+$ Tregs enhanced tumor rejection and improved therapeutic responses to cancer vaccines by promoting the function of CD8$^+$ CTLs[58]. The mechanisms by which Tregs inhibit the function of CD8$^+$ CTLs are incompletely understood (see chapter 9 for more Treg details). Interestingly, the immunologic consequences of loss of IL-2 signaling, as demonstrated in mice with targeted deletion of IL-2 or the IL-2 receptor that develop a generalized inflammatory syndrome and often die of autoimmune colitis[56–59], are further evidence of the importance of IL-2 not only as an activator

of immune responses but also as an important element of immune tolerance. This view of IL-2 as a regulatory cytokine, rather than a purely stimulatory T cell growth factor, suggests that the use of IL-2 in the clinical setting needs to be re-evaluated. In support, IL-2 can increase Tregs and alter their trafficking patterns in humans (see discussion below, and chapter 9 for details). An important area of further investigation will be a more careful analysis of the dosing, schedule, and kinetics of IL-2 administration on specific T cell subsets.

7.3.4 Interleukin-12 Family

Interleukin-12 (IL-12) is a heterodimeric cytokine containing a 35 kD and a 40 kD subunit that signals through a receptor of the Type I family of cytokine receptors. IL-12 is produced mainly by phagocytic cells in response to antigenic stimulation, leading to cytokine production, primarily IFN-γ, from NK and T cells[60]. IL-12 also acts as a growth factor for activated NK and T cells, promotes $CD4^+$ T cell differentiation into Th1 $CD4^+$ T cells and enhances the activity of $CD8^+$ CTLs[61]. IL-12 has demonstrated antitumor activity in murine models of melanoma, colon carcinoma, mammary carcinoma, and sarcoma[62–72]. Experimental investigation of the mechanism of IL-12 activity using mice with molecularly targeted defects suggests that the effector cells involved in the antitumor immune response to IL-12 differ by species, by tumor model and by dose and schedule of IL-12 as well as other cytokines and elements of the immune microenvironment. For example, in the B16 murine melanoma model, a significant role for NK cells has been demonstrated in mediating antitumor immunity with high doses of IL-12[20]. In contrast, antitumor responses at low doses of IL-12 appear to be mediated by NKT cells[70]. IL-12 also elicits anti-angiogenic effects that require IFN-γ and are mediated by IFN-γ-inducible protein 10 (IP-10), a chemokine induced in a variety of cells in response to IFN-γ and lipopolysaccharide[71].

7.3.5 GM-CSF Family

GM-CSF, a heterogeneously glycosylated 14–35 kD polypeptide, was initially identified as a mediator of hematopoiesis and monocyte-macrophage differentiation[73]. GM-CSF is also a highly pleiotropic cytokine and is closely related to IL-3, which also stimulates multi-lineage myelopoiesis, and IL-5, the predominant growth factor for eosinophils, by way of a common β chain on the GM-CSF receptor[74]. The receptors for GM-CSF, like those for IL-3 and IL-5, are composed of two subunits, a ligand-specific α chain and a common β chain. GM-CSF is produced by monocytic cells and T cells and promotes the maturation of DC. The

potential for GM-CSF to stimulate immune responses has been shown in many tumor models, including a murine melanoma in which transgenic expression of GM-CSF provided protection to subsequent tumor challenge in over 90 % of the animals[75], and promising results have been observed in other tumors when used alone and in combination with other immunomodulators such as checkpoint-blocking antibodies[76, 77]. The antitumor activity of GM-CSF appears to be related to its ability to activate macrophages and DC[78, 79]. GM-CSF also matures DCs leading to upregulation of co-stimulatory molecules and CD1d receptors[80]. Initial studies suggested that $CD4^+$ and $CD8^+$ T cells mediated GM-CSF-stimulated antitumor immunity, but recent models using CD1d-deficient mice support a critical role for NKT cells in GM-CSF antitumor immune responses[81]. More recently, it has been suggested that GM-CSF may serve a more regulatory role in the induction of DC-mediated T cell immunity through complex interactions with milk fat globule-8 (MFG-8), a glycoprotein on APCs that contributes to the control, under various biologic conditions, of immunologic responses resulting from their interactions with T cell subsets[82].

7.3.6 Interleukin-10

Interleukin-10 (IL-10) is a homodimeric 17–20 kDa glycoprotein with an α-helical tertiary structure that signals through a JAK–STAT complex, the specific components of which vary with the target cell type. The IL-10 receptor is a member of the IFN receptor family and has two subunits, an α subunit that is primarily expressed on immune cells, with the highest density on monocytes and macrophages, and a β subunit that is found ubiquitously. IL-10 is produced by many different cells of the immune system, including T and B lymphocytes, monocytes, DC, and NK cells[83]. While IL-10 generally functions as an immunosuppressive cytokine, polarizing T cell responses towards the Th2 phenotype associated with other suppressive cytokines like IL-4, IL-13, and TGF-β, IL-10 can also have stimulatory effects in certain circumstances, including the stimulation of macrophage phagocytosis and NK cytotoxicity while suppressing inflammatory cytokines, antigen-presentation, and T cell response[84–87]. IL-10 can act as a growth factor for malignant B cells such as the plasma cell clone of myeloma and other B cell lymphoproliferative diseases[88]. There are also preclinical data suggesting that one mechanism of antitumor activity induced by CTLA-4 blockade (covered in chapter 8) might be through a decrease in IL-10 secretion[89].

Various tumor cells can produce IL-10, including cells from non-small cell lung cancers, melanomas, gliomas, leukemias, and lymphomas[90–94]. Furthermore, increased IL-10 production has been observed in tumor-infiltrating lymphocytes (TIL) from patients with aggressive malignancies such as advanced non-small cell lung cancer and in peritoneal monocytes from patients with malignant ascites from advanced ovarian cancer[95, 96]. Constitutional IL-10 promoter polymorphisms have

been associated with susceptibility to certain malignancies, suggesting that this cytokine may play a critical role in some aspect of tumor immunosurveillance[97–99].

7.4 Cytokine Therapy in Clinical Practice

Two cytokines, IFN-α and IL-2, have been approved by the FDA for the treatment of cancer. Both cytokines are approved as single agents and have been widely explored as components of various combination regimens. This section focuses on these approved agents.

7.4.1 Interferon-α

IFN-α has been approved for the adjuvant treatment of high-risk melanomas well as for hairy cell leukemia and AIDS-related Kaposi's sarcoma. It is also a component of approved anti-angiogenic therapy in combination with bevacizumab for advanced renal cancer. IFN-α has been particularly effective as therapy for hematologic malignancies including hairy cell leukemia and chronic myelogenous leukemia. For the treatment of hairy cell leukemia, IFNα-2b given at 2 million units/m^2 subcutaneously three times a week for 1 year resulted in an overall response rate of 77 % with a complete response rate of 5 %. This relatively low dose is well-tolerated. Patients with an intact spleen appear to achieve an even greater complete response rate of 25–35 % in follow-up studies, suggesting the importance of early initiation of cytokine therapy in this disease. While relapses are common in hairy cell leukemia following IFN-α therapy, retreatment provides remissions in most patients[100, 101]. Nevertheless, the advent of nucleoside analogs, with a complete response rate close to 90 % and durable remissions in most patients, has relegated IFN-α therapy to second-line treatment in patients with refractory disease or in those with contraindications to nucleoside analog drugs[102].

IFN-α is the only currently approved adjuvant therapy for patients with high-risk stage II or stage III melanoma based on data from a cooperative group, multi-institutional clinical trial. In this trial, patients with primary lesions greater than 4 mm or those with regional lymph node involvement were treated following lymph node dissection with 1 year of IFN-α2b. The regimen consisted of 20 million units/m^2/day intravenously 5 days per week for 4 weeks followed by 10 million units/m^2/day subcutaneously three times a week for an additional 48 weeks. An overall improvement in median relapse-free survival from 1 to 1.7 years and median overall survival from 2.8 to 3.8 years was reported[103]. A follow-up intergroup clinical trial that also included a low-dose IFN-α cohort and did not require surgical staging for clinically node-negative patients also reported an improvement in median and overall relapse-free survival for high-dose IFN-α over observation (no benefit for low-dose therapy) but failed to demonstrate any improvement in overall survival[104]. The reason for these disparate outcomes is not clear but has been attributed to the subsequent off-protocol

use of IFN-α following nodal relapse in the observation group. A subsequent large cooperative group trial compared a novel ganglioside GM2 vaccine to the same one-year high-dose IFN-α therapy and was halted early when patients receiving IFN-α alone demonstrated a significant increase in both relapse-free and overall survival over those who were randomized to receive vaccine[105]. Although questions have been raised as to the possibility that the vaccine actually worsened the outcome, its inferior performance compared with IFN-α provided continued support for the use of IFN-α routinely in the adjuvant setting.

Subsequent long-term follow-up data regarding patients in the cooperative group trials detailed above revealed that the tails of the curves for relapse-free and particularly overall survival no longer demonstrate significant benefits for IFN-α over the comparators[106]. In a more recent meta-analysis of 14 randomized clinical trials enrolling 8,122 patients over an 18-year period, IFN-α was associated with a significant improvement in disease-free survival in 10 of 17 comparisons and improved overall survival in 4 of 14 comparisons[107]. A long-acting form of IFN-α that is chemically modified with a polyethylene glycol moiety (PEG-IFN) to increase its serum half-life was recently approved for the adjuvant therapy of high-risk melanoma. While PEG-IFN showed a favorable effect on relapse-free but not on overall survival[108], there was a significant survival benefit for patients with an ulcerated primary and microscopic nodal metastasis, a finding which led to the current trial of PEG-IFN for this cohort of patients. Recent data demonstrating the antitumor activity and survival benefit of the CTLA4 immunologic checkpoint blocking agent ipilimumab (see chapter 8) also provided the basis for the ongoing US intergroup trial comparing unmodified IFN-α (using the regimen detailed above) with two different doses of ipilimumab for patients with high-risk stage III and resected stage IV melanoma (http://clinicaltrials.gov/ct2/show/NCT01274338?term=interferon+and+ipilimumab+and+adjuvant+and+melanoma&rank=1). The proof of concept associating favorable outcomes between IFN-α adjuvant therapy and the development of selected immune-related outcomes such as thyroid dysfunction, vitiligo, and serologic evidence of connective tissue diseases was provided by a report from the Hellenic Oncology Group in 2006[109]. Although subsequent analyses from the USA and European studies did not find this association[110], the ongoing study of IFN-α vs. ipilimumab will attempt to identify these and/or other correlates of autoimmunity as evidence of benefit and will study pretreatment host and tumor factors to look for predictive biomarkers in all three cohorts.

Experience with IFN-α administration has resulted in established guidelines for recognition and management of toxicities and side effects. The toxicity profile of IFN-α is usually dose-related, and most side effects can be managed without discontinuation of treatment. Constitutional symptoms including fever, fatigue, headaches, gastrointestinal symptoms, and myalgias are quite common and will likely occur in 80 % or more of patients. IFN-α also produces increases in blood levels of hepatic enzymes in some patients, particularly during the high-dose intravenous period when patients should be monitored frequently. Therapy should be held and dosing decreased for those with hepatic enzyme elevations during therapy. Thrombocytopenia, leukopenia, and neutropenia are common and can also

be readily managed with dose reductions although rarely transfusion may be required[111, 112]. More serious are the neuropsychiatric issues, which include depression (45 %), confusion (10 %), and mania (<1 %)[111–113]. In some studies of IFN-α, depression was highly significant and rare suicides were reported[113]. Permanent alterations of the immune system have also been reported, including common development of vitiligo and hypothyroidism and rare occurrence of sarcoidosis, lupus, rheumatoid arthritis, polymyalgia rheumatica, and psoriasis[114–116]. In view of these observations and those detailed above, it is likely that the mechanisms of IFN-α in melanoma and the possible association with selected parameters of altered immune control suggest that it may be possible to identify underlying tumor or patient factors predictive of benefit prior to initiating therapy and to avoid the toxicities of IFN-α in those predicted to have no benefit. To date, attempts to find such predictors among the polymorphisms in the HLA system and single nucleotide polymorphisms of the checkpoint protein CTLA-4 have not been fruitful.[117, 118]

7.4.2 Interleukin-2

IL-2 plays a pivotal role in the treatment of patients with metastatic melanoma and renal cell carcinoma. Malignant melanoma is a tumor of melanocytes. Many primary cutaneous melanomas exhibit histologic regression coincident with infiltration of T cells and NK cells at the time of clinical detection[119–121]. In general, melanoma has not been responsive to cytotoxic chemotherapy. Thus, early work focused on the generation of effective immune responses in melanoma patients. Early work in the Surgery Branch of the National Cancer Institute found that adoptively transferred IL-2-activated peripheral blood mononuclear cells with the phenotype and functional characteristics of activated NK cells, supported with concomitant administration of IL-2 in high doses, resulted in significant tumor regression in patients selected for normal organ function and good performance status[122]. Further investigation of these encouraging results suggested that therapeutic benefit could be seen in a subset of patients treated with high doses of IL-2 alone.

High-dose IL-2 induces objective clinical responses in 15–20 % of patients with advanced melanoma and durable complete responses in 5–7 % of these patients[123, 124]. In order to reduce IL-2-related side effects, a variety of modifications to high-dose IL-2 regimens have been tested in patients with melanoma, including alterations of dose, schedule, and route as well as chemical alterations of IL-2 molecular structure that alter its cellular targets. Other modulations, including the addition of toxicity modulators such as drugs with anti-inflammatory properties or anti-angiogenic agents, have also been tested. Unfortunately, none of these modifications has led to an improved therapeutic index, and current strategies are revisiting the concept of investigating host and tumor biologic and immunologic properties that might predict benefit and allow for selection of patients with an improved therapeutic index. Additional efforts are underway to develop combinations of

immunomodulators that combine cytokines with other agents to enhance antitumor immunity. IL-2 continues to play an important role as a T cell growth factor in adoptive T cell therapies (as a component of the T cell expansion procedure and administered following cell infusions) but is likely to be supplanted or augmented by other cytokines such as IL-15, IL-21, or IL-7 as their advantages and possible synergies become better understood.

Metastatic renal cancer, particularly the clear-cell histology that comprises the majority of cases, is another tumor that is inherently resistant to cytotoxic agents and has shown responsiveness to immune modulators such as IFN-α and IL-2. The response rate with high-dose intravenous bolus IL-2 is around 20–25 % for metastatic renal cell carcinoma patients, similar to that seen in melanoma patients and with a similar rate of durable complete response in the 7 % range. In renal cell carcinoma, it has been possible to demonstrate activity with lower doses of IL-2 as well as combination with IFN-α, although durable complete responses appear to be more likely with the high-dose intravenous regimen. Two large trials have directly compared high-dose IL-2 with the combination of IL-2 plus IFN-α. One of these trials also had an IFN-α alone cohort and demonstrated that while the combination had superior activity, its toxicities outweighed any enhancement of activity. The other trial compared high-dose intravenous bolus IL-2 with an outpatient regimen of subcutaneous IL-2 plus IFN-α and showed similar overall response rates but superiority in response and survival for patients in the most unfavorable groups (liver metastasis, bone metastasis, and primary tumor in place)[125]. Further evidence of benefit for high-dose IL-2 was provided by another randomized trial comparing two intravenous bolus regimens of IL-2 (high-dose and low-dose) and one low-dose subcutaneous IL-2 regimen in patients with metastatic renal cell carcinoma. The objective response rate in the high-dose intravenous arm was twice that of both low-dose arms (21 % vs. 11 % and 10 %)[126]. The overall clinical responses with high-dose IL-2 have been relatively durable, with median response durations of 24–54 months and with over 80 % of complete responders being long-term survivors[127]. Thus, high-dose intravenous bolus IL-2 should remain in the armamentarium of the experienced clinical oncologist for advanced renal cell carcinoma. Interactions and optimal sequencing of IL-2 with tyrosine kinase inhibitors, now widely used for the frontline and subsequent treatment of advanced renal cancer, need to be better understood to provide the optimal therapy for all patients with this disease.

The toxicity profile of IL-2 is largely associated with a capillary leak syndrome, which is characterized by hypotension, tachycardia, and peripheral edema secondary to third space fluid accumulation. In addition, IL-2 can cause constitutional symptoms such as fever, chills, and fatigue, gastrointestinal side effects such as nausea, vomiting, anorexia, and diarrhea, and transaminase elevation and cholestasis[128]. In addition to hypotension, IL-2 can also induce pulmonary edema, cardiac arrhythmias, myocarditis, reversible renal and hepatic dysfunction, pruritus, electrolyte abnormalities, thrombocytopenia, anemia, and coagulopathy. Rarely IL-2 can also induce confusion, disorientation, or visual hallucinations. Although early studies with IL-2 reported a 2 % mortality rate, generally related to gram-positive sepsis, current IL-2 centers

that routinely use prophylactic antibiotics report no mortality[129, 130]. In experienced centers, IL-2-related toxicity can usually be easily managed and all side effects are reversible upon cessation of treatment.

7.5 Cytokine Therapy in Clinical Development

7.5.1 Interferon-α and Related Cytokines

Though not approved as a single agent for renal cell carcinoma, numerous clinical trials have evaluated the effects of single agent IFN-α in patients with metastatic disease. While response rates in the range of 10–15 % were reported in the older literature, recent randomized trials have shown lower activity at tolerable doses (see below), and only a modest suggestion of a dose–response relationship up to 10 million units/m^2/day, but at the expense of reduced quality of life[131]. Older trials combining IFN-α with chemotherapy and/or other cytokines reported promising activity that has not been corroborated in phase III trials, but two large randomized trials were recently reported that showed dramatic increases in the progression-free survival for a combination of bevacizumab (blockade of vascular endothelial growth factor) with IFN-α compared with IFN-α alone. Further evidence of the role for as-yet unidentified factors in the immune and angiogenic milieu was provided by the demonstration that cytoreductive nephrectomy increased survival in patients presenting with metastatic renal cancer treated with IFN-α alone or following surgery, although the objective responses to IFN-α were under 10 % in these trials[132, 133].

With the advent of a series of well-tolerated and active molecularly targeted agents for advanced renal cancer, the use of this expensive and somewhat toxic regimen that requires parenteral administration has waned.

IFN-α has also been investigated in patients with metastatic melanoma. Although single agent response rates have been low, IFN-α has also been tested in combination with chemotherapy and IL-2 in various biochemotherapy regimens[134–137]. In studies combining cisplatinum, vinblastine, and dacarbazine with IFN-α and low-dose IL-2, inconsistent clinical benefit has been reported[134]. A phase III randomized trial, however, failed to show an improvement in overall survival for this biochemotherapy regimen when compared to chemotherapy alone[135], and other comparisons of complex, toxic regimens containing chemotherapy and one or both cytokines (IL-2 and/or IFN-α) have also failed to demonstrate a survival benefit in advanced melanoma. Because of their high initial regression rates, these regimens are sometimes used for otherwise robust patients with rapidly progressive melanoma to achieve control of symptomatic disease but are not recommended for routine use. The US cooperative groups recently completed a large trial of adjuvant therapy for high-risk, resected stage III melanoma comparing a similar 5-drug biochemotherapy regimen with the 1-year regimen of adjuvant IFN-α (http://clinicaltrials.gov/ct2/show/NCT00006237?term=S0008&rank=2), but the study results have not yet matured, and it is likely that

regardless of the outcome, both therapies will have been replaced by regimens selected for patient- and tumor-specific factors, some of which are already under investigation or development for study in the adjuvant setting.

7.5.2 Interleukin-2 Combination Regimens

To improve the clinical effectiveness and therapeutic index of IL-2, studies combining IL-2 with other immunomodulatory agents have been widely evaluated in melanoma. Based on the expectation that cytotoxic T cell responses to selected immunodominant peptides from melanoma antigens could be enhanced by exogenous cytokine, a study of high-dose IL-2 plus an HLA-A2-restricted gp-100 peptide was performed in HLA-A2-positive patients with metastatic melanoma. The encouraging results (objective response rate of 42 % among 31 patients in this small phase II trial[138]) led to a confirmatory phase III clinical trial was designed to compare the combination of the modified gp100 peptide vaccine and high-dose IL-2 to IL-2 alone. While the results of this trial showed an improvement in progression-free survival and a small benefit in overall survival for the combination[139], its confirmation would require a larger trial. Furthermore, a randomized phase II trial testing different schedules of IL-2 and peptide was completed in parallel with the phase III trial and did not demonstrate sufficient activity in any of the schedules to justify further development[140]. Current therapies that appear to have a superior therapeutic index and to be more widely available (not requiring a specific HLA type as peptide vaccines do) are likely to temper enthusiasm for this approach.

7.5.3 IL-2 Predictive Factors and Patient Selection

There has been intense interest in the discovery of predictive biomarkers for better selection of patients likely to respond to IL-2 therapy for both renal cell carcinoma and melanoma. A defined polymorphism in the CCR5 gene (CCR5Δ32) was associated with decreased survival following IL-2 administration in patients with stage IV melanoma compared to patients not carrying the deletion.[141] Increased pretreatment serum vascular endothelial growth factor and fibronectin levels were associated with a poor response to IL-2 and a decreased overall survival[142]. Preliminary studies suggested that elevated levels of carbonic anhydrase IX in renal cell carcinoma patients conferred a better response to IL-2 therapy compared to patients with tumors demonstrating normal or low levels[143, 144], but larger trials that included additional characteristics raised the possibility of more discriminating markers that remain under active investigation. Other studies have focused on assessing the number, phenotypic characteristics, and functional status of

$CD4^+FoxP3^+$ Tregs in melanoma and renal cell carcinoma patients undergoing standard high-dose IL-2 administration. While the number of Tregs increased after exposure to IL-2 and remained elevated in patients with disease progression, patients who responded to IL-2 demonstrated a decrease in Tregs to normal levels within 4 weeks of completing IL-2 treatment[145]. Ultimately, it will be essential to identify predictive factors (specific for the intervention and not simply prognosticators for the natural history of the disease) in sufficient numbers to be validated in large patient cohorts.

7.5.4 Investigational Cytokines for Treatment of Malignancy

IL-15: IL-15, a member of the small four α-helix bundle family of cytokines, is one of the several IL-2-related cytokines that signal through the γ_c receptor subunit and have recently entered clinical investigation for cancer and hematologic malignancies[146]. While both IL-2 and IL-15 provide early stimulation for T cell proliferation and activation, IL-15 acts to block IL-2-induced apoptosis[147, 148]. IL-15 also supports the persistence of memory $CD8^+$ T cells, which may be important for maintaining long-term antitumor immunity[149–152]. IL-15 has demonstrated significant therapeutic activity in several preclinical murine models of cancer.[153] These effects are mediated through direct activation of $CD8^+$ effector T cells in an antigen-independent manner[151]. Importantly, IL-15 must be presented by a cell-bound α-receptor (see Fig. 7.2) to $CD8^+$ T cells and NK cells expressing the common β- and γ receptors[154]. Recombinant IL-15 has just entered phase I trials in human subjects and is expected to have value in a variety of immunotherapeutic strategies both in vivo and in ex vivo strategies for adoptive cell therapies.

IL-18: IL-18, a 24 kDa, non-glycosylated polypeptide, was initially identified as IFN-γ-inducing factor and is structurally related to IL-1β[155–157]. IL-18 stimulates IFN-γ secretion by NK and $CD8^+$ T cells and enhances their cytotoxicity[158–160]. Other functions of IL-18 include macrophage activation, development of Th1 helper $CD4^+$ T cells, increased expression of FasL on lymphocytes, and promotion of angiogenesis[161, 162]. Phase I clinical trials documented the safety of IL-18 and found increased levels of serum IFN-γ and GM-CSF in patients after receiving intravenous IL-18. The clinical responses have been modest with only 2 objective responses in 26 patients in one trial and three stable disease patients in another study[163, 164]. Additional investigation will be required to discover a niche for IL-18 in cancer immunotherapy strategies.

IL-12: IL-21 is a Type I cytokine with close homology to IL-2, IL-4, and IL-15 that shares with these cytokines plus IL-7 and IL-9 the γ_c receptor subunit and a cytokine-specific α-receptor. IL-21 is produced primarily by activated $CD4^+$ T cells. IL-21 signaling is distinguished from that of other γ_c cytokines by activating primarily STAT1 and STAT3[165]. IL-21 is produced by activated $CD4^+$ T cells and has pleiotropic effects, including the promotion of $CD4^+$ and $CD8^+$ T cell proliferation

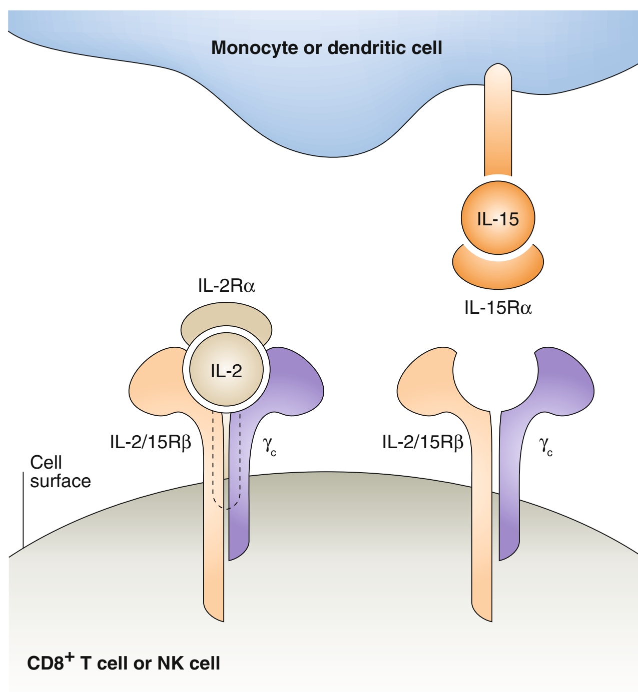

Fig. 7.2 The complex of IL-15 bound to its unique α receptor on the surface of antigen-presenting cells ligates the shared IL-2/IL-15 β receptor and common γ chain to form the IL-15 signaling complex on T and NK cells.

and enhancement of $CD8^+$ T cell and NK cell cytotoxicity without promoting activation-induced cell death[166–168]. Although the role of IL-21 in Th1/Th2 differentiation is unclear, it is required for normal humoral responses[169]. IL-21 has demonstrated therapeutic activity in murine tumor models of melanoma and has recently entered phase I clinical trials with modest preliminary results[170–172]. Like the other γ_c cytokines, IL-21 may find an important role in ex vivo strategies for adoptive T cell therapies and/or in conjunction with other elements of multicomponent immunotherapy strategies.

IL-12: IL-12 has been studied as a therapeutic cytokine for cancer in extensive preclinical investigations and a variety of clinical settings. It also exhibits anti-angiogenic effects mediated by IFNs, particularly IFN-γ, and by the chemokine IP-10[72]. Based on these provocative preclinical studies, IL-12 was evaluated in patients with metastatic melanoma or renal cancer. In a phase I clinical trial, the objective response rate was less than 5 %. Although the response rates were low, patients who did respond had sustained serum levels of IFN-γ, IL-15, and IL-18,

suggesting that sustained IFN-γ production might result in better responses[173, 174]. In a phase I trial combining IL-12 with low doses of IL-2, sustained levels of IFN-γ and expansion of NK cells were observed, although only one patient achieved a partial response[175]. IL-12 appears to have the potent ability to induce counter-regulatory cytokines such as IL-10 that may abrogate its immunostimulatory properties, depending on the dose and schedule of administration, but it could have promise as a component of the immune adjuvant in certain tumor vaccine strategies as well as in locoregional delivery vehicles such as plasmid electroporation[176].

7.5.5 GM-CSF and Related Cytokines

Recombinant GM-CSF was approved by the FDA to shorten the time to neutrophil recovery and reduce the incidence of bacterial infections following induction chemotherapy in patients with acute myelogenous leukemia. GM-CSF has also been used to mobilize hematopoietic progenitor cells into the peripheral blood for leukapheresis collection and enhance engraftment and myeloid reconstitution after autologous and allogeneic bone marrow transplantation. Based on extensive pre-clinical work demonstrating a variety of immunostimulatory properties for this molecule, in particular through its transgenic expression in tumor cells to create a promising tumor vaccine[177], this cytokine has undergone extensive testing in a variety of immunotherapeutic strategies. Single agent GM-CSF has antitumor activity in melanoma when injected directly into metastatic lesions[178, 179]. Recombinant GM-CSF has also been used as a subcutaneous injection in patients with resected stage III and IV melanoma based on a nonrandomized historical comparison suggesting a prolongation of disease-free and overall survival in patients who received GM-CSF for 2–3 years following surgery[180]. However, a recently completed randomized, placebo-controlled study of recombinant GM-CSF in patients with limited stage IV and bulky stage III melanoma following resection of all detectable lesions failed to show a clinical benefit either alone or when combined with a melanoma peptide vaccine in those patients whose HLA type permitted study of the vaccine[181]. Among many strategies currently under evaluation with this cytokine, GM-CSF has also been expressed in recombinant viral vectors for intratumoral administration and has shown sufficient promise in Phase II trials to justify the design of a randomized trial that is described below[182].

7.5.6 Interferon-β

IFN-β is a Type I IFN produced not only by leukocytes but also by some tumors[183]. Nevertheless, its therapeutic potential in immunomodulatory strategies has been demonstrated both for suppression of autoimmune reactivity (four different formulations of the drug are approved for multiple sclerosis) as well as for immunostimulation in the treatment of malignancy in a number of preclinical

models. In comparative analyses of the antitumor effects of the Type I IFNs, IFN-β is more potent than IFN-α in inducing antiproliferative effects in preclinical cancer models[184–186]. In spite of its higher antiproliferative potential compared to IFN-α, the clinical use of IFN-β in cancer therapy has been limited by its low bioavailability and sustained side effects that may be overcome by delivery in different routes and schedules.

7.5.7 Alternative Strategies for Cytokine Delivery

A variety of innovative strategies for delivery of therapeutic cytokines have been promising in the treatment of malignancy. These include a variety of cytokine-antibody fusion molecules (immunocytokines), recombinant viral vectors to deliver cytokine genes, DNA plasmid vaccines encoding a cytokine molecule, transgenic expression of cytokines in whole tumor cells, chemical conjugation to polyethylene glycol (PEGylation) to improve the kinetics of the cytokine, and the use of single or combination cytokines to promote the ex vivo development of cell-based immunotherapy.

7.5.8 Cytokine-Antibody Fusion Molecules

A cytokine-antibody fusion molecule is a genetically engineered fusion protein consisting of a chimeric antibody with a functional cytokine and an antigen-binding site designed to deliver cytokines to the tumor microenvironment. The prototype fusion molecule has utilized various antigen-binding moieties fused to recombinant human IL-2[187]. The therapeutic potential of this approach has been demonstrated using a fusion construct encoding the anti-GD2 ganglioside-binding site and IL-2 against a human neuroblastoma tumor in an SCID mouse model. In this system, local IL-2 delivery through the fusion molecule resulted in enhanced effector T cell responses and increased tumor cell lysis compared to systemic IL-2 delivery. The fusion molecule was also more potent than equivalent doses of recombinant human IL-2 in prolonging survival, and in another study, supported proliferation of lymphokine-activated killer cells. Treatment also resulted in the accumulation of the fusion molecule in the tumor, which slowed tumor growth and induced a significant immune response. This effect was more pronounced when the bifunctional molecule was injected directly into the tumor, highlighting the importance of local delivery[188, 189]. Phase I and II clinical trials of this recombinant fusion molecule in both adult melanoma and pediatric neuroblastoma patients have demonstrated its safety in patients at doses and schedules that are able to induce immune activation[190, 191]. Other novel immunocytokines entering clinical trials include a molecule containing an antigen-specific T cell receptor fused to two functional molecules of IL-2 (http://clinicaltrials.gov/ct2/show/NCT01029873?term=altor+and+melanoma&rank=).

7.5.9 *Recombinant Viruses as Delivery Systems for Tumor Immunotherapy*

The expression of cytokines by recombinant viruses provides another strategy for increasing the immunogenicity of antigen-specific vaccines and for local delivery of cytokines to the tumor microenvironment (see chapter 6 for many additional details). An attenuated oncolytic herpes simplex virus Type 1 encoding GM-CSF was shown to replicate selectively in tumor cells, leading to the production of local GM-CSF with the potential to augment tumor-specific immunity. The virus was attenuated by deletion of pathogenic viral genes, leading to enhanced replication of the virus in tumor cells through increased expression of the herpes US11 promoter and to increased antigen presentation in HSV-infected cells. Local GM-CSF enhances dendritic cell maturation and uptake of necrotic tumor cells, and induction of T cell immunity. Phase I studies demonstrated an acceptable safety profile with low-grade fever as the major side effect[192]. A phase II multi-institutional clinical trial tested the vector by direct injection into accessible melanoma lesions in patients with unresectable stage IIIc or IV melanoma[193]. Fifty patients were treated, and a 26 % overall objective response rate (that included some regressions outside of the injected metastases) was reported. There was a correlation between clinical regression and increased tumor MART-1-specific $CD8^{+}$ T cells and a decrease in $CD4^{+}FoxP3^{+}$ Tregs at the tumor site[194]. Based on these results, a prospective, randomized phase III clinical trial of the virally encoded cytokine injected interalesionally compared with subcutaneous administration of GM-CSF in patients with metastatic melanoma is currently underway[182].

Recombinant vaccinia viruses have also shown promise against a variety of tumors using in vivo murine tumor models. When tumors were injected directly with the oncolytic vector vaccinia virus, there was significant local cytokine production by DC and T cells[195]. Vaccinia virus-induced inhibition of T cell proliferation was seen but could be reversed by adding IL-2 and IL-12 to the vaccinia constructs, and the vaccinia-cytokine strategy led to profound local tumor regression[196]. This suggests that intratumoral vaccinia-cytokine gene constructs can retard tumor growth by targeting the immune system through tumor-infiltrating DC and T cells.

In contrast to the oncolytic effects of vaccinia virus, the vectors can also be used to encode tumor-associated antigens. A vaccinia virus encoding human prostate-specific antigen (rV-PSA) was administered intradermally with or without subcutaneous GM-CSF in a phase I trial to 33 men who had failed standard therapy. Vaccinia was shown to be safe and efficacious at inducing PSA-specific T cell responses and lowered or stabilized the PSA level in about half of the patients for 6 to greater than 21 months[197]. In another trial, seven patients with unresectable cutaneous melanoma received twice-weekly intradermal and/or subcutaneous injections of a recombinant vaccinia-GM-CSF virus into tumor metastases. The vaccinia-GM-CSF virus was safe and led to tumor regression in a few patients[191].

Recombinant vaccinia-GM-CSF virotherapy delivered intratumorally remains promising and may lend itself to further immunomodulatory strategies using other

interventions like the delivery of other genes and/or the addition of immune checkpoint blockers. Details of viruses in tumor immunotherapy are found in chapter 6.

7.5.10 Cell Engineering Approaches

Genetic engineering of tumor cells, APCs and effector lymphocytes is being developed as a way of delivering cytokines into the tumor microenvironment in an optimally defined space and time to prime host antitumor immunity against natural tumor antigens that have been processed and presented in immunogenic fashion[199]. Improvements in in vitro technology have facilitated the identification of tumor antigens and the isolation and expansion of antigen-reactive T cells. In these approaches, whole irradiated autologous or allogeneic tumor cells have been shown to secrete cytokines for sufficient periods of time to prime effective immune responses, and the possibility of introducing genes encoding accessory molecules not ordinarily expressed by the tumor has also been explored. Cytokines secreted locally in this fashion can also provide proliferative and survival signals to antigen-presenting and effector cells that further promote immune responses[200]. It has been shown that irradiated tumor cells in a vaccine expressing murine GM-CSF stimulate potent, long-lasting, and specific antitumor immunity, requiring both $CD4^+$ and $CD8^+$ cells[201]. Clinical trials of allogeneic-irradiated whole cell tumor vaccines encoding GM-CSF have been reported, with the theoretical potential that an allogeneic source of such an engineered tumor cell vaccine could provide an "off the shelf" consistent and well-characterized source of the therapeutic agent, but the best overall choice of genetically engineered tumor cell vaccine remains to be determined.

7.5.11 DNA Vaccination

The use of DNA vaccines encoding tumor antigens and/or cytokines has been extensively studied in animal tumor models with promising results. A DNA vaccine encoding human tyrosinase has been approved for the treatment of canine melanoma[202]. There are also many murine studies supporting the potential therapeutic benefit of DNA vaccines encoding cytokines alone, in combination with other cytokines, or in combination with tumor antigens[203, 204], and these strategies are in development for testing in human cancer.

7.5.12 Cytokine PEGylation

Conjugation of the polymer polyethylene glycol (PEG) to proteins is termed PEGylation. This process can significantly decrease protein clearance from plasma and increase the in vivo half-life, providing a method for enhancing exposure to

specific proteins and potentially avoiding toxicities associated with high peak concentrations of the unmanipulated protein[205]. To date, PEGylation has been successfully applied to two cytokines: PEG-interferonα-2a (PEG-IFN-α2a) and PEG-granulocyte colony-stimulating factor (PEG-G-CSF). IFN-α2a and PEG-IFN-α2a are virtually indistinguishable clinically, and both formulations elicit IFN response genes with equal efficiency while inhibiting tumor development with equal potency[206]. While PEG-IFN-α2a has similar activity to that of unmodified IFN-α2a in the adjuvant therapy of melanoma as well as in other diseases and the greater convenience associated with less frequent dosing and avoidance of the intravenous period of dosing, its use has been limited for the most part to European centers. In the USA, PEG-IFN is now rarely used for chronic myelogenous leukemia due to the superior therapeutic index of tyrosine kinase inhibitors; PEG-IFN is approved in combinations with antiviral agents for the treatment of chronic viral hepatitis[207].

7.5.13 *Ex vivo Cytokines for the Immunotherapy of Cancer*

Cytokines are important for the ex vivo generation of cell populations used in the vaccination and/or adoptive therapy of cancer. This is an important area of current investigation, as new combinations of cytokines are being tested for their impact on subsequent therapeutic responses. The two most widely evaluated approaches thus far have been in the generation of antigen-loaded dendritic cell vaccines and the generation of antigen-specific T cells for adoptive cellular therapy.

Dendritic cell (DC)-based vaccination therapy for cancer is a very promising strategy, since DCs are the most potent agents to prime T cells. Cytokines are used in several aspects of DC-based vaccine strategies, including their generation from peripheral blood monocytes obtained by leukapheresis (most commonly with IL-4 plus GM-CSF) and their maturation to potent APCs (for example, with TNF-α and IL-1β[27]). The safety of using autologous DC vaccines has been reported in clinical trials enrolling over 1,000 cancer patients exposed to a wide variety of types of DC products, route, and schedule of administration[208]. While pooled results suggest modest activity with simpler regimens, current strategies are focused on optimizing DC-based vaccines by exposing them to defined and precisely timed stimulants as well as different methods of introducing antigens and co-stimulatory molecules, including via RNA encoding these elements[209–211].

In addition to melanoma, DC vaccines stimulated with cytokines have shown promise in the treatment of prostate cancer. Using an autologous DC pulsed with a fusion protein containing prostate acid phosphatase as the antigen and GM-CSF to stimulate DC antigen-presentation, a recent double-blind, placebo-controlled multi-institutional phase III clinical trial was carried out in 512 men with castration-resistant prostate cancer who were randomized 2:1 to vaccine or placebo (autologous DC not loaded with the antigen-cytokine construct). The results of this trial included a 22 % reduction in the risk of death for vaccinated patients compared to placebo, representing a 4.1 month improvement in median survival (25.8 vs.

21.7 months). In this trial, over 60 % of the vaccinated patients exhibited antibody titers against the prostate acid phosphatase compared to only 2 % in the placebo group[212]. The results of his trial led to the FDA approval of this vaccine (sipuleucel-T) for prostate cancer in 2010, consistent with other data from trials performed during the same time interval showing encouraging results for similar immunomodulatory therapies for advanced prostate cancer[213]. Other malignancies with the potential for treatment based on promising preliminary data include lymphoma and renal cancer, but the optimal strategies remain to be identified.

The demonstration that TIL from metastatic lesions in patients with melanoma could be grown ex vivo with IL-2 and that these cells could mediate HLA-restricted tumor recognition suggested that adoptive transfer of TIL might be a powerful therapeutic strategy[214]. The generation of T cells for transfer generally utilizes ex vivo cytokines, most commonly IL-2, which is also commonly administered to patients following cell infusions to provide proliferative and survival signals to the adoptively transferred cells. In early studies at the NCI Surgery Branch, 86 patients with metastatic melanoma were treated with autologous TIL and high-dose IL-2[215]. Fifty-eight patients also received a preparative dose of cyclophosphamide (25 mg/kg) prior to T cell transfer. An objective clinical response was seen in 31 % in the TIL only group and 35 % in the cyclophosphamide-treated group. Following the recognition that T cell survival may be prolonged in the lymphopenic host, a series of clinical trials utilizing more profound lymphodepleting regimens were tested. In one study of 43 melanoma patients, a non-myeloablative chemotherapy regimen consisting of cyclophosphamide (60 mg/kg) given on 2 consecutive days followed by fludarabine (25 mg/m^2) for 5 days was used prior to T cell infusion. In this study an objective clinical response rate of 49 % was reported[216]. Another trial utilized an additional preparative treatment of whole body irradiation (200 cGy total) and an objective response rate of 52 % was reported[217]. A third trial intensified the total radiation dose to 1,200 cGy and a response rate of 72 % was seen[218].

The role of cytokines in supporting the adoptive transfer of T lymphocytes has been recognized in murine models and is widely utilized in clinical protocols. While high-dose IL-2 has generally been utilized to maintain persistence of adoptively transferred T cells, low doses may be sufficient to maintain the persistence of antigen-specific CD8 cells[219]. There is also evidence that IL-15 may be superior to IL-2 due to its superior profile in maintaining memory CD4$^+$ and CD8$^+$ T cells and its lack of promotion of CD4$^+$CD25$^+$Foxp3$^+$ regulatory T cells or activation-induced cell death, in contrast with IL-2[220]. Further studies are needed to understand better the optimal use of these cytokines in T cell persistence, migration, and homeostatic repopulation to improve therapeutic effectiveness of adoptive T cell approaches. Adoptive T cell transfers are discussed in detail in chapter 3.

7.5.14 Conclusions and Future Directions

The cytokines represent a large, diverse family of intercellular mediators that regulate and influence the immune system to maintain homeostasis, defend against pathogenic

organisms and mediate other important immune functions. The cytokines are also critical for tumor immunosurveillance and have demonstrated therapeutic antitumor activity in murine models and in the clinical treatment of several human cancers. A better understanding of the molecular signaling pathways used by cytokine receptors and the temporal and kinetic pattern of receptor expression have led to the therapeutic application of cytokines in a variety of settings. Single agent IFN-α and high-dose IL-2 have been approved for the adjuvant therapy of melanoma and the treatment of metastatic renal cell carcinoma and melanoma, respectively. Other members of the IL-2-related cytokine family are under intense scrutiny for additional antitumor activity based on encouraging murine tumor models. In addition, several innovative strategies have been developed that utilize cytokines to promote effective antitumor immunity, including bifunctional molecules such as antibody-cytokine fusions, expression of cytokines in recombinant viral vectors, DNA or irradiated whole tumor cells as vaccines, by PEGylation to enhance the kinetics and for ex vivo manipulation of cells, such as DC and adoptively transferred T cells.

There is little doubt that future studies will evolve at both the basic and clinical levels. A better understanding of how cytokines mediate tumor regression vs. progression by impacting effector and regulatory cell populations is an important area for development. The potential to block immunosuppressive cytokines has been demonstrated in preclinical studies but has not yet been translated into clinical trials. A major effort to identify predictive biomarkers will also be important as better patient selection might be one way to increase the clinical benefit of cytokine therapy while identifying new strategies for individual patients. The combination of cytokines with other treatment modalities is also expected to yield important insights. The cytokines have already proven to be useful in the treatment of cancer and will likely continue to play a major role in the development of immunotherapy for the treatment of cancer.

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Chapter 8
Immune Co-signaling to Treat Cancer

Margaret K. Callahan, Jedd D. Wolchok, James P. Allison, and Padmanee Sharma

Abstract The past two decades have been marked by a growing understanding of the co-stimulatory and co-inhibitory pathways that are critical to the generation of an effective, well-regulated immune response. Capitalizing on an increasingly nuanced appreciation for the role that these molecules play in anti-tumor immune responses, a diversity of novel therapies to treat human cancers are being explored. The ground-breaking success of anti-CTLA-4 therapy in the treatment of advanced melanoma has set the stage for the clinical development of agents targeting a diversity of co-stimulatory and co-inhibitory molecules. Herein, we review the

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co-signaling molecules that regulate T cell activation with a focus on their potential role(s) in anti-tumor immune responses. Where available, pre-clinical and clinical studies evaluating the anti-tumor activity of agents targeting these molecules are presented.

8.1 Introduction

Induction of an antigen-specific T cell response is a complex, highly regulated process. Studies performed in the 1970s and 1980s led to the development of the "two signal" model of T cell activation[1–4]. In this model, T cell activation requires both antigen-specific stimulation via the T cell receptor (TCR) (signal 1) as well as a co-stimulatory signal (signal 2). TCR signaling in the absence of co-stimulation leads to T cell anergy rather than activation[2]. Research over the subsequent decades has largely validated the two signal model, and has added layers of complexity to this framework. It is now clear that a variety of co-signaling molecules, both co-stimulatory and co-inhibitory, are required to orchestrate an antigen-specific immune response, governing both the activation and regulation of this process.

Cancer immunotherapy seeks to harness the immune system to treat cancer. Based upon the two signal model of immune activation, proposed immunotherapies have focused on enhancing antigen-specific (signal 1) and co-stimulatory (signal 2) pathways involved in anti-tumor immune responses. Characterization of the "signal 1" necessary for anti-tumor immune responses led to the identification of a variety of tumor antigens, including many with exquisite specificity for their tumor of origin[5]. Co-signaling molecules play several roles in modulating anti-tumor immunity. First, co-stimulatory molecules are the requisite "signal 2" necessary for productive anti-tumor immunity. Initial studies of co-stimulatory molecules like B7-1 (CD80) and CD28 demonstrated the potential for co-signaling to enhance anti-tumor immunity. Immunotherapies that augment "signal 2" are presently under clinical investigation. Secondly, co-inhibitory molecules have proven to be important checkpoints that limit anti-tumor immunity. Thus, approaches to block co-inhibitory molecules like cytotoxic T lymphocyte-associated antigen (CTLA-4) and programmed death (PD)-1 have shown great promise in enhancing anti-tumor responses. Underscoring the translational potential in developing novel anti-tumor agents by targeting co-signaling molecules, a human CTLA-4 blocking antibody, ipilimumab, has recently been approved by the Food and Drug Administration (FDA) for the treatment of advanced melanoma.

In this chapter, we will briefly review the co-stimulatory and co-inhibitory molecules presently defined. We will then review each co-signaling molecule individually, highlighting its role in anti-tumor immunity and its potential as a target for clinical development. Given the scope of this subject, and its rapid expansion in the last few years, we will focus most heavily on the agents in the most advanced stages of clinical development, those targeting CTLA-4 and PD-1.

8.2 Co-signaling Molecules

Co-signaling molecules expressed on T cells fall broadly into two categories: co-stimulatory molecules that enhance T cell activation or co-inhibitory molecules that constrain T cell activation. More than a dozen unique co-signaling molecules have been characterized to date (Table 8.1). Each of these molecules has its own pattern of expression, defined affinity for a ligand or ligands, and specific patterns of downstream signaling. Some of the ligands for co-signaling molecules have their own signaling capacity, and this bidirectional communication adds yet another layer of intricacy to an already complex web of interactions.

Most co-stimulatory molecules fall into two major families. The tumor necrosis factor (TNF) receptor superfamily includes co-stimulatory molecules OX40 (CD134), CD27, 4-1BB (CD137), CD30, GITR, HVEM, and CD40. The immunoglobulin superfamily (IGSF) includes co-stimulatory molecules CD28 and ICOS. One recently identified co-stimulatory molecule, TLT-2 (TREM-like transcript 2) breaks this mold as a member of the triggering receptor expressed on myeloid (TREM) cells receptor family[6]. All of the co-inhibitory molecules defined thus far are members of the IGSF, including CTLA-4, PD-1 (CD279), BTLA, and LAG-3.

8.3 CD28/CTLA-4/CD80/CD86

CD28, CTLA-4 (CD152), B7-1 (CD80), and B7-2 (CD86) are the canonical co-signaling molecules regulating T cell activation and inhibition. The first evidence that co-signaling could be manipulated as an effective cancer therapy came out of initial studies of these molecules[7, 8]. Subsequent studies led to the development of novel immunotherapies like the CTLA-4 blocking antibody ipilimumab, which has demonstrated a benefit in overall survival for patients with metastatic melanoma[9].

8.3.1 Biology

CD28 is the original and defining member of the immunoglobulin family of co-stimulatory receptors, first identified on T cells in 1980[10, 11]. Ligands for CD28, B7-1 (CD80), and B7-2 (CD86) were cloned in 1991 and 1993 respectively[12–15]. These molecules are expressed on most antigen presenting cells (APCs). B7-2 is expressed constitutively at low levels and rapidly upregulated during APC activation, whereas B7-1 is inducibly expressed later on[15–17]. Ligation of CD28 by B7-1 or B7-2, in concert with TCR engagement, provides the co-stimulation necessary for naïve T cell activation resulting in cytokine production, proliferation, and inhibition of activation-induced cell death (AICD)[18, 19]. CD28$^{-/-}$ knockout

Table 8.1 Co-stimulatory and co-inhibitory molecules for T cell activation

Co-signaling molecule	Expression	Ligand(s)	Expression
Inhibitory receptors in the immunoglobulin superfamily			
CTLA-4	Activated T cells	CD80, CD86	Antigen presenting cells
PD-1	Activated T cells	PD-L1	T cells, B cells, macrophages, NK cells, DCs, mast cells, cardiac endothelium, lung, small intestine, keratinocytes, islet cells of the pancreas, syncytiotrophoblasts, and many tumor cell types
	B cells		
	Monocytes		
	NKT cells		
	DCs	PD-L2	DCs, macrophages, mast cells, and the B1 subset of B cells
BTLA	T cells, B cells, and NK cells	HVEM	T cells, B cells, NK cells, DCs, and monocytes
LAG-3	T cells, NK cells, DCs, macrophages, liver, lung, bladder, testes, prostate, uterus, intestine, kidney, pancreas, and breast	MHC II	Antigen presenting cells
Co-stimulatory receptors in the immunoglobulin superfamily			
CD28	Naïve T cells	CD80	Antigen presenting cells
		CD86	
ICOS	Upregulated in Activated T cells including $CD4^+$, Th1, Th2, Th17, Tfh, and Treg cells	ICOSL	Activated monocytes and DCs
Co-stimulatory receptor in the TREM receptor family			
TLT-2	$CD8^+$ T cells, induced on activated $CD4^+$ T cells	B7-H3	T cells, NK cells, DCs, macrophages, liver, lung, bladder, testes, prostate, uterus, intestine, kidney, pancreas, and breast
Co-stimulatory receptors in the TNFR family			
OX40	Activated T cells	OX40-L	Activated B cells, activated T cells, DCs, vascular epithelial cells
CD27	NK, B cells, naïve $CD4^+$ T cells, naïve $CD8^+$ T cells	CD70	Activated T cells, B cells, DCs
CD30	Activated T cells, B cells, NK cells	CD30L	Resting B cells, activated T cells
4-1BB	Activated T cells, DCs, NK cells, eosinophils, microglia	4-1BBL	Activated macrophages, DC, B cells

HVEM	Resting T cells, monocytes, immature DC	LIGHT	Activated T cells, monocytes, NK cells, immature DCs
		BTLA	T cells, B cells, and NK cells.
		CD160	Cytotoxic lymphocytes
		gD	NA
		LTa	T cells
GITR	Upregulated on activated T cells, constitutively expressed on Tregs	GITRL	B cells, macrophages, DCs
CD40	B cells, macrophages, DCs, endothelial cells, fibroblasts	CD40L	Activated T cells, B cells, platelets

and B7-1$^{-/-}$B7-2$^{-/-}$ double knockout mice have markedly compromised T cell responses, underlining the indispensable role that CD28 plays in vivo[20–22].

CTLA-4 was cloned in 1987 and its similarity to CD28, also a member of the immunoglobulin gene family, was recognized early on[23]. However, it took several years to appreciate the unique role of CTLA-4 in attentuating T cell activation[24–26]. Like CD28, CTLA-4 binds to B7-1 and B7-2, albeit with higher afinity[27]. Experiments using cross-linking antibodies to ligate TCR, CD28, and CTLA-4 suggested that CTLA-4 acts as a negative regulator of CD28-mediated co-stimulation[24, 28]. CTLA-4 engagement on activated T cells inhibits cytokine synthesis and restricts cell proliferation[24, 26, 29–31]. Characterization of CTLA-4$^{-/-}$ knockout mice established a critical negative regulatory function for CTLA-4 in vivo. These mice develop a profound, hyperproliferative lymphocyte expansion, which is lethal within 3 weeks after birth[32–34].

8.3.2 CTLA-4 Mechanisms of Inhibition

On a cellular level, CTLA-4-mediated inhibition of T cell activation relies on several overlapping mechanisms. First, CTLA-4 competes with CD28 for interaction with the co-stimulatory molecules B7-1 and B7-2. The orchestration of this competition is temporospatially controlled by differences in both the subcellular localization and patterns of expression of CD28 and CTLA-4. CD28 is constitutively expressed, whereas CTLA-4 expression is up-regulated after T cell activation, reaching a maximal level after 2–3 days[26]. Upon TCR engagement and the formation of an immunologic synapse between T cell and APC, a complex pattern of subcellular trafficking of CD28 and CTLA-4 ensues[35–39]. CD28, already present on the cell surface, is rapidly recruited to the synapse and provides a co-stimulatory signal upon engagement of B7. CTLA-4 is expressed at low levels on resting T cells and is sequestered in intracellular vesicles. Upon T cell activation, CTLA-4 is mobilized to the cell surface and is subsequently recruited to the synapse[35, 36, 38, 40]. Once in the synapse, CTLA-4 out-competes CD28 to bind B7, forms a lattice-like network in association with B7-1, and effectively excludes CD28 from the immunological synapse[39, 41, 42]. Secondly, CTLA-4 engagement impacts multiple intracellular pathways. Inhibitory signaling is thought to be mediated by (1) association with intracellular phosphatases like Src homology 2 (SH2) domain-containing phosphatase-1 (SHP-1), SHP-2, and protein phosphatase 2A (PP2A), (2) blockade of lipid-raft expression, and (3) disruption of microcluster formation (reviewed by Rudd et al.[19]). Downstream sequelae include inhibition of protein tyrosine kinases Lck, Fyn, and ZAP-70 and reduced expression of transcription factors like nuclear factor-kB (NF-kB) and activator protein-1 (AP-1)[43–50].

Adding layers of complexity to the CTLA-4/B7 interaction, CTLA-4 also transmits suppressive signals via B7 to the APC. This “reverse” signaling results in the induction of indoleamine 2,3-dioxygenase (IDO), an enzyme that degrades

tryptophan into byproducts that inhibit T cell proliferation[51, 52]. Additionally, B7 molecules expressed on T cells provide another target for regulation via T cell to T cell interaction between CTLA-4 and B7[53, 54].

8.3.3 CTLA-4 on Regulatory T Cells

CTLA-4 is constitutively expressed by natural and inducible Foxp3^{+} regulatory T cells (Tregs)[55]. Moreover, CTLA-4 appears to be necessary for Treg function in some, but not all, experimental systems[56–61]. Evidence supporting a role for CTLA-4 in Treg function comes from mice that have a conditional deletion of CTLA-4 in the Treg compartment. These mice develop a spontaneous lymphoproliferative disorder and die 10 weeks after birth, a phenotype similar to, but less severe than CTLA-4$^{-/-}$ knockout mice[62].

Understanding the precise mechanism(s) of CTLA-4 activity in vivo is an area of active investigation. Two distinct, but not mutually exclusive, hypotheses to explain CTLA-4 activity have gained experimental support: a cell intrinsic and a cell extrinsic mechanism. In the cell intrinsic model, CTLA-4 acts *in cis* on activated T cells to oppose the co-stimulatory signal provided by CD28 and B7 interaction, as described above. In support of this model, conventional CD4^{+} and CD8^{+} T cells that do not express CTLA-4 have a higher proliferative capacity in vitro and in vivo[31, 34, 63–66]. The cell extrinsic activity of CTLA-4, focusing on a role for Tregs, has been more challenging to define. Early experiments demonstrating that CTLA-4$^{-/-}$ T cells transferred into RAG1/2$^{-/-}$ hosts could be inhibited by concomitant transfer of wild-type (wt) T cells suggested *trans*-regulation by CTLA-4-sufficient wt cells[56, 67], later defined as CD4^{+}CD25^{+} Tregs[68]. Additionally, comparing the phenotypes of CTLA-4$^{-/-}$ knockout mice with the conditional Treg CTLA-4$^{-/-}$ knockout mice implied that both conventional T cells and Tregs contribute to this phenotype[62].

8.4 Applications for Tumor Immunotherapy

The co-signaling pathways involving CD28, B7-1, B7-2, and CTLA-4 have proven fruitful ground for enhancing our understanding of the role of co-stimulatory and co-inhibitory molecules in anti-tumor immunity. These observations have served as a platform for the development of novel immunotherapies, several of which show great promise in their clinical application (Table 8.2).

Table 8.2 Targeting co-stimulatory and co-inhibitory molecules in the development of novel anti-cancer therapies

Target	Agent(s) in clinical development	Stage
CTLA-4	Blocking Ab Ipilimumab (MDX-010) (BMS)	FDA approved
	Blocking Ab tremelimumab (Pfizer)	Phase III
PD-1	Blocking Ab MDX-1106/BMS-936558 (BMS)	Phase III
	Blocking Ab CT-011 (CureTech)	Phase II
	Blocking Ab MK-3475 (Merck)	Phase I
	Fc-fusion protein of PD-L2 AMP-224 (Amplimmune)	Phase I
PD-L1	Blocking Ab MDX-1105/BMS-936559 (BMS)	Phase I
	Blocking Ab MPDL3280A (Genentech)	Phase I
LAG-3	Soluble LAG-3 IMP321 (immutep SA)	Phase I
GITR	Agonist Ab TRX518 (Tolerex)	Phase I
CD40	Recombinant CD40L	Phase I
	Agonist Ab CP-870,893 (Pfizer)	Phase I
	Agonist Ab SGN-40 (Seattle Genetics)	Phase II
	Agonist Ab HCD122 (Novartis)	Phase I
4-1BB	Agonist Ab BMS-663513 (BMS)	Phase II

8.4.1 B7-1/B7-2

The first successful modification of T cell co-signaling to treat cancer was accomplished through overexpression of B7-1 on tumor cells. In initial experiments, melanoma cell lines transfected with B7-1 were found to regress spontaneously in a $CD8^+$ T cell-dependent process[7, 8, 69, 70]. Animals previously challenged with these B7-1-overexpressing tumors were found to be immune to subsequent challenge with B7-1 negative tumors. A similar pattern was later seen with B7-2-expressing tumors[71]. This work was subsequently expanded to murine lymphoma, hepatoma, and prostate cancer tumor cell lines[70, 72, 73].

8.4.2 Clinical Trials

Studies in mice paved the way for several clinical trials using autologous or allogeneic tumor cell lines transfected with B7-1 as a vaccine strategy. Phase I and II studies in renal cell carcinoma, and phase I studies in melanoma, breast carcinoma, and non-small cell lung cancer have been completed[74–79]. These studies demonstrated an increase in tumor-associated immune responses, but limited clinical benefit. As an alternative approach, B7-1 molecules have been included in strategies for DNA vaccination, often in combination with tumor antigens or other

co-stimulatory molecules. Two phase I studies treating patients with metastatic colon cancer with a vaccine incorporating human carcinoembryonic antigen (CEA) and B7-1 in a recombinant canarypox virus vaccine demonstrated generation of tumor antigen-specific immune responses, but showed modest clinical benefit[80, 81]. Using a similar approach, the B7-1 gene has been combined with genes for cell adhesion molecules ICAM-1 and LFA-3 called TRiad of Co-stimulatory Molecules (TRICOM) and this combination has been tested in several studies[82–91]. One phase I study combining TRICOM with CEA in patients with adenocarcinoma expressing CEA demonstrated the strategy to be safe and reported a complete response in one patient of the 58 treated[90]. A second phase I study combining TRICOM with two tumor antigens, CEA and MUC-1, demonstrated evidence of induced immune responses, and a partial response in one patient of the 25 treated on the study[84]. In an alternative approach, intralesional delivery of a B7-1 expressing virus has been evaluated[78, 92, 93]. In a study of 13 patients with metastatic melanoma, local delivery of a recombinant vaccinia virus expressing TRICOM (rvTRICOM) demonstrated clinical benefit, with 5/13 (38.5 %) patients showing an objective response of the treated lesion by Response Evaluation Criteria in Solid Tumors (RECIST) criteria. Additionally, there was evidence of systemic response in two patients with one partial response and one complete response[94].

8.4.3 CTLA-4

Based upon the evidence that CTLA-4 functions to attenuate immune responses, it was proposed that blockade of CTLA-4 could enhance immune responses against tumors by inhibiting this "checkpoint"[95]. This hypothesis was initially validated using transplantable murine tumor lines of colon carcinoma and fibrosarcoma[96–100]. These experiments demonstrated that established tumors could be rejected by administration of a CTLA-4 blocking antibody. This observation has since been validated for transplantable tumors of many types including prostate carcinoma, breast carcinoma, melanoma, ovarian carcinoma, lymphoma, and others (Table 8.3)[96–100]. In some poorly immunogenic tumors, such as the B16 melanoma and the SM1 mammary tumor, CTLA-4 monotherapy was not effective, but combination therapy with GM-CSF-expressing tumor cells, peptide, or DNA vaccines had synergistic activity[102, 103, 108]. CTLA-4 blockade has since shown activity in combination with conventional cancer therapies including surgery[109], radiation[105, 110], chemotherapy[101] cryoablation, and radiofrequency ablation[111]. CTLA-4 has also been combined successfully with a diversity of immunotherapies[102–104, 106–108, 112–116].

Experience with CTLA-4 blockade in mouse models of transplantable tumors demonstrated that enhancing co-signaling could stimulate anti-tumor immunity to eradicate established tumors. Treated mice were resistant to subsequent tumor challenge, confirming the generation of a memory response. Side effects of CTLA-4 blockade, such as skin depigmentation and prostatitis were seen in mouse models of

Table 8.3 Treatment with anti-CTLA-4 antibodies in mouse tumor models

Treatment	Tumor	Response(s)	Reference
Antibody alone	Colon carcinoma (51 BLiml 0) Fibrosarcoma (Sa1N)	Reduced growth or rejection of established tumors. Generation of tumor-specific memory	Leach et al.[96]
Antibody alone	Prostate carcinoma (TRAMPC1)	Reduced growth or rejection of established tumors	Kwon et al.[97]
Antibody alone	Fibrosarcoma (CSA1M) Ovarian carcinoma (OV-HM)	Reduced growth or rejection of established tumors. Generation of tumor-specific memory	Yang et al.[98]
Antibody plus GM-CSF secreting cellular vaccine	Mammary carcinoma (SM1)	Rejection of established, poorly immunogenic tumors. Generation of tumor specific memory. Tumor rejection dependent upon CD4+ and CD8+ T cells	Hurwitz et al.[112]
Antibody plus chemotherapy (melphalan)	Plasmocytoma (MOPC-315)	Rejection of established, poorly immunogenic tumors	Mokyr et al.[101]
Antibody plus GM-CSF secreting cellular vaccine	Melanoma (B16)	Rejection of established, poorly immunogenic tumors. Generation of tumor specific memory. Tumor rejection dependent upon CD8+ and NK1.1+ cells	Van Elsas et al.[108]
Antibody alone or Antibody plus surgery	Prostate carcinoma (C2)	Reduced growth or rejection of established tumors. Increased survival and decrease metastatic burden in mice treated in adjuvant setting	Kwon et al.[109]
Antibody alone	Lymphoma (A20) expressing model antigen HA	Enhanced antigen-specific T cell priming	Sotomayor et al.[100]
Antibody alone	Thymoma (EL-4) expressing model antigen ovalbumin	Reduced tumor growth. Enhanced expansion of antigen specific CD8+ T cells, dependent upon CD4+ cells and IL-2	Shrikant et al.[99]
Antibody plus GM-CSF secreting cellular vaccine	Spontaneous prostate tumor (TRAMP)	Reduced tumor incidence. Development of autoimmunity (prostatitis)	Hurwitz et al.[114]
Antibody plus DC vaccine and anti-VEGFR antibody	Colon cancer (CT-26)	Increased tumor rejection alone and in combination with other therapies	Pedersen et al.[583]
Antibody plus GM-CSF secreting cellular vaccine plus depletion of Tregs	Melanoma (B16)	Reduced growth or rejection of established tumors. Development of autoimmunity (depigmentation). Enhanced	Sutmuller et al.[584]

(continued)

Table 8.3 (continued)

Treatment	Tumor	Response(s)	Reference
		generation of tumor specific T cells	
Antibody plus peptide plus CpG adjuvant	Melanoma (B16)	Delayed tumor growth and increase survival. Survival dependent upon CD4+ and CD8+ T cells	Davila et al.[102]
Antibody plus xenogenic DNA vaccination	Melanoma (B16)	Reduced growth or rejection of established tumors. Enhanced tumor-antigen specific T cell responses	Gregor et al.[103]
Antibody plus p53 expressing viral vaccine	Fibrosarcoma (MethA)	Reduced growth or rejection of established tumors. Anti-tumor effect dependent upon CD8+ T cells and IFN-gamma	Espenschied et al.[585]
Antibody plus p53 expressing viral vaccine	Mammary carcinoma (11A-1) Colon carcinoma (MC-38)	Reduced growth or rejection of established tumors.	Daftarian et al.[104]
Antibody plus local radiation	Mammary carcinoma (4T1)	Increased overall survival. Decreased lung metastasis. Anti-tumor effect dependent upon CD8+ T cells	Demaria et al.[105]
Antibody plus GM-CSF secreting cellular vaccine	Melanoma (B16)	Increased ratio of effector/Treg within tumor	Quezada et al.[117]
Antibody plus peptide pulsed dendritic cells vaccine	Thymoma (EL-4) expressing model antigen ovalbumin	Reduced growth or rejection of established tumors. Generation of specific memory response against model antigen and with evidence of epitope spread to additional tumor antigens.	Met et al.[106]
Antibody plus GM-CSF secreting cellular vaccine	Melanoma (B16)	Maximal anti-tumor response requires synergist effects on effector and Treg populations	Peggs et al.[118]
Antibody plus Flt-3 secreting cellular vaccine	Melanoma (B16) Prostate carcinoma (C2)	Reduced growth or rejection of established tumors. Enhanced tumor infiltration of CD8+ T cells	Curran et al.[116]
Antibody plus Her2/neu expressing viral vaccine	Mammary carcinoma (D2F2/E2)	Rejection of established tumors. Anti-tumor effect dependent upon CD4+ and CD8+ T cells	Gao et al.[107]

melanoma and prostate cancer respectively, and prefigured some of the immune-related side effects of CTLA-4 blockade later seen in humans[108, 113, 114].

8.5 Mechanism of Activity: CTLA-4 Blockade and Tumor Immunity

Defining the mechanism(s) of CTLA-4 activity in vivo, and by extension, the mechanism of anti-tumor immune activity mediated by CTLA-4 blockade, is an area of active investigation. The relative contributions of cell intrinsic versus cell extrinsic effects of CTLA-4 have been a matter of debate. In several mouse models, anti-tumor immunity generated by CTLA-4 blockade relies most heavily upon the effector T cell compartment, arguing in support of the cell intrinsic model[113, 117, 118]. Utilizing a transgenic mouse expressing human CTLA-4, Peggs et al. were able to assess the relative contributions of CTLA-4 expression independently on effector T cells versus Tregs. These studies demonstrated an absolute requirement for effector T cells, but not Tregs in anti-CTLA-4-mediated tumor immunity. However, concomitant blockade of both effector T cells and Tregs was required for maximal anti-tumor effects[118]. Thus, the cell intrinsic effects of CTLA-4 blockade predominate, but cell extrinsic effects also contribute to anti-tumor immunity.

8.5.1 Clinical Trials

CTLA-4 blocking antibodies for use in humans were developed based on the preclinical activity seen in mouse models. Both ipilimumab (formerly MDX-010, Bristol-Myers Squibb, Princeton, NJ) and tremelimumab (formerly CP-675, 206 or ticilimumab, Pfizer, New York, NY) are fully human antibodies against CTLA-4[119–121]. Ipilimumab is an IgG1 with a plasma half-life of 12–14 days. Tremelimumab is an IgG2 with a plasma half-life of approximately 22 days. Both of these agents have been most widely tested in patients with metastatic melanoma, where durable clinical responses have been well documented. On March 25, 2011, the US FDA approved ipilimumab for the treatment of patients with unresectable or metastatic melanoma.

8.5.2 Ipilimumab in the Treatment of Melanoma

In a pilot study of ipilimumab reported in 2002, 17 patients with unresectable melanoma were treated with single dose of 3 mg/kg. There were two partial responses, which were durable. The treatment was tolerated well, with only a mild rash noted[122]. A second pilot study of single-dose ipilimumab enrolled

Table 8.4 Selected anti-CTLA-4 antibody clinical trials

Reference	Patients	Treatment arms	Response rates	irAEs	Median OS
Robert[130]	502 patients with previously untreated metastatic melanoma	Ipilimumab plus dacarbazine v. dacarbazine plus placebo Ipilimumab 10 mg/kg q3 weeks × 4 doses, then q 3 months	Ipilimumab + dacarbazine BORR 15.2 % DCR 33.2 % 4 CR, 34 PR, 45 SD Dacarbazine + Placebo BORR 10.3 % DCR 30.2 % 2 CR, 24 PR, 50 SD	For Ipilimumab + dacarbazine 77.7 % irAEs 41.7 % Grade III/IV irAEs For Dacarbazine + Placebo 38.2 % irAEs 6.0 % Grade III/IV irAEs	Ipilimumab + dacarbazine 11.2 months 47.3 % at 1 year 28.5 % at 2 years 20.8 % at 3 years Dacarbazine + Placebo 9.1 months 36.3 % at 1 year 17.9 % at 2 years 12.2 % at 3 years
Hodi[9]	676 patients with previously treated, unresectable stage III or IV melanoma	Ipilimumab v. gp100 peptide vaccine v. combination Ipilimumab 3 mg/kg q3 weeks × 4 doses	Ipilimumab alone BORR 10.9 % DCR 28.5 % 2 CR, 13 PR, 24 SD Ipilimumab + gp100 BORR 5.7 % DCR 20.1 % 1 CR, 22 PR, 58 SD gp100 alone BORR 1.5 % DCR 11.0 % 0 CR, 2 PR, 13 SD	Ipilimumab alone 61.1 % 14.5 % Grade III/IV Ipilimumab + gp100 58.2 % 10.2 % Grade III/IV gp100 alone 31.8 % 3 % Grade III/IV	Ipilimumab alone 10.1 months 45.6 % at 1 year 23.5 % at 2 years Ipilimumab + gp100 10.0 months 43.6 % at 1 year 21.6 % at 2 years gp100 alone 6.4 months 25.3 % at 1 year 13.7 % at 2 years
Hersh et al.[129]	72 patients with chemotherapy naïve metastatic melanoma	Ipilimumab with or without dacarbazine Ipilimumab 3 mg/kg q3 weeks × 4 doses	Dacarbazine + Ipilimumab BORR 14.3 % DCR 37.1 % 2 CR, 3 PR, 8 SD	Dacarbazine Ipilimumab 65.7 % 17.1 % Grade III/IV	Dacarbazine + Ipilimumab 14.3 months 62 % at 1 year 24 % at 2 years 20 % at 3 years

(continued)

Table 8.4 (continued)

Reference	Patients	Treatment arms	Response rates	irAEs	Median OS
		Dacarbazine 250 mg/m^2 daily for 5 days q 3 weeks up to 6 cycles	Ipilimumab BORR 5.4 % DCR 21.6 % 0 CR, 2 PR, 6 SD	Ipilimumab 53.8 % 7.7 % Grade III/IV	Ipilimumab 11.4 months 45 % at 1 year 21 % at 2 years 9 % at 3 years
Wolchok[128]	217 patients with previously treated metastatic melanoma	Ipilimumab 0.3 mg/kg v. 3 mg/kg v. 10 mg/kg q3 weeks × 4 doses, then q 3 months	10 mg/kg BORR 11.1 % DCR 29.2 % 2 CR, 6 PR, 13 SD 3 mg/kg BORR 4.2 % DCR 26.4 % 0 CR, 3 PR, 16 SD 0.3 mg/kg BORR 0 % DCR 13.7 % 0 CR, 0 PR, 10 SD	10 mg/kg 70 % 25 % Grade III/IV 3 mg/kg 65 % 7 % Grade III/IV 0.3 mg/kg 26 % 0 % Grade III/IV	10 mg/kg 11.4 months 48.6 % at 1 year 29.8 % at 2 years 3 mg/kg 8.7 months 39.3 % at 1 year 24.2 % at 2 years 0.3 mg/kg 8.6 months 39.6 % at 1 year 18.4 % at 2 years
Weber et al.[587]	115 patients with previously treated metastatic melanoma	Ipilimumab with or without budesenide 10 mg/kg q3 weeks × 4 doses, then q 3 months	Budesenide + Ipilimumab BORR 12.1 % DCR 31 % 1 CR, 6 PR, 11 SD Ipilimumab alone BORR 15.8 % DCR 35 % 0 CR, 9 PR, 11 SD	Budesenide + Ipilimumab 81 % 29% Grade III 12% Grade IV Ipilimumab alone 84 % 15% Grade III 12% Grade IV	Budesenide + Ipilimumab 17.7 months 55.9 % at 1 year Ipilimumab alone 19.3 months 62.4 % at 1 year

seven patients with melanoma and two patients with ovarian cancer. All patients had previously been treated with tumor vaccines; five patients were previously treated with a GM-CSF secreting tumor vaccine and four patients were previously treated with a melanoma antigen vaccine. Evidence of treatment efficacy was seen in patients who had received ipilimumab after receiving the GM-CSF tumor vaccine. Tumor necrosis was demonstrated in three patients with melanoma and reduction or stabilization of the tumor marker CA-125 was seen in three patients with ovarian cancer. The treatment was well tolerated, and, again, a rash was noted[123]. Next, ipilimumab was tested in combination with a gp100 peptide vaccine in a phase I study of melanoma. An initial 14 patients were treated with ipilimumab at a dose of 3 mg/kg given every 3 weeks for 4 doses. Three objective responses, two complete responses and one partial response, were reported. However, six patients developed grade III/IV toxicities which included colitis, dermatitis, hypophysitis, and hepatitis. Subsequently, a similar study enrolling 56 patients with stage IV melanoma previously vaccinated with gp100 peptide compared two different dosing schedules of ipilimumab: 3 mg/kg every 3 weeks versus a 3 mg/kg initial dose followed by 1 mg/kg on subsequent doses. There was no difference in response rate or toxicity between these two groups, and the dosing regimen of 3 mg/kg every 3 weeks for four doses was adopted in several subsequent studies. The overall response was 12.5 % including two complete responses. Grade III toxicities were seen in 25 %, with a similar pattern including colitis (7), dermatitis (4), uveitis (1), enterocolitis (1), hepatitis (1), and hypophysitis (1). All toxicities were considered to be related to immune activation, a clustering of side effects later labeled immune-related adverse events (irAEs). A positive correlation between irAEs and response to therapy was noted with 36 % of patients with irAEs experiencing a clinical response compared with only 5 % of patients without irAEs[124]. A similar phase I trial in melanoma combining ipilimumab with peptide vaccine against gp100, MART-1, and tyrosinase demonstrated similar toxicities and detected antigen-specific immune responses[125]. Maker et al. combined IL-2 with ipilimumab in a phase I/II trial enrolling 36 patients. There was a response rate of 22 %, interpreted as an additive effect between the two agents[126].

Subsequent studies further explored the question of dosing for ipilimumab. Weber et al. treated 88 patients with unresectable stage III/IV melanoma with ipilimumab over a dose range of 2.8–20 mg/kg comparing single versus multiple dose schedules. The overall response rate was 4.5 % and an additional 16 % of patients had prolonged stable disease (median 194 days)[121]. Downey et al. reported a response rate of 17 % for a phase II trial of 139 patients treated with ipilimumab at doses of 3–9 mg/kg. Sixty-two percent of patients had an irAE of any grade and having an irAE was associated with objective response ($P = 0.0004$)[127]. A dose–response relationship was clearly defined in a double-blind phase II study comparing ipilimumab at doses of 0.3, 3, and 10 mg/kg every 3 weeks, followed by maintenance doses administered every 12 weeks. The highest dose cohort, 10 mg/kg, had the greatest response rate (11 %), followed by 3 mg/kg (4.2 %), and 0.3 mg/kg (0 %). The irAEs followed a similar pattern[128]. Lastly, a randomized phase II study comparing ipilimumab at a dose of 3 mg/kg with or without dacarbazine demonstrated a trend favoring combination therapy. There was a

14.3 % response rate for the 35 patients treated with the combination compared with a 5.3 % response rate for the 37 patients treated with ipilimumab alone. The combination had only a slightly higher incidence of irAEs (65.7 % versus 53.8 %)[129].

A randomized, double-blind, phase III study examining 676 patients treated with ipilimumab at a dose of 3 mg/kg every 3 weeks for four doses compared with patients treated with peptide vaccine alone or peptide vaccine plus ipilimumab demonstrated a best overall response rate of 10.9 % among patients treated with ipilimumab alone and a benefit in overall survival (10.0 months for ipilimumab plus peptide vaccine versus 10.1 months for ipilimumab alone versus 6.4 months for peptide vaccine alone) favoring ipilimumab treatment[9]. In this trial, survival rates for ipilimumab-treated patients were 45.6 % at 1-year and 23.5 % at the 2-year mark. A second randomized, placebo-controlled, phase III clinical trial comparing dacarbazine plus ipilimumab at a dose of 10 mg/kg every 3 weeks for four doses, followed by maintenance ipilimumab versus dacarbazine combined with placebo in treatment naïve patients with metastatic melanoma also reported a benefit in overall survival[130]. In this trial, survival rates for ipilimumab-treated patients were higher than patients treated with dacarbazine alone at 1 year (47.3 % versus 36.3 %), 2 years (28.5 % versus 17.9 %), and 3 years (20.8 % versus 12.2 %).

8.5.3 Ipilimumab in Cancers Other Than Melanoma

Outside of melanoma, the largest clinical trial experience with ipilimumab is in the treatment of metastatic prostate cancer. In a pilot study, 14 patients with castrate-resistant metastatic prostate cancer received a single dose of 3 mg/kg[131]. Two patients had a biochemical response according to consensus criteria (>50 % decrease in PSA). An additional eight patients had a decline in PSA that was <50 %. A phase II study expanding to multiple doses of ipilimumab randomized 43 patients with castrate-resistant metastatic prostate cancer to receive ipilimumab 3 mg/kg every 4 weeks for four doses alone or in combination with a single dose of docetaxel[132]. Six patients, three in each arm, had a biochemical response; there were no radiographic responses and five patients had grade III/IV irAEs. Several additional studies combining ipilimumab with GM-CSF, radiation, and a DNA vaccine (PSA-TRICOM) have reported promising results[133–135]. A phase II study comparing hormone therapy with or without ipilimumab in the treatment of advanced prostate cancer has completed accrual (NCT00170157). At present, two phase III trials of ipilimumab in prostate cancer are ongoing. The first study targets patients who have received prior chemotherapy treatment with docetaxel. This study is a randomized, double-blind study comparing ipilimumab with placebo in patients who have received radiotherapy (NCT00861614). A second study includes only patients with castrate-resistant prostate cancer who are chemotherapy naïve. This phase III double-blind study randomizes patients to receive ipilimumab or placebo (NCT01057810).

Ipilimumab has also been tested in several additional malignancies including non-small cell lung cancer, renal cell carcinoma, pancreatic cancer, and hematologic malignancies. The largest of these so far is a phase II study combining ipilimumab with chemotherapy in patients with stage IIIb/IV non-small cell lung cancer. The study accrued 203 patients with chemotherapy-naïve non-small cell lung cancer who were randomized 1:1:1 to receive either chemotherapy alone or ipilimumab combined with chemotherapy in two different schedules. There was a statistically significant ($P = 0.024$), but very modest (<1 month) delay in progression-free survival between the arm receiving chemotherapy alone and one of the combination arms, and a nonsignificant trend toward improved overall survival for the ipilimumab arm[136]. Small studies treating patients with non-Hodgkin lymphoma relapsed hematological malignancies after allogeneic bone marrow transplant, and renal cell carcinoma have shown some promise[137–139]. A phase II study of 27 patients with metastatic pancreatic cancer demonstrated minimal activity[140].

8.5.4 Ipilimumab in the Adjuvant Setting

Experience with ipilimumab in the adjuvant setting has been limited. Sanderson et al. reported the results of the phase I study of 19 patients with high risk, resected stage III or IV melanoma treated with ipilimumab in combination with a vaccine of peptides from gp100, MART-1, and tyrosinase. Within this study, eight patients experienced irAEs, with four grade III/IV irAEs. For the 8 patients who experienced irAEs, 3/8 (37.5 %) developed relapse of disease, whereas 9/11 (81.8 %) patients without irAE experienced relapse[125]. Ipilimumab has also been administered preoperatively in the adjuvant setting to patients with localized bladder cancer. In a study of six patients with localized urothelial cancer of the bladder, Liahou et al. demonstrated the feasibility and tolerability of an ipilimumab regimen of a single 3 mg/kg dose given prior to radical cystoprostatectomy[141]. This approach, later expanded to an additional six patient treated with ipilimumab at a 10 mg/kg dose, has allowed the investigation of surgically resected tumor tissue after ipilimumab therapy, facilitating immune monitoring of the tumor microenvironment[141–143]. A phase II study of ipilimumab in combination with hormone therapy administered in the neoadjuvant setting for patients with early stage prostate cancer prior to radical prostatectomy is presently open for accrual (NCT01194271). A randomized, double-blind study comparing ipilimumab to placebo in the adjuvant setting for patients with stage IIIB or IIIC melanoma is also presently accruing (NCT00636168).

8.5.4.1 Tremelimumab

Clinical testing for tremelimumab began in 2002 with a phase I dose escalation study. This study accrued 39 patients with metastatic solid tumors (34 with melanoma) and treated over a dose range of 0.01–15 mg/kg. Objective responses for patients with melanoma were observed in four patients; all objective responses were at doses of 3 mg/kg or above[120]. A pattern of irAEs comparable to those seen in patients treated with ipilimumab was reported. A phase I study compared a schedule of 10 mg/kg given monthly to 15 mg/kg given every 3 months, and reported an overall response rate of 16.7 % (5/30)[144]. A phase II dose-finding study also compared the 10 and 15 mg/kg regimens[145]. This study, reporting a 10 % overall response rate, identified the regimen of 15 mg/kg every 3 months as superior, based upon comparable response rates and a lower rate of irAEs. A larger phase II study, enrolling 251 patients treated with the preferred 15 mg/kg regimen, reported an overall response rate of 6.6 %[146]. On the basis of these results, a randomized, open-label phase III trial comparing tremelimumab with the standard of care (dacarbazine or temozolomide) was opened. The study was halted after an interim analysis failed to demonstrate a benefit (overall survival 10.7 months versus 11.7 months)[147]. Recently updated interim results from the study demonstrate a nonsignificant trend favoring tremelimumab for overall survival ($P = 0.14$)[148].

8.5.4.2 Tremelimumab in Cancers Other Than Melanoma

Several small studies of tremelimumab in solid malignancies outside of melanoma have now been reported, with primarily disappointing results. In a phase I study, 26 patients with advanced, hormone-responsive breast cancer received tremelimumab in combination with exemestane[149]. The regimen was tolerated, but the best overall response was stable disease for 12+ weeks, seen in 11/24 patients. The utility of tremelimumab in non-small cell lung cancer was evaluated in a randomized, open-label, phase II study which accrued 87 patients[150]. Patients were randomized to receive either tremelimumab or best supportive care after a course of standard chemotherapy. There was no significant difference in relapse rate at current follow-up. A phase I study combining tremelimumab with sunitinib for the treatment of renal cell carcinoma encountered unexpected renal toxicities as the dose-limiting toxicity[151]. A phase II study of tremelimumab in hepatocellular carcinoma is ongoing (NCT01008358).

8.5.4.3 Lessons Learned from Clinical Trials of CTLA-4 Blockade

To date, more than 10,000 patients have been treated with CTLA-4 blockade. As a monotherapy, CTLA-4 blockade is capable of inducing objective responses in approximately 5–15 % of patients with metastatic melanoma. CTLA-4 blockade

has also shown activity in several other malignancies including prostate cancer, renal cell carcinoma, and non-small cell lung carcinoma. For ipilimumab, two phase III clinical trials demonstrated an improved overall survival for patients with metastatic melanoma. Additional phase III studies in melanoma and prostate cancer are outstanding. Observations from clinical trials to date have consistently highlighted two areas where anti-CTLA-4 blockade is unique when compared to conventional chemotherapies: (1) side effects related to treatment and (2) kinetics of response to treatment.

8.5.4.4 Immune-Related Adverse Events

For some patients, the potent ability of CTLA-4 blockade to activate the immune system results in inflammatory manifestations characterized as irAEs. The most clinically significant irAE is enterocolitis which can range in severity; grade III/IV enterocolitis is seen in ~15 % of patients treated with ipilimumab at 10 mg/kg[128]. Pathological evaluation is consistent with a panenteritis that resembles an autoimmune enteropathy[152]. With vigilance and early intervention with corticosteroids and/or anti-TNF therapy, colitis symptoms are readily treatable and rarely result in life-threatening complications[153]. Notably, colitis treatments do not appear to compromise the anti-tumor activity or duration of response to ipilimumab[127]. Additional irAEs occur with variable frequency. At a dose of 10 mg/kg, observed rates of irAEs (any grade, grade III/IV) are as follows: rash/pruritus (47–68 %, 0–4 %), hepatitis (3–9 %, 3–7 %), hypophysitis (4–6 %, 1–5 %), uveitis (<1 %), pancreatitis (1–3 %), and cytopenias (<2 %)[125, 153–157]. Hepatitis, pancreatitis, uveitis, and cytopenias respond well to steroid treatment. Hypophysitis, if detected early, may respond to steroids, although hormone supplementation is often necessary[157].

Overlap between patients who develop irAEs and those who derive clinical benefit from CTLA-4 blockade was noticed in early studies. For example, in a phase I study of 14 patients with metastatic melanoma, Phan et al. reported that 3/3 responding patients (1 complete response, 2 partial responses) had grade III/IV toxicities, whereas only 3/11 (27 %) of non-responders had similar toxicities[158]. The correlation between grade III/IV irAEs and clinical response has since been substantiated in several larger analyses[121, 124, 127, 154, 156, 159]. The presence of grade III/IV irAEs correlates with higher rates of clinical response. Conversely, irAEs are more frequent among clinical responders. It should be emphasized, however, that high-grade irAEs are not required for clinical response; nor does a high-grade irAE guarantee a clinical response. The factors that determine the focus (anti-tumor, autoimmune, or both) of immune responses activated by CTLA-4 are an area of active investigation.

8.5.4.5 Kinetics of Response: Immune-Related Response Criteria

Standard criteria for evaluating responses to chemotherapeutic agents under investigation, such as the RECIST and WHO criteria were developed to promote objectivity in clinical trials reporting and facilitate comparisons between studies. These guidelines are based upon patterns of responses to chemotherapeutic agents that correlate with clinical outcomes. Following RECIST guidelines, any increase in tumor size and/or development of new lesions is defined as progressive disease. Early observations from phase I and II clinical trials with CTLA-4 blockade suggested that the patterns of responses to immunotherapy may differ significantly from standard responses to chemotherapies. In an analysis of 487 patients treated on three multicenter phase II clinical trials of ipilimumab, Wolchok et al. identified four patterns of response to treatment of ipilimumab, all associated with favorable survival[160]. These patterns include (1) Decreased baseline lesions without evidence of new lesions, (2) Durable stable disease, (3) Response after an initial increase in total tumor burden, and (4) Response in the presence of new lesions. Based upon these observations, new guidelines for monitoring responses to immune therapy, immune-related response criteria (irRC) were proposed. The major distinguishing feature of the irRC guidelines is that new lesions are incorporated into a "total tumor burden"; thus a new lesion in the context of an overall decrease in tumor burden would not be defined as progressive disease. These criteria may better reflect the underlying biology of immune-mediated anti-tumor activity. Prospective validation of irRC is ongoing.

8.5.4.6 Biomarkers for CTLA-4 Blockade

Immunological monitoring has been an integral part of the completed and ongoing clinical trials of ipilimumab and tremelimumab. To date, immune monitoring has identified several endpoints that may correlate with a variety of clinical parameters (Table 8.5). Most of these biomarkers have been identified in small, retrospective analyses and larger, prospective studies are needed.

In the largest evaluation of biomarkers in patients treated with ipilimumab reported to date, the rate of rise in absolute lymphocyte count (ALC) was found to correlate with clinical benefit[161]. This analysis was performed in two parts. In the first part, pooled data from three studies of patients with unresectable stage III/IV melanoma (379 patients) was analyzed retrospectively. For the 55 patients (15.8 %) with evidence of clinical activity, there was a positive correlation with mean rate of ALC change ($P = 0.0013$). This correlation was then tested and confirmed in a prospective fashion in 64 additional patients ($P = 0.00042$). The significance of the ALC was highlighted in a second, independent study of 51 patients[159]. Patients with an ALC ≥ 1000 after the second dose of ipilimumab had a higher rate of clinical benefit (complete response + partial response + stable disease) at week 24 (52 % versus 0 %; $P = 0.01$). Likewise, the high absolute lymphocyte count group had

Table 8.5 Potential biomarkers of clinical activity in patients treated with CTLA-4 blockade

Study population	Biomarker	Endpoint	Reference
Patients with MM pooled from four clinical trials receiving ipilimumab at a dose of 0.3, 3, or 10 mg/kg			Berman et al.[161]
379 patients pooled from 3 clinical trials (retrospectively analyzed)	Mean rate of ALC change	CB P 0.0013	
64 patients (prospectively analyzed)	Mean rate of ALC change	CB $P = 0.00042$	
51 patients with MM treated with ipilimumab at a dose of 10 mg/kg[a]	ALC 1,000 wk 7	CB wk 24 $P < 0.01$ OS $P < 0.0001$	Ku et al.[159]
35 patients with MM treated in 4 trials with ipilimumab at a dose of 10 mg/kg	Mean rate of ALC change Mean rate of CD8 change	CB P < 0.0001 CB $P = 0.0294$	Yang et al.[162]
14 patients with MM treated with ipilimumab at a dose of 10 mg/kg	Persistent increase in CD4 + ICOShigh cells	CB P 0.004 OS $P = 0.03$	Carthon et al.[143]
75 patients with stage IIIC/IV melanoma treated with MART-1/gp100/tyrosinase peptides plus ipilimumab at a dose of 3 or 10 mg/kg	IL-17 secreting CD4+ T cells	FFR $P = 0.049$	Weber et al.[155]
35 patients with MM treated with ipilimumab at a dose of 3 or 10 mg/kg[b]	Intratumoral FoxP3 expression (33 patients)	CB P 0.014	Hamid et al.[588]
	Intratumoral IDO expression (35 patients)	CB $P = 0.012$	
10 patients with MM treated with tremelimumab at a dose of 15 mg/kg	Resistance of CD4+ T cells to Treg-mediated inhibition	PFS $P = 0.001$ OS $P = 0.035$	Menard et al.[587]
10 patients with MM treated with tremelimumab at a dose of 15 mg/kg	Resistance of CD4 + CD45RO + cells to Tregs in vitro	PFS $P = 0.001$ OS $P = 0.035$	Menard et al.[587]

[a]The study analyzed 51 patients, only 41 were evaluable for ALC at wk 7 and CB wk 24.
[b]Only 33 patients were evaluable for FoxP3 expression.
CB clinical benefit (CR + PR + SD); *MM* metastatic melanoma; *CR* complete response; *NS* not significant; *PR* partial response; *ALC* absolute lymphocyte count; *SD* stable disease; *PBMC* peripheral blood mononuclear cells; *OS* overall survival; *wk* week; *PFS* progression-free survival; *mo* month; *NS* not significant; GVAX, irradiated, autologous tumor cells engineered to secrete GM-CSF; *FFR* freedom from response

higher rates of 6-month (75 % versus 0 %) and 12-month (47 % versus 0 %) overall survival. Further characterization of ALC as a biomarker points to $CD8^+$ T cells as the pertinent lymphocyte subset[162].

The correlation between CTLA-4 blockade and inducible co-stimulator (ICOS) was first described in an analysis of six bladder cancer patients receiving ipilimumab in the preoperative setting[141]. The design of this study permitted analysis of both peripheral blood cells and tumor tissue. Analysis of multiple parameters identified a positive correlation between ipilimumab treatment and frequency of $CD4^+$ cells expressing high levels of ICOS in both peripheral blood and tumor samples. Subsequently, in the same patient population, prostate tissue removed during radical cystoprostatectomy was analyzed[142]. In both normal prostate tissue and prostate adenocarcinoma, the same pattern of increased frequency of $CD4^+ICOS^{hi}$ cells after ipilimumab treatment was observed. This association was also reported in a recent phase I study of tremelimumab given in combination with exemestane in hormone responsive metastatic breast cancer[149]. Lastly, retrospective analysis of peripheral blood mononuclear cells in 14 patients with metastatic melanoma patients treated with ipilimumab identified increased frequency of $CD4^+ICOS^{hi}$ T cells, sustained over a period of 12 weeks, as a correlate of overall survival[143].

Characterization of antigen-specific immune responses during CTLA-4 blockade has been performed for several cancer-related antigens including NY-ESO-1, MAGE, Melan-A, MART-1, gp-100, tyrosinase, PSA, PAP, and PSMA. Antigen-specific immune responses to NY-ESO-1 have been the most extensively characterized and may be correlated with clinical activity. The largest study thus far to specifically characterize NY-ESO-1 responses in the setting of CTLA-4 blockade examined 15 patients with metastatic melanoma treated with ipilimumab[163]. Within this group, 5/8 (62.5 %) clinical responders demonstrated antibody, or $CD4^+$ or $CD8^+$ T cell responses to NY-ESO-1. By comparison, only 1/7 (14.3 %) non-responders developed a $CD4^+$ T cell response. Among patients who had antibody responses, NY-ESO-1 specific antibody titer increased with ipilimumab treatment. Similarly, patients who developed NY-ESO-1 specific T cell responses after CTLA-4 blockade demonstrated a more robust, polyfunctional T cell response after treatment. These findings implicate the development of polyfunctional NY-ESO-1 specific T cells as a surrogate of a broader anti-tumor immune compartment and/or as direct mediators of anti-tumor immunity.

8.5.5 PD-1/PD-L1/PD-L2

8.5.5.1 Biology

Programmed death-1 (PD-1, CD279) is a member of the IGSF of molecules involved in regulation of T cell activation. PD-1 acquired its name “programmed

death" when it was identified in 1992 as a gene upregulated in a T cell hybridoma undergoing cell death[164]. PD-1 is expressed more broadly than other IGSF members. It is not expressed on naive or resting lymphocytes, but is expressed on activated $CD4^+$ and $CD8^+$ T cells, B cells, monocytes, NKT cells and DCs[165, 166]. The structure of PD-1 is composed of one IGSF domain, a transmembrane domain, and in intracellular domain containing a immunoreceptor tyrosine-based inhibitory motif (ITIM) and a immunoreceptor tyrosine-based switch motif (ITSM)[167–169]. PD-1 has two binding partners: PD-L1 (B7-H1, CD274) and PD-L2 (B7-DC, CD273), distant relatives of the B7-1 and B7-2 molecules. PD-L1, discovered in 1999, is expressed quite broadly, on both hematopoietic and non-hematopoietic lineages[170, 171]. It is found on T cells, B cells, macrophages, NK cells, DCs, and mast cells. It has also been described on peripheral tissues including cardiac endothelium, lung, small intestine, keratinocytes, islet cells of the pancreas, and syncytiotrophoblasts in the placenta as well as a variety of tumor cell types[172–186]. PD-L1 is constitutively expressed on many hematopoietic cells, but may be upregulated in hematopoietic and non-hematopoietic cells. Regulation of PD-L1 is mediated in part by type I and type II interferons[172, 173, 178, 187, 188]. PD-L2 was identified in 2001[189, 190]. Its expression is far more restricted and is confined to hematopoietic cells. PD-L2 is inducibly expressed on dendritic cells, macrophages, mast cells, and the B1 subset of B cells[172, 174, 191, 192]. PD-L1 and PD-L2 have distinct patterns of induction; under some circumstances, Th2 cytokines preferentially upregulate PD-L2 while Th1 cytokines upregulate PD-L1[193].

8.5.5.2 Inhibitory Signaling Mediated by PD-1/PD-L

Engagement of PD-1 on T cells inhibits activation with downstream effects on cytokine production, proliferation, cell survival, and transcription factors associated with effector T cell function[171, 194–198]. Inhibitory signaling by PD-1 is thought to depend upon the cytosolic ITSM domain, which associates with phosphatases SHP-1 and SHP-2[199, 200]. While CTLA-4 and PD-1 are both inhibitory receptors, they fulfill distinct roles and mediate their effects through distinct mechanisms[201]. For example, PD-1 inhibits activation of the serine threonine kinase Akt via its effect on the phosphoinositide 3-kinase (PI3K) pathway, whereas CTLA-4 inhibits Akt in a PI3K independent manner[194, 200, 202]. Additionally, "reverse signaling" via PD-L1and PD-L2 molecules expressed on dendritic cells can enhance or inhibit dendritic cell activation as measured by dendritic cell maturation and cytokine production[203–208]. Adding another layer of complexity to the PD-1/PD-L pathway, B7-1 has recently been identified as a ligand for PD-L1, but not PD-L2[209]. The B7-1:PD-L1 interaction delivers an inhibitory signal to B7-1 or PD-L1 expressing T cells. Some apparent contradictions between studies of PD-1/PD-L interaction may be resolved by factoring in the bidirectional signaling between these molecules[186].

Studies of PD-$1^{-/-}$ knockout and PD-$L^{-/-}$ knockout mice support unique roles for PD-1:PD-L1 interactions in mediating peripheral tolerance and preventing

autoimmunity[186]. The phenotype of the PD-1$^{-/-}$ knockout mouse depends upon the genetic background, but manifestations of spontaneous autoimmunity have been reported including dilated cardiomyopathy and glomerulonephritis[210, 211]. Furthermore, PD-1- and PD-L1-deficient mice are more prone to autoimmunity in several mouse models[212–216].

8.5.5.3 PD-1/PD-L in Human Cancers

PD-L1 and PD-L2 are expressed on many human tumors including urothelial, ovarian, breast, cervical, colon, pancreatic, gastric cancers as well as melanoma glioblastoma, and non-small cell lung cancer[175, 177, 180–185, 217–220]. In addition, PD-L1 and PD-L2 have been detected on hematologic malignancies including Hodgkin lymphoma, primary mediastinal B cell lymphoma, angioimmunoblastic T cell lymphoma, multiple myeloma, acute myeloid leukemia chronic lymphocytic leukemia, and adult T cell leukemia/lymphoma[178, 221–224]. Expression of PD-L has been correlated with prognosis in many these malignancies, fueling the hypothesis that PD-L expression is a mechanism for tumor immune evasion[217, 218, 225, 226]. Additionally, PD-1 is highly expressed on lymphocytes infiltrating human tumors and circulating tumor-specific T cells, a phenotype correlated with impaired T cell function[227–230]. Together these findings suggest that interrupting PD-1:PD-L interaction could be an effective anti-cancer therapy.

8.5.5.4 Preclinical Anti-tumor Activity

In mice, PD-L1 overexpression on murine squamous cell carcinoma and mastocytoma cells diminished anti-tumor immune response and enhanced tumor growth, an effect that was reversible with PD-L1 blocking antibody[225, 231]. Conversely, in a model utilizing TCR transgenic T cells specific for a model tumor antigen, PD-1$^{-/-}$ T cells demonstrated enhanced anti-tumor activity[232]. Additional preclinical studies of PD-1:PD-L blockade in murine tumor models have yielded promising results in several tumor models including ovarian carcinoma, squamous cell carcinoma, melanoma, mastocytoma, hepatoma, and acute myeloid leukemia[225, 231, 233–239]. Strategies for blockade of PD-1:PD-L interaction include blocking antibodies against PD-1, PD-L1, PD-L2, as well as soluble PD-1. PD-1 blocking antibodies have been combined to good effect with several immunotherapy strategies including CpG, 4-1BB agonist antibody, CTLA-4 blocking antibody, irradiated tumor cells, GM-CSF secreting tumor cells and adoptive T cell therapy[115, 233, 240–243]. For example, in a mouse model of melanoma, combination of CTLA-4 blockade and PD-1 blockade demonstrated synergistic anti-tumor activity[240].

8.5.5.5 Clinical Trials

Several anti-PD-1 and anti-PD-L1 antibodies have been developed (see Table 8.2).

The anti-PD-1 antibody BMS-936558 is a fully human IgG4 antibody, which has demonstrated activity in vitro, and a serum half-life of 20 days at the highest doses tested[227, 248]. In a first-in-human dose escalation phase I trial of BMS-936558, 39 patients with advanced solid tumors were treated with escalating doses of 0.3, 1, 3, or 10 mg/kg, with a dose expansion cohort at 10 mg/kg[248]. The treatment was well tolerated with a single serious adverse event of grade III inflammatory colitis in a patient treated with five doses of 1 mg/kg. One patient developed a grade II hypothyroidism, and two patients developed arthropathies. There was a response rate of 7.7 % overall with one complete response (colorectal cancer, 3 mg/kg) and two partial responses (melanoma and renal cell carcinoma, both 10 mg/kg). A second phase I study investigating a different schedule of biweekly dosing of BMS-936558 demonstrated a higher response rate of 37.5 % (6/16 patients). Patients with advanced solid tumors were treated with BMS-936558 at doses of 1, 3, or 10 mg/kg biweekly for up to 48 doses. Responses included five partial responses (melanoma, renal cell carcinoma, non-small cell carcinoma) and one complete response (renal cell carcinoma). One possible significant adverse event was reported: myelodysplastic syndrome in a patient previously treated with cytotoxic chemotherapy. Most side effects were grade I/II and included: fatigue (56.3 %), nausea (25 %), diarrhea (18.8 %), pruritus (18.8 %) and xerostomia (18.8 %). The results of a second large Phase I study of BMS-936558 were recently reported.[589] In this study, a total of 296 patients with advanced melanoma, colorectal cancer, renal-cell cancer (RCC), prostate cancer or non-small-cell lung cancer (NSCLC) were enrolled. The profile of toxicities were similar to those previously reported. Notable clinical activity was observed in patients with melanoma, NSCLC, and RCC. For melanoma and RCC, objective response rates of 28% and 27% respectively were observed. For NSCLC, an overall response rate of 18% was observed with a 33% response rate in those with squamous histology and a 12% response rate in those with nonsquamous histology. A subset of 42 patients were tested for pre-treatment tumor expression of PD-L1 by immunohistochemistry and a positive correlation between PD-L1 expression and objective response was observed.

BMS-936558 appears to have promising activity in multiple solid tumor and additional studies are ongoing[249].

These include a phase I study in advanced solid tumors being conducted in Japan (NCT00730639), a dose escalation study combining ipilimumab with BMS-936558 for patients with metastatic melanoma (NCT01024231), a study combining peptide vaccine with BMS-936558 in unresectable melanoma (NCT01176461), phase II and phase III studies in RCC (NCT01354431 and NCT01668784), and several studies in NSCLC including soon to open phase III studies (NCT01673867 and NCT01642004).

BMS-936559 is a fully human PD-L1-specific igG4 monoclonal antibody with a mutated hinge region (S228P). The results of a phase I, dose-ranging study of BMS-936559 were recently published (Brahmer J et al. Safety and Activity of Anti-PD-L1 Antibody in Patients with Advanced Cancer). In this study, 207 patients with advanced solid tumors were treated with BMS-936559 at doses of 0.3 to 10 mg/kg. Activity in patients with advanced melanoma, NSCLC, ovarian cancer, and RCC was documented with objective response rates of up to 17 % and (in melanoma).

CT-011 is a humanized IgG1 antibody with a half-life in the range of 9–17 days based on the initial pharmacokinetic study[244]. CT-011 has demonstrated anti-tumor activity in preclinical models of hematological and solid malignancies[245–247]. In a pilot study of 17 patients with hematological malignancies, patients were treated with a single dose of CP-011 ranging from 0.2 to 6 mg/kg. The maximal tolerated dose was not achieved and the doses tested were safe and well tolerated. The most frequent adverse event was diarrhea (11.8 %). Clinical benefit was reported for 33 % of the study population with one patient with follicular B cell lymphoma achieving a clinical response. Based on these results, a phase II study combining CP-011 (3 mg/kg) with rituximab for the treatment of patients with relapsed follicular lymphoma is presently recruiting patients (NCT00904722). A second phase II study treating patients with relapsed diffuse large B cell lymphoma with adjuvant CT-011 (1.5 mg/kg) after autologous stem cell transplant is also underway. CP-011 has also shown promising activity for the treatment of multiple myeloma. In a preclinical study of primary NK cells and multiple myeloma cells derived from patients with multiple myeloma, Benson et al. observed expression on PD-1 (on NK cells) and PD-1L (on multiple myeloma cells) ex vivo. Furthermore, they found that in vitro, CP-011 enhanced NK-mediated cytotoxicity against autologous multiple myeloma cells. This effect was further augmented by the addition of lenolidomide, which caused downregulation of PD-L1 on primary multiple myeloma cells. A phase II study of CP-011 combined with a dendritic cell vaccine following autologous stem cell transplant for patients with multiple myeloma is ongoing (NCT01067287). Additional studies in colon cancer (NCT00890305), hepatocellular carcinoma (NCT00966251), and acute myelogenous leukemia (NCT01096602) are active.

While clinical experience with PD-1 or PD-L1 blocking antibodies is limited, some tentative patterns appear to be emerging. First, unsurprisingly, PD-1 blockade appears to be associated with irAEs similar to those described in CTLA-4 blockade: colitis/diarrhea, endocrinopathies, and dermatitis/pruritus. The irAE of arthropathies seen in two patients treated with PD-1 blockade have not been frequently reported in patients treated with CTLA-4 blockade. Notably, both patients treated with PD-1 blockade who developed arthropathies had potentially predisposing risk factors: history of Lyme arthritis and polymyalgia rheumatica and preexisting antinuclear antibody titer. While the maximally tolerated dose for PD-1 blocking antibodies has not yet been reached, doses that confer clinical benefit have been achieved. At these doses, the frequency and/or severity of irAEs appear to be less than for CTLA-4 blockade. However, there has been no formal comparison between CTLA-4 blockade and PD-1 blockade and many fewer patients have

been treated with PD-1 blockade. PD-1 blockade as a monotherapy or in combination is a particularly promising strategy and larger studies are merited.

8.5.6 BTLA/HVEM/CD160/LIGHT

8.5.6.1 Biology

B and T lymphocyte attenuator (BTLA, CD272) is another recently discovered co-inhibitory molecule of the IGSF, both structurally and functionally related to CTLA-4 and PD-1. BTLA was independently identified as an expressed sequence tag (EST) in a Th1 polarized TCR transgenic T cell population and as a transcript expressed in activated thymocytes[250, 251]. BTLA is expressed constitutively on T cells, B cells, and dendritic cells. Naïve T cells express low levels of BTLA, which may be increased upon activation or induction of anergy[252]. BTLA expression on dendritic cells is upregulated upon stimulation with lipopolysaccharide[251]. Unlike CTLA-4 or PD-1, BTLA does not appear to be expressed on $CD4^{+}CD25^{+}$ Tregs[252]. Initial reports suggested that BTLA is preferentially expressed on Th1 but not Th2 cells. However, later studies described BTLA expression on both populations[252–254]. The cytoplasmic domain of BTLA contains two ITIM domains that associate with the phosphatases SHP-1 and SHP-2, consistent with the described inhibitory function of BTLA signaling[255].

Herpesvirus entry mediator (HVEM, TNFRSF14) serves as a shared ligand for inhibitory receptors BTLA and CD160 as well as TNF superfamily members LIGHT and lymphotoxin-alpha[250, 256, 257]. HVEM was discovered in a screen for proteins that allowed entry of herpes simplex virus-1 (HSV-1) into CHO cells and its first defined binding partner was the viral gD protein[258–261]. HVEM is expressed widely on hematopoietic and nonhematopoietic cells including T cells, B cells, NK cells, dendritic cells, and monocytes. HVEM levels are higher on resting cells and decrease with B or T cell activation[262, 263]. Whereas HVEM signaling via BTLA transmits an inhibitory signal, engagement of HVEM expressed on T cells by its alternative ligand LIGHT enhances T cell proliferation and cytokine production[264–268]. HVEM transmits an activating signal via recruitment of the cytosolic TNFR-associated factor (TRAF) 2 and 5 molecules that activate downstream signaling through the NF-kB and AP-1 pathways[259, 260].

LIGHT (LT-g, CD258) is an acronym for "homologous to lymphotoxins, exhibits inducible expression, and competes with HSV glycoprotein D for HVEM, a receptor expressed by T lymphocytes"[257]. LIGHT belongs to a family of TNF ligands, and is closely linked with related molecules CD70 and 41BBL on human chromosome 19. LIGHT interacts with HVEM, lymphotoxin-β, and a decoy receptor, DcR3/TR6. LIGHT expression is tightly regulated. It is expressed on T cells, dendritic cells, NK cells, and B cells at specific stages of cell differentiation. LIGHT is rapidly and highly induced on activated T cells, especially $CD8^{+}$ T cells[257, 262]. Conversely, naïve dendritic cells express high levels of LIGHT

which is downregulated upon dendritic cell maturation[269]. LIGHT exhibits a reciprocal pattern of expression with its binding partner HVEM[262]. It functions to enhance T cell activation both directly through engagement of HVEM on T cells and indirectly by engagement of HVEM on dendritic cells, which stimulates their maturation[257, 260, 265, 266, 269–272].

CD160 was identified and later cloned based on its recognition by a monoclonal antibody raised against a human NK leukemia cell line[273, 274]. CD160 was first recognized as a weak binding partner for classical and non-classical MHC I molecules[275–278]. Later, HVEM was identified as a ligand for the CD160 receptor. CD160 expression is limited to cytotoxic lymphocytes including NK cells, NKT cells, and subsets of $CD8^+$ and $CD4^+$ T cells. CD160 is upregulated upon activation and particularly enriched among $CD8^+$ T cells in the late stages of chronic viral infection[279]. The consequences of CD160 ligation are still being dissected. In cultured human $CD4^+$ and $CD8^+$ T cells, cross-linking of CD160 with monoclonal antibodies (5D.10A11 and 5D.8E10) inhibits T cell activation[280–282]. Inhibitory signaling via CD160 depends upon HVEM engagement[280]. In apparent contradiction, engagement of CD160 by MHC I multimers or the anti-CD160 Ab (BY55) enhances NK and $CD8^+$ T cell cytotoxic activity and cytokine production[277, 278, 283]. CD160 is a GPI-anchored protein; the downstream signaling employed by CD160 to deliver inhibitory or activating signals is an area of ongoing investigation.

For this grouping of molecules, there are a number of potential pairings each with potentially unique outcomes. For example, HVEM signaling through BTLA delivers an inhibitory message whereas LIGHT engagement of HVEM delivers a co-stimulatory signal. Broadly, an axis of inhibitory signaling (BLTA/CD160/HVEM) and an axis of stimulatory signaling (LIGHT/HVEM) have been defined. The inhibitory effects of BTLA engagement were initially characterized using cultured T cells, with $CD4^+$ T cells demonstrating greater sensitivity to inhibition by anti-BTLA antibodies[251, 254, 256, 273, 280, 281, 284, 285]. The phenotype of $BTLA^{-/-}$ knockout mice is consistent with an inhibitory role for BTLA. Aged mice develop a spontaneous autoimmune hepatitis and increased levels of anti-nuclear antibodies[286]. Additionally, studies of BTLA-deficient mice support a role for BTLA in peripheral tolerance and regulation of inflammation and autoimmunity[250, 286–289]. LIGHT-deficient mice have reduced T cell activation and expansion, in agreement with the co-stimulatory function of LIGHT[266, 290–292]. Conversely, mice with transgenic expression of LIGHT on T cells develop spontaneous autoimmunity[267, 293, 294]. Interestingly, the phenotype of HVEM knockout mice most closely resembles the BTLA-deficient mice, not the LIGHT-deficient mice. HVEM-deficient mice have increased susceptibility to autoimmunity in several mouse models including EAE and autoimmune hepatitis[295]. This finding suggests that in vivo the HVEM:BTLA interaction may be more functionally important than the HVEM:LIGHT interaction.

8.5.6.2 Relevance in Human Tumors

The areas of overlap between BTLA/CD160/HVEM/LIGHT and human malignancy are still being defined and a clear and cohesive picture has yet to emerge. Early studies described that engagement of LIGHT by tumors expressing HVEM can deliver a pro-apoptotic signal that suppresses tumor growth[296]. In some lymphoma cells, stimulation with LIGHT via HVEM increased sensitivity to Fas-mediated apoptosis[297]. For patients with follicular lymphoma, acquired mutations in HVEM have been correlated with worse prognosis[298]. On the other hand, in chronic lymphocytic leukemia, CD160 expression has been identified as a pro-survival factor[299]. Recently, in a study of patients with melanoma, persistent expression of BTLA was identified as a unique marker of tumor-specific CD8^{+} T cells in vivo[300, 301]. BTLA expression was associated with compromised T cell activity. Furthermore, vaccination with CpG led to downregulation of BTLA expression on tumor-specific T cells and restoration of their function.

8.5.6.3 Preclinical Studies

With a myriad of possible interactions and outcomes, the exploitation of therapies that enhance anti-tumor immunity are in early stages of development for these molecules. Potential reagents that block inhibitory signals or enhance activating signals will have to be tested carefully to identify any unintended or unanticipated interactions. Two strategies that have been tested preclinically in mice are the ectopic expression of LIGHT as a stimulator of enhanced anti-tumor activity, and administration of soluble BTLA as a blockade of inhibitory signaling. In mouse models of fibrosarcoma and mastocytoma, overexpression of LIGHT in tumor cells leads to T cell-dependent tumor rejection, an effect mediated in part by the changes in the tumor stroma[268, 302–305]. Additional studies of ectopic LIGHT expression have shown activity in mouse models of melanoma, colon cancer, and breast cancer[306]. In a murine model of mammary carcinoma adenoviral expression of LIGHT in the primary tumor generated anti-tumor immunity that controlled metastatic disease[306]. One study has tested the effect of BTLA blockade on tumor immunity. In a mouse model of cervical carcinoma, expression of soluble BTLA enhanced the anti-tumor activity of an hsp70 cancer vaccine[307].

8.5.7 LAG-3/MHC II

8.5.7.1 Biology

LAG-3 (CD223) is a member of the IGSF initially identified as a molecule expressed on an NK cell line, F5[308]. LAG-3 is expressed on activated CD4^{+} and

CD8$^+$ T cells, NK cells, and activated B cells[309, 310]. The LAG-3 gene is located on human chromosome 12, adjacent to the CD4 coding region. LAG-3 shares a common structure and common ligand, MHC II, with CD4. LAG-3 has an affinity for MHC II that is several-fold higher than CD4 for MHC II[311, 311]. LAG-3 contains 4 IgG domains: a small area in the D1 domain is necessary for binding to MHC II[313]. LAG-3 has a short intracellular tail containing a unique motif (KIEELE) responsible for its inhibitory functions[314].

The significance of LAG-3:MHC II engagement is still an area of active investigation. LAG-3 has been described as a negative regulator of T cell activation. LAG-3-blocking antibodies enhance murine and human T cell proliferation and cytokine production in vitro[229, 312, 315]. Cross-linking of an agonist LAG-3 antibody inhibits T cell proliferation and cytokine production in vitro[316]. The immunologic phenotype of LAG-3$^{-/-}$ knockout mice is subtle and initially only a deficiency in NK cell-mediated killing was reported[317]. Later studies demonstrated that TCR transgenic LAG-3-deficient T cells have reduced expansion upon antigen exposure[314]. This phenotype was abrogated by disruption of the intracellular KIEELE sequence, the motif responsible for inhibitory signaling. Additionally, closer analysis of LAG$^{-/-}$ knockout mice revealed that aged mice have double the number of CD4$^+$ and CD8$^+$ T cells of their wild-type counterparts, supporting a role for LAG-3 in homeostatic proliferation[318]. LAG-3 may play a special role in anergic and/or Treg populations. Using microarray analysis, Huang et al. identified LAG-3 as a gene highly expressed in population of TCR transgenic CD4$^+$ T cells that develop anergic/regulatory features upon transfer into an antigen-bearing host. They further reported that LAG-3 blocking antibodies inhibit the regulatory capacity of this population of cells in vitro and in vivo and ectopic expression of LAG-3 confers regulatory function in vitro[319]. It is unclear how this population of LAG-3 expressing cells with regulatory activities relates to classically defined CD4$^+$CD25$^+$Foxp3$^+$ Tregs. A subsequent study postulated that LAG-3 expression defines a subset of CD4$^+$CD25^{hi}Foxp3$^+$ Tregs that have potent inhibitory activity and are enriched in patients with melanoma or colorectal carcinoma, both in peripheral blood, and within the tumor[320]. In a separate report of patients with tuberculosis, an antigen-specific population of CD8$^+$LAG-3$^+$CD25$^+$Foxp3$^+$ cells had potent suppressive activity[321]. The effect of “reverse” signaling via LAG-3 to APCs expressing MHC II has been challenging to define. Initial studies found that engagement of MHC II on APCs by a soluble LAG-3-Ig fusion protein enhanced dendritic cell maturation, upregulated co-stimulatory molecules, and increased cytokine production, leading to the description of LAG-3 as a dendritic cell activator[322, 323]. Subsequent studies using co-culture of dendritic cells with CD4$^+$CD25$^+$Foxp3$^+$ Tregs found that they inhibit dendritic cell activation by expressing membrane-bound LAG-3, suggesting disparate effects for membrane-bound LAG-3 compared with the soluble LAG-3-Ig fusion protein[324, 325].

8.5.7.2 Preclinical Studies

The approaches to manipulating LAG-3:MHC II interaction for enhanced anti-tumor immune activity fall in to two categories. First, blockade of inhibitory T cell signaling mediated by LAG-3 engagement of MHC II enhances T cell activation and anti-tumor activity. This has been demonstrated in a mouse model of spontaneous prostate cancer, TRAMP, where LAG-3 blocking antibodies alone enhanced intratumoral accumulation and effector function of tumor-specific $CD8^{+}$ T cells[326]. In this study, a combination of LAG-3 blocking antibody and a tumor vaccine led to a reduction in tumor grade and an increase in activated intratumoral $CD8^{+}$ T cells, consistent with the generation of an anti-tumor immune response. In a second approach, administration of a soluble LAG-3-Ig fusion protein can enhance T cell activation and anti-tumor immunity. For example, $CD8^{+}$ T cell responses to a model antigen, ovalbumin, are enhanced in vivo by the administration of LAG-3-Ig[327]. In a study of three murine tumor cells lines (kidney, sarcoma, and mammary), Prigent et al. found that LAG-3-Ig, either transduced into the tumor cells or administered by injection, reduced tumor growth in vivo[328]. Additionally, and in apparent contradiction to studies of LAG-3 blockade, transfection of transmembrane human or mouse LAG-3 gene into the same tumors slowed tumor growth and enhanced tumor rejection. This effect was more marked for the human LAG-3, which might have represented a novel rejection antigen. Lastly, in a mouse model of spontaneous HER-2/neu^{+} mammary carcinoma, co-administration of LAG-3-Ig with a DNA vaccine prevented carcinogenesis[329].

8.5.7.3 Clinical Trials

The clinical development of agents targeting LAG-3 has thus far been limited to a single reagent, IMP321 (Immutep, Paris, France), a soluble LAG-3-Ig fusion protein consisting of the human LAG-3 proteins fused to human IgG1 Fc. IMP321 has been described as a dendritic cell adjuvant[330]. The first two phase I studies of IMP321 tested its safety when administered with commercially available vaccines against influenza and hepatitis B[331–333]. These studies demonstrated IMP321 to be safe and to enhance responses to vaccine antigens. The first study of IMP321 in cancer patients was a phase I dose escalation study in patients with metastatic renal cell carcinoma. In a study of 21 patients treated with doses of 0.05–30 mg IMP321 given biweekly, the treatment was well tolerated, but there were no objective responses[334]. There was, however, some indication of activity with an improvement in progression-free survival seen among patients who received a dose above 6 mg. At 3 months after treatment, 7/8 patients treated with the higher dose had stable disease whereas 3/11 patients treated with the lower dose had stable disease ($P = 0.015$). A second phase I study of IMP321 combined with standard chemotherapy as a first line agent for metastatic breast cancer reported promising preliminary results[335]. Thirty patients were treated in

three cohorts of 0.25, 1.25, or 6.25 mg of IMP321 in combination with paclitaxel. The therapy was again well tolerated and a response rate of 50 % compares favorably with historical controls of paclitaxel alone.

While LAG-3 appears to be a promising molecule for anti-cancer therapy, studies to date highlight several areas where our understanding of this molecule is incomplete. Thus far, LAG-Ig, a dendritic cell adjuvant, is one approach for the treatment of cancer that is under clinical development. Preclinical studies suggest that LAG-3 blocking antibodies may warrant further exploration.

8.5.8 B7-H3/TRT-2/B7-H4

8.5.8.1 Biology

B7-H3 (CD276) was identified in 2001 as a B7 homolog sharing 20–27 % identity with other B7 family members[336]. Low levels of B7-H3 expression can be detected in peripheral tissues including liver, lung, bladder, testes, prostate, uterus, intestine, kidney, pancreas, and breast[336, 337]. Higher levels of B7-H3 expression may be induced on T cells, NK cells, dendritic cells, and macrophages[336, 338, 339]. B7-H3 has been described as having both co-stimulatory and co-inhibitory effects on T cell responses. The co-stimulatory properties of this molecule were first described when a B7-H3-Ig fusion protein was found to increase T cell proliferation and cytokine production in vitro[336]. The co-stimulatory receptor for B7-H3 was identified in 2008 as TLT-2 (TREM-like transcript 2) a member of the TREM (Triggering Receptor Expressed on Monocyte) receptor family[6]. TLT-2 is expressed constitutively on $CD8^+$ T cells and is induced on activated $CD4^+$ T cells[6]. Additionally, TRT-2 has been detected on B cells, monocytes, and macrophages[340, 341]. Consistent with TLT-2's function as a co-stimulatory receptor, overexpression of TLT-2 on T cells leads to enhanced cytokine production upon stimulation with B7-H3[6]. Alternatively, experimental evidence also has identified co-inhibitory functions of B7-H3. For example, recombinant B7-H3 protein inhibits anti-CD3 antibody-induced T cell proliferation and cytokine production in vitro. This effect is associated with inhibited activation of transcription factors NFAT, NF-kB, and AP-1[338, 342, 343]. This duality in B7-H3 function suggests that another, as yet undiscovered counter-receptor for B7-H3 may exist. The phenotype of B7-H3$^{-/-}$ knockout mice provides support for both co-stimulatory and co-inhibitory functions of B7-H3 in vivo. In models of induced airway hypersensitivity and induced EAE, B7-H3-deficient mice had more severe symptoms, supporting a co-inhibitory role for B7-H3[338]. On the other hand, B7-H3$^{-/-}$ knockout mice have increased survival of cardiac and islet allografts, supporting a co-stimulatory role for B7-H3[344].

B7-H4 (B7×, B7S1) was discovered in 2003 and thus far has been described solely as a co-inhibitory molecule[343, 345, 346]. Its partnering receptor has not yet been identified. Expression of B7-H4 is more restricted than B7-H3, and in non-malignant tissue protein expression has been detected only in epithelial cells of the

female genital tract, kidney, lung, and pancreas[347]. B7-H4 expression has also been described on activated T cells, B cells, monocytes, macrophages, and myeloid dendritic cells[343, 345, 346, 348–350]. In vitro, B7-H4 acts to inhibit $CD4^+$ and $CD8^+$ T cell proliferation and cytokine production and blockade of B7-H4 in vivo enhances T cell responses[343, 345, 346]. B7-$H4^{-/-}$ knockout mice have a subtle phenotype; on the BALB/c background they have modestly enhanced Th1 responses and lower burdens of *Leishmania major* upon infection[351].

8.5.8.2 Relevance in Human Tumors

Both B7-H3 and B7-H4 have been detected in human cancers, where they provide useful prognostic information. Their expression in most, but not all cases, is a poor prognostic feature. B7-H3 and B7-H4 have been detected in non-small cell lung cancer and correlated with reduced numbers of tumor infiltrating lymphocytes and increased likelihood of metastases[352]. In renal cell carcinoma, B7-H3 and B7-H4 may be expressed on tumor cells or tumor vasculature and, in either case, expression correlates with poorer prognosis[353, 354]. Furthermore, higher levels of soluble B7-H4 detected in the serum of patients with renal cell carcinoma is associated with advanced stage of disease[355]. For patients with prostate cancer, expression of B7-H3 or B7-H4 is associated with metastatic spread, increased risk of recurrence, and increased risk of death[356, 357]. Soluble and membrane-bound forms of B7-H3 have also been detected in patients with colorectal cancer, where they are associated with lower levels of tumor infiltrating lymphocytes and higher tumor grade[358]. B7-H4 has also been detected several additional tumor types including ovarian, breast, endometrioid, and pancreatic cancers[359–362]. In all cases reported, B7-H4 expression is associated with reduced tumor infiltrating lymphocytes and poorer outcomes. Curiously, in one study of 102 patients, expression of B7-H3 in gastric cancer was noted as a positive prognostic factor associated with increased survival[363].

8.5.8.3 Preclinical Studies

Preclinical studies of anti-tumor therapies based upon targeting B7-H3 or B7-H4 are in early stages of development and a defined strategy for targeting these molecules has yet to emerge. The data thus far does support a role of B7-H3 in modulating anti-tumor immunity. In several mouse models, including lymphoma, mastocytoma, hepatocellular, and colon cancer, overexpression of B7-H3 leads to slowed tumor growth or enhanced tumor rejection. The anti-tumor immunity generated by ectopic B7-H3 expression depends upon $CD8^+$ T cells and NK cells, but not $CD4^+$ T cells[364–367]. These findings stand in apparent contradiction to most human studies where higher levels of B7-H3 expression correlate with poorer prognosis, highlighting the areas of uncertainty regarding the role of B7-H3 as both and positive and negative regulator of T cell responses.

8.5.9 CD40/CD40L(CD154)

8.5.9.1 Biology

CD40 is a member of the TNFR superfamily that acts as a modulator of humoral and cell-mediated immunity. It was initially detected on a panel of transformed B cell lines and bladder cancers[368]. CD40 expression has since been described in B cells, macrophages, dendritic cells, endothelial cells, thymic epithelial cells, hematopoietic progenitors, and activated T cells[369–371]. CD40 has also been detected in hematologic malignancies including non-Hodgkin lymphoma, Hodgkin lymphoma, and multiple myeloma, and in solid tumors including bladder, renal, pancreatic, nasopharageal, cervical, breast, prostate and lung cancers[372–378]. Its ligand, CD40 ligand (CD40L, CD154), is expressed on activated T cells, B cells, and platelets[379–382]. During an inflammatory response, additional cell types also may express CD40L including monocytes, vascular endothelial cells, and smooth muscles cells[383–386].

Binding of CD40 with its counter-receptor CD40L results in bidirectional signaling. The signal transduction pathways employed upon CD40 ligation depend, in part, on the cell expressing CD40. For example, in dendritic cells, recruitment of TRAF6 to the cytoplasmic tail of CD40 is necessary for activation of p38 MAPK and JNK, and ultimately the production of inflammatory cytokines like IL-12[387]. On the other hand, in B cells, TRAF2,3 binding is necessary for phosphorylation of p38 MAPK and JNK.

CD40 participates in a diversity of immune functions in vivo including immunoglobulin class switching, B cell memory generation, germinal center formation, and cytokine production by endothelial cells, monocytes, B cells, and dendritic cells. CD40 plays a major role in "licensing" dendritic cells, allowing for maturation, upregulation of co-stimulatory molecules, cytokine production, and consequently enhanced priming and expansion of T cell responses[388]. Patients with germ line mutations in either CD40 or CD40L define a subclass of hyper-IgM syndrome, a combined immunodeficiency syndrome, and have increased susceptibility to opportunistic infections[389–391].

8.5.9.2 CD40 Expression on Human Tumors

CD40 expression has been detected on human tumor cells including many hematologic malignancies and solid tumors. CD40 has been detected on most B cell malignancies including chronic lymphocytic leukemia, non-Hodgkin lymphoma, Hodgkin lymphoma, and multiple myeloma[392, 393]. CD40 may be expressed in solid tumors and has been described in bladder, renal, pancreatic, prostate, colon, lung, breast, and cervical cancers. In melanoma, CD40 expression is relatively common. In a study of 97 short-term metastatic melanoma cell cultures, CD40 expression was detected on 42 % of melanomas. In a study of hepatoma specimens and derived

cell lines, CD40 expression was detected in 40 % of hepatomas, but not in normal hepatocytes[394]. Lastly, in a study of ovarian cancer, CD40 expression was detected in 60 % of paraffin embedded samples and 73 % of fresh ovarian cancer samples, but not on normal ovarian tissue. Additionally, CD40 expression correlated significantly with ovarian cancer staging[395].

8.5.9.3 Preclinical Studies

The activities of anti-CD40 antibodies have been tested both in vitro and in vivo. For some tumors, anti-CD40 antibodies have direct anti-tumor activity, potentiating cell death, and inhibiting cell growth[396]. A direct anti-tumor effect has been observed in B cell malignancies, and breast and ovarian carcinomas[395, 397–399]. For some CD40-expressing cancers like melanoma, CD40 engagement has no direct effect[386]. Numerous studies utilizing murine tumor models have characterized the immunologically mediated anti-tumor effects of targeting CD40. In one early study, transgenic expression of CD40L in a poorly immunogenic neuroblastoma cell line, neruo-2a, led to reduced tumor growth, enhanced survival and protection against subsequent tumor challenge[400]. These effects were dependent upon $CD8^+$ T cells and supported a role for CD40 ligation in generating anti-tumor immunity. In several subsequent studies, overexpression of CD40L in several tumor types including melanoma, myeloma, and lung, colon, and mammary carcinoma have confirmed and expanded this initial observation[400–404]. As an alternative approach to CD40 ligation, anti-CD40 agonist antibodies have been tested in several transplantable murine tumor models including renal carcinoma, lymphoma, mesothelioma, and fibrosarcoma[405–413]. Collectively, these studies have elucidated the importance CD40 ligation in overcoming tolerance via effects on $CD4^+$ and $CD8^+$ cytotoxic T cells[407–409]. Additionally, the activity of anti-CD40 agonist antibodies has been demonstrated in a mouse model of spontaneous pancreatic carcinoma[414]. In this model, the activity of the anti-CD40 agonist antibody was dependent upon macrophages. Further preclinical studies of anti-CD40 antibodies have demonstrated synergy in combination with chemotherapy, radiation, peptide vaccination, IL-2, CTLA-4 blocking antibody, or DR5 agonist antibody plus 41BB agonist antibody[405, 406, 410–413].

8.5.9.4 Clinical Trials

Several approaches to targeting CD40 have undergone phase I evaluation for the treatment of patients with advanced cancers[373, 415, 416]. The first therapy to be developed was a trimer of recombinant human CD40L (rhuCD40L)[417]. In a phase I study of 32 patients with advanced solid tumors or non-Hodgkin lymphoma, two partial responses were reported, one in a patient with non-Hodgkin lymphoma and one with laryngeal cancer. The patient with laryngeal cancer later achieved a complete response. The dose-limiting toxicity, grade III/IV transaminitis, was seen in 57 % of patients treated at the highest dose. Three agonist anti-CD40 antibodies

have since been developed: CP-870,893 (Pfizer, New London, CT), SGN-40 (Seattle Genetics, Bothell, WA), and HCD122 (Novartis/XOMA, Berkeley, CA).

CP-870,893 is a fully human IgG2 antibody. In a phase I, single dose, dose escalation study, CP-870,893 was administered to 29 patients with advanced solid tumors[418]. At the highest dose, three dose-limiting toxicities were observed, venous thromboembolism and grade III headache. The most common adverse event was grade I–II cytokine release syndrome, seen in 55 % of patients. There were four partial responses (14 %) reported, all in patients with metastatic melanoma. In a second phase I study, CP-870,893 was administered in weekly doses to 27 patients with advanced solid tumors[419]. Again, cytokine release syndrome was the most common adverse event, and dose-limiting toxicities included grade III cytokine release syndrome and grade III urticaria. There were no responses reported in the study; seven patients (26 %) had stable disease. Correlative immune studies demonstrated T cell depletion in 50 % of the patients treated at the maximum tolerated dose, perhaps suggesting one explanation for the lack of observed responses. In a third phase I study, 21 patients with unresectable pancreatic cancer were treated with CP-870,893 in combination with gemcitabine, and four partial responses (19 %) were reported[414]. Additional studies with CP-870,893 are ongoing (NCT01103635, NCT01008527).

The remaining two antibodies are in earlier stages of clinical testing and testing has been confined to hematologic malignancies. SGN-40 (dacetuzumab) is a fully humanized IgG1 antibody. It has been tested in three phase I studies in patients with non-Hodgkin lymphoma, chronic lymphocytic leukemia, or multiple myeloma, respectively[420]. In the first study, 50 patients with refractory or recurrent non-Hodgkin lymphoma were treated with escalating doses of SGN-40 administered weekly[420–422]. No dose-dependent adverse events were reported and a maximum tolerated dose was not established. One complete response and five partial responses were reported. In the subsequent phase I studies in chronic lymphocytic leukemia and multiple myeloma, SGN-40 was similarly tolerated, but no objective responses were observed[421, 422]. Presently, ongoing studies are testing SGN-40 in combination with chemotherapies or immunotherapies (NCT00664898, NCT00529503, NCT00655837, NCT00556699). HCD122 is a fully humanized IgG1 antibody. Data available from a phase I study of HCD122 in advanced B cell malignancies suggests that it is well tolerated and has potential, with two partial responses (8 %) reported out of 24 evaluable patients with chronic lymphocytic leukemia or multiple myeloma[416].

8.5.10 GITR/GITRL

8.5.10.1 Biology

Glucocorticoid-induced TNFR family-related gene (GITR) was originally identified in a dexamethasone-treated murine T cell hybridoma[423]. It shows

significant homology with other co-stimulatory TNFR family members including OX40, 4-1BB, CD40, and CD27[423, 424]. GITR is expressed at low levels on naïve $CD4^+$ and $CD8^+$ T cells, but is upregulated upon T cell activation[425, 426]. In addition, it is expressed constitutively at high levels on Tregs, and further upregulated upon activation[426–428]. Its binding partner, GITR ligand (GITR-L), is expressed in an inducible fashion on macrophages, dendritic cells, and B cells[426, 429–431]. The cytoplasmic tail of GITR contains several TRAF domains, and downstream signaling appears to be mediated via interaction with TRAF 1, 2, and 3[432, 433]. Stimulation of GITR may result in the activation of downstream targets including NF-kB, p38 MAPK, JNK, and ERK[423, 425, 434]. GITR has also been shown to interact with Siva, a death domain containing protein that can induce apoptosis in some cells[435].

In $CD4^+$ and $CD8^+$ effector T cell populations, ligation of GITR, by GITR-L or agonist antibodies, during TCR-mediated activation enhances proliferation and effector function[425, 426, 436, 437]. This effect is especially pronounced under conditions of suboptimal stimulation. The co-stimulatory effects of GITR and CD28 are synergistic, suggesting non-overlapping functions of these two molecules[437]. Conversely, blocking GITR activation with anti-GITR-L antibodies inhibits T cell proliferation[438]. In Tregs, engagement of GITR abrogates suppressor activities, but enhances proliferation in vitro[426–428, 439]. In vivo, GITR agonist antibodies have been shown to exacerbate autoimmunity and inflammation in several mouse models including models of colitis, diabetes, experimental autoimmune encephalomyelitis, and graft versus host disease[427, 436, 440–442]. Unexpectedly, $GITR^{-/-}$ knockout mice show enhanced T cell proliferation and sensitivity to AICD, suggesting a more complicated role of GITR in lymphocyte proliferation and survival[439].

8.5.10.2 Preclinical Studies

The immune potentiating effects of GITR ligation have been tested in several mouse models and provided preclinical support for the development of an agonist antibody presently being tested in a Phase I clinical trial. GITR agonist antibodies were first tested in mice and shown to be potent stimulators of anti-tumor immunity. Mice treated with the GITR agonist antibody DTA-1 were able to eradicate established MethA fibrosarcoma or CT26 colon carcinoma tumors. This effect was dependent upon IFN-γ, $CD4^+$, and $CD8^+$ T cells[443]. In another transplantable tumor model, the poorly immunogenic B16 melanoma, treatment with DTA-1 was able to induce concomitant immunity that allowed rejection of distal sites of disease[444]. In the B16 model, tumor rejection was dependent upon $CD4^+$, $CD8^+$, and $NK1.1^+$ cells as well as IFN-γ and Fas ligand, but independent of perforin and $CD25^+$ cells[445]. Additionally, DTA-1 treatment results in a reduced ratio of intratumoral Treg/effector T cells in tumor bearing mice[446, 447]. Lastly, GITR agonist antibodies have been tested and proven to have synergistic T cell activating and anti-tumor effects when combined with vaccines[448, 449].

8.5.10.3 Clinical Development

On the basis of this preclinical activity, GITR was identified as a promising area for research by the National Cancer Institute[450]. A humanized, Fc-disabled, anti-human GITR monoclonal antibody, TRX518 has been developed by Tolerx (Cambridge, MA, USA) and is presently being tested in a phase I clinical study for patients with previously treated metastatic melanoma (NCT01239134).

8.5.11 4-1BB (CD137)/4-1BBL

8.5.11.1 Biology

A member of the TNF-receptor superfamily, 4-1BB (CD137) was originally identified as an RNA transcript selectively upregulated in activated T lymphocytes[451, 452]. 4-1BB is also detected on dendritic cells, NK cells, smooth muscle, and vascular endothelium in tumors[453–455]. 4-1BB functions as a co-stimulatory molecule that enhances proliferation, cytokine production, survival, and effector function particularly in the $CD8^+$ T cell population[453, 456–461]. Its binding partner, 4-1BB ligand (4-1BB-L), is expressed on activated macrophages, dendritic cells, and B cells as well as on myeloid progenitors and hematopoietic stem cells[459, 462–464]. Expression levels of 4-1BB are regulated and are low in resting cells, but are upregulated under conditions of inflammation[465–467]. Upon engagement of its ligand, 4-1BB recruits adaptor molecules TRAF1 and TRAF2 to a cytoplasmic domain, initiating several activating, pro-survival signal transduction cascades. Downstream activation events include enhanced activation of members of MAPK pathways and NF-kB pathway, upregulation of Bcl-2 family members, and downregulation of the pro-apoptotic molecule Bim[468–475].

Early studies demonstrated that 4-1BB engagement both in vitro and in vivo provides co-stimulatory signals to both $CD4^+$ and $CD8^+$ T cells, with an especially potent effect on $CD8^+$ T cell proliferation and survival[460, 461, 476, 477]. Mice deficient in 4-1BB or 4-1BB-L have a fairly subtle immunological phenotype, suggesting that 4-1BB ligation is not an absolute requirement for the normal development of lymphocytes, or for the generation of several types of immune responses. However, in some settings, such as response to influenza virus, a lack of 4-1BB stimulation results in a suboptimal anti-viral immune response[478]. Moreover, regardless of the necessity for 4-1BB, augmented 4-1BB signaling during antigenic stimulation enhances the magnitude of the subsequent antigen-specific immune response[479]. Unexpectedly, 4-1BB agonist antibodies have been observed to diminish the severity of autoimmunity in several mouse models including experimental autoimmune encephalomyelitis, rheumatoid arthritis, and systemic lupus erythematosus[480–482]. An explanation for this apparent paradox is presently

under investigation, and the diminished generation of T cell-dependent humoral responses after treatment with anti-4-1BB antibody may offer an explanation[483].

8.5.11.2 Preclinical Studies

Increased signaling through 4-1BB can enhance anti-tumor immune responses. The potential of an agonist anti-4-1BB antibody to stimulate anti-tumor immunity was first tested in the treatment of two transplantable murine tumors: the sarcoma Ag104A, and the mastocytoma P815. Antibody-induced regression of established tumors required $CD4^+$ and $CD8^+$ T cells as well as $NK1.1^+$ cells[455, 484]. Subsequent studies using either 4-1BB agonist antibodies, 4-1BB-L, or transfection of the 4-1BB gene, demonstrated enhanced anti-tumor immune responses in a variety of transplantable tumor types including melanoma, fibrosarcoma, lymphoma, and colon carcinoma[485–494]. In a study of the mouse fibrosarcoma B10.2, anti-4-1BB-mediated tumor rejection depended upon $CD8^+$ T cells, CD40 and IFN-γ[487]. Promising activity for anti-4-1BB antibodies has been demonstrated in combination with dendritic cell vaccines, adoptive T cell therapy, chemotherapy, or antibodies against CTLA-4, PD-1, or CD40[412, 495–500]. Notable toxicities observed in the preclinical testing of 4-1BB agonist antibodies in mice include hepatotoxicity, skin toxicities, and hematopoietic toxicities[500, 501]. The hepatotoxicity observed in mice was an elevation in blood levels of liver transaminases associated with liver-infiltrating $CD8^+$ T cells. The phenomenon was transient, resolved with cessation of treatment, and was dependent upon $CD8^+$ T cells and TNF-α. In contrast to mice, preclinical studies in monkeys treated with the fully human anti-4-1BB IgG4 antibody, BMS-663513, showed no evidence of toxicities, hepatic or otherwise[501, 502]. Hematologic toxicities included lymphopenia, thrombocytopenia, and anemia[500].

8.5.11.3 Clinical Development

BMS-663513 is a fully human IgG4 agonist antibody against human 4-1BB. It was initially tested in a Phase I-II multidose, dose escalation study of 115 patients with advanced or metastatic solid tumors[503]. The most common toxicity reported was transaminitis seen in 15 % of patients. Other common toxicities included leukopenia, neutropenia, and thrombocytopenia. Clinical activity, including partial responses and sustained stable disease, was reported in all dose levels. Based on these findings, a randomized, open-label, phase II study of patients with previously treated stage IV melanoma was initiated. This study was terminated early due to an unusually high incidence of grade IV hepatitis[501, 504]. A second antibody targeting 4-1BB, PF-05082566 (Pfizer, Groton, CT, USA), a fully human IgG2 antibody, is presently under investigation.[590]

8.5.12 OX40/OX40-L

8.5.12.1 Biology

OX40 (CD134) is another co-stimulatory member of the TNF receptor superfamily. It was initially characterized as a determinant expressed on CD4$^+$ T cell blasts[505]. It is expressed on activated, but not resting or naïve T cells, and may be expressed on both CD4$^+$ and CD8$^+$ lymphocytes[505–510]. OX40 is expressed on CD4$^+$CD25$^+$ Tregs in both humans and mice[511, 512]. Additionally, OX40 may also be detected on neutrophils and NKT cells[513, 514]. The ligand for OX40 (OX40-L, CD252) was initially identified as a 34-kDa glycoprotein expressed on human T cell leukemia virus type-1 transformed cells, and consequently named GP34. It was adopted into the TNF superfamily based on sequence homology to other family members[515–517]. Subsequently, GP34 was identified as a ligand for OX40 and renamed OX40-L[518, 519]. OX40-L is expressed on APCs including dendritic cells, B cells, and macrophages[520, 521]. Its expression may be upregulated by inflammatory stimuli[521]. Expression of OX40-L has also been detected on activated T cells, vascular endothelial cells, and airway-smooth muscle cells[522, 523, 524].

For effector CD4$^+$ and CD8$^+$ T cells, OX40 engagement appears to play an important co-stimulatory role in the later stages of T cell activation, enhancing proliferation and survival. The interaction of OX40 with OX40-L leads to the recruitment of TRAF molecules to the cytoplasmic domain of OX40 and activates pro-survival signals through NF-kB, BCL-2, BCL-xL, and survivin. OX-40 signaling also enhances cytokine production by activated CD4$^+$ T cells. The phenotype of OX40- and OX40-L-deficient mice support a role for OX40 signaling in T cell proliferation and survival, resulting in enhanced clonal expansion of effector and memory populations, especially for CD4$^+$ T cells[525–528].

For Tregs, which constitutively express OX40, OX40 engagement has been shown to block suppressor function in vitro. OX40 appears to play several roles in Treg function in vivo, perhaps reflecting differences in the two major Treg subpopulations: natural Tregs (nTregs) that develop in the thymus and inducible Tregs (iTregs) that develop in the periphery. In mouse models of inflammatory bowel disease, graft-versus-host disease, and skin transplant, OX40 ligation has been described as blocking the suppressive function of nTregs[511, 529, 530]. Furthermore, OX40 ligation appears to antagonize the generation of new iTregs[529, 531, 532]. However, OX40 can convey both positive and negative signals to Tregs, depending upon the context. In mouse models of experimental autoimmune encephalomyelitis and colitis, OX40 was found to play an important role in Treg expansion, accumulation in inflamed tissue, and in controlling autoimmunity[533, 534].

8.5.12.2 Preclinical Studies

In mouse models of transplantable tumors, strategies to augment OX40 signaling to enhance anti-tumor immunity have shown promise. Treatment of tumor-bearing

mice with either agonist antibodies to OX40 or soluble OX40-L has shown activity in mouse models of sarcoma, melanoma, glioma, lymphoma, mammary, renal prostate, and colon cancer[535–542]. Mice that survived tumor challenge after OX40 treatment developed protective tumor-specific memory[535]. The efficacy of OX40 ligation depends upon the inherent immunogenicity of the tumor, with poorly immunogenic tumors showing an inferior response[536]. The anti-tumor activity elicited by OX40 ligation appears to primarily reflect enhanced $CD4^+$ T cell function, which indirectly promotes $CD8^+$ T cell activity by augmenting $CD4^+$ T cell help[543]. OX40 engagement for anti-tumor therapy also may impact Tregs. In a model where intratumoral injection of OX40 agonist antibody led to tumor rejection, OX40 expression was required on both Treg and effector T cells[544]. In a second model, combination therapy with OX40 antibody plus chemotherapy led to intratumoral depletion of Tregs via induction of apoptosis. Lastly, OX40 agonist therapy has also shown promise in combination with GM-CSF, IL-2, chemotherapy, surgery, radiation, and adoptive immunotherapy[537, 545–548].

8.5.12.3 Clinical Studies

OX40 targeted therapy for the treatment of human malignancies is in the early stages of testing. A mouse monoclonal antibody agonist to human OX40 was developed and initially tested in non-human primates[549]. Subsequently, this antibody was tested in a first-in-humans, phase I, single-dose, dose escalation study including 30 patients with advanced malignancies. Toxicities were mild and included fatigue and transient lymphopenia[550]. A humanized anti-OX40 antibody and a human OX40-L-immunoglobulin fusion protein are also presently in development[550, 551].

8.5.13 ICOS/ICOS-L

Inducible co-stimulator (ICOS, CD278), a member of the IGSF, is structurally related to CD28 and CTLA-4. ICOS was initially identified in 1999 as a molecule expressed by activated human T cells with 39 % sequence similarity to CD28[552]. As its name suggests, ICOS is co-stimulatory molecule rapidly upregulated upon TCR engagement and CD28 co-stimulation[553]. ICOS may play an especially important role in T cell survival, proliferation, and generation of memory[554]. ICOS partners exclusively with ICOS ligand (ICOS-L, B7h, B7RP-1, CD275), a molecule initially identified in a screen of genes induced by NF-kappaB/Rel transcription factors[555–557]. ICOS-L is expressed constitutively on B cells, macrophages and dendritic cells and can be induced on non-lymphoid cells exposed to inflammatory cytokines[556, 557]. When compared to CD28 signaling, engagement of ICOS appears to activate similar downstream events. Both CD28 and ICOS function as activators of PI3K and MAP kinases, and PI3K is necessary for some aspects of ICOS-mediated activation including cytokine secretion[558–561]. However,

the intracellular domains of CD28 and ICOS have several distinctive features that may explain subtle differences in signaling. For example, a unique YMFM motif in ICOS recruits a particular variant of PI3K which is an especially potent stimulator of Akt, an important T cell survival factor[562, 563].

Genetically deficient mice and transgenic mice have defined important roles for ICOS in vivo[555, 557, 564]. ICOS is an important factor in T cell-dependent B cell responses via its indispensible role in the development of follicular T helper cells[565–571]. ICOS also plays an important role in the development and effector functions of Th1, Th2, and Th17 cells and in development of a robust memory T cell compartment[554, 571–577]. ICOS is also constitutively expressed on Tregs and plays an important role in their development and in stimulating production of IL-10, a key regulatory cytokine[578, 579].

8.5.13.1 ICOS—A Clinical Connection?

The first studies correlating ICOS expression with clinical activity came from the monitoring of six patients with bladder cancer who were treated with ipilimumab in the neoadjuvant setting. Patients treated with ipilimumab prior to surgical resection for advanced bladder cancer. In this setting, ICOS was identified as a biomarker upregulated on peripheral and tumor infiltrating T cells[141]. This correlation was subsequently observed in tumor samples from patients with prostate, and breast cancer as well[143, 149]. Furthermore, in a retrospective analysis of melanoma patients treated with ipilimumab, increased frequency of $CD4^{+}ICOS^{hi}$ T cells, sustained over a period of 12 weeks, correlated positively with increased overall survival[143].

Several additional studies are beginning to suggest that ICOS expression and activation may be a relevant to understanding immune cell functions in human tumors in the absence of CTLA-4 blockade. For example, in a study examining tumor infiltrating Tregs in human melanomas, the authors observed high ICOS expression on tumor infiltrating Tregs when compared to Tregs found in the peripheral blood of healthy donors[580]. In this study, high ICOS expression on Tregs corresponded to high levels of suppressor activity in vitro. In contrast, in a study of genes expressed in metastatic lesions of patients with melanoma, expression of ICOS had a positive correlation with survival[581]. Lastly, in a study by Paulos et al., cultured human cells activated with ICOS co-stimulation had increased production of IL-17 and IFN-γ and increased anti-tumor activity when adoptively transferred into mice bearing human tumors[582]. Thus, it seems likely that ICOS may play multiple roles in the interaction between immune cells and tumors, reflecting its diverse expression pattern in multiple immune cell subtypes.

8.5.13.2 Closing Remarks

The past two decades have been marked by a growing understanding of the co-stimulatory and co-inhibitory pathways that are critical to the generation of

an effective, well-regulated immune response. Capitalizing on an increasingly nuanced appreciation for the role that these molecules play in anti-tumor immune responses, novel therapies to treat human cancers have been developed. A number of these molecules are currently being targeted in early-stage clinical trials, and some, like anti-CTLA-4 therapy, have been granted clinical approval. Given the complexity of the generation and regulation of anti-tumor immune responses, combining therapies that target co-signaling molecules with conventional therapies like chemotherapy or radiation or experimental therapies like vaccination and adoptive T cell therapy, are already showing promise. Combinations of therapies that augment anti-tumor immunity via distinct mechanisms are most likely to shown synergy.

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Chapter 9
Managing Regulatory T Cells to Improve Cancer Immunotherapy

Tyler J. Curiel

Abstract Regulatory T cells (Tregs) are increased in peripherally circulating blood cells and in the solid tumor masses of patients afflicted with many different cancer histologies. Cancer Tregs not only are capable of impeding endogenous protective anti-tumor immunity from optimal functioning but are also capable of impeding the efficacy of anti-cancer immunotherapy. Tumor-associated Tregs represent heterogeneous populations, differing by their origins and in their mechanisms used to impede anti-tumor immunity. Their properties can differ compared to those in peripheral circulation. Most studies now report that Treg content in the tumor inversely correlates with survival or therapeutic response, but a few reports suggest that Tregs are beneficial to patients with certain types of cancers. Therapeutic strategies to manage Treg capacity to mediate immune dysfunction include depletion, regulatory functional blockade, differentiation blockade, altering trafficking, differentiation diversion, or raising the threshold of anti-cancer effector cells for Treg-mediated regulation. Several clinical trials have shown the feasibility and relative safety of managing Tregs in human cancer, although treatment effects are modest. This chapter will review contemporary knowledge of Tregs in cancers, including origins, mechanisms of action, interactions with other immune cells and strategies for therapeutic management, addresses the major questions facing the field and suggests additional important areas for future research. The focus is on $CD4^{+}CD25^{+}Foxp3^{+}$ Tregs, but other cancer-associated regulatory cells will be addressed in brief.

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9.1 Introduction

Malignancies post significant immunologic challenges for the host. On the one hand, they are antigenic and pathological, and should thus be amenable to immune destruction. On the other hand, irrespective of how abnormal the malignant cells have become, they nonetheless derive from self-tissues. Thus, the powerful host machinery of peripheral tolerance intervenes to prevent autoimmune (anti-tumor) attack indistinguishable from pathologic autoimmunity, consequently impeding what could otherwise be clinically protective anti-tumor immunity. Tumors also employ a devastating array of other immune escape features, discussed below and in detail in other chapters (see especially Chaps. 2, 4, 8, and 10).

Many potentially self-reactive T cells are deleted in the fetal thymus in central tolerance. However, imperfections in central tolerance prevent removal of all potentially self-reactive T cells, some of which enter peripheral tissues including blood, lymph nodes and gut, posing life-long risks for development of autoimmune problems. Those self-reactive T cells not eliminated through central tolerance must thus be dealt with using additional strategies, including peripheral immune tolerance. In peripheral immune tolerance, a potentially autoimmune attack in progress is sensed and inhibited typically before clinically apparent pathologic consequences arise. Sakaguchi and others elegantly showed that a subset of T cells within the $CD4^{+}CD25^{+}$ population were key mediators of peripheral tolerance[1].

Nonetheless, activated T cells, including anti-tumor effector T cells can also express the identical $CD4^{+}CD25^{+}$ phenotype. Thus, Tregs cannot usually be identified by flow cytometric phenotype alone. We now know that the forkhead/winged helix nuclear transcription factor Foxp3 regulates Treg differentiation and function[2–4]. Thus, Tregs usually express high Foxp3, although not all $Foxp3^{+}$ T cells are Tregs[5]. Additional phenotypic features of Tregs (mouse and human) include high expression of CTLA-4 and GITR, and expression of additional markers including CD62L, CCR7, LAG3, CD103, and others, and low expression of CD127, IL-2, IL-17, and interferon (IFN)-γ[6]. Nonetheless, these features are also common to many activated non-Treg $CD4^{+}$ T cells. Appropriate Treg identification with confirmatory functional studies continues to confound interpretations of clinical and preclinical data on potential immunopathogenic functions of Tregs in various settings.

Data derived from small animal models and from human patients have established that Tregs are numerically increased in peripheral blood and the solid tumor masses of epithelial carcinomas, lymphomas and sarcomas, and in lymph nodes draining these tumors[7–24]. Initial Treg work focused on cells in blood circulation as they were easy and safe to access. Tregs circulate in increased numbers in the blood of patients during the blood phase of their hematologic malignancies, such as in acute myelogenous leukemia[25]. $CD4^{+}CD25^{+}FOXP3^{+}$ T cells are found in brain metastases in human melanoma and non-small cell

carcinoma, and in metastatic brain lesions in mouse models for breast and colon cancer, and metastatic melanoma[26], suggesting that Treg management strategies could also be effective in tumors in the central nervous system. It is now clear that the numbers, phenotypes, and functions of Tregs determined from studies of peripherally circulating cells might not reflect accurately local events in the tumor microenvironment. Immunological and clinical implications of these compartment-specific differences are still incompletely understood.

9.1.1 Categorizing Tregs

Although Tregs have been categorized in different schemes, a useful and durable concept was proposed by Jeff Bluestone[27]. In this scheme, Tregs arising in the thymus through homeostatic processes are defined as natural Tregs (nTregs), and are thought primarily to function as mediators of peripheral tolerance against autoimmune attack. Adaptive or induced Treg (iTregs) are induced extrathymically during inflammation or extrathymic T cell activation (including antigen encounter) under tolerizing conditions, likely to help control inflammation, among other functions (Fig. 9.1). nTregs appear to regulate immune processes through direct cell-to-cell contact despite producing IL-10 and TGF-β. iTregs regulate immunity through a variety of mechanisms including both cell-to-cell contact as well as soluble factors, reflecting their heterogeneous origins. nTregs and iTregs are phenotypically indistinguishable despite differences in in vivo function and mechanisms of action, although recently the nuclear transcription factor Helios was reported to identify nTregs relatively exclusively in both mice and humans[28]. It is not yet clear whether Helios will be useful to distinguish nTregs versus iTregs in inflammatory conditions, such as in cancer. This inability to distinguish nTregs from iTregs has impeded progress in understanding contributions to normal and pathologic process from each. A recent, pivotal study from the Rudensky lab[29] describes a mouse deficient in iTreg generation owing to experimental deletion of the CNS1 region of the *foxp3* gene that his group showed to be critical for iTreg generation[30]. These studies confirmed a role for iTregs in regulating inflammation and for nTregs in mediating autoimmune protection, although much additional work is required for a fuller understanding of their physiologic roles in a variety of contexts.

Remarkably, yet predictably, additional Treg subsets have been identified based on functional attributes, including subsets specialized to inhibit specific immune functions such as Th2 or Th17 $CD4^+$ T cell function. An exhaustive review of Treg differentiation pathways and factors was recently published[6], which is an excellent reference for additional reading.

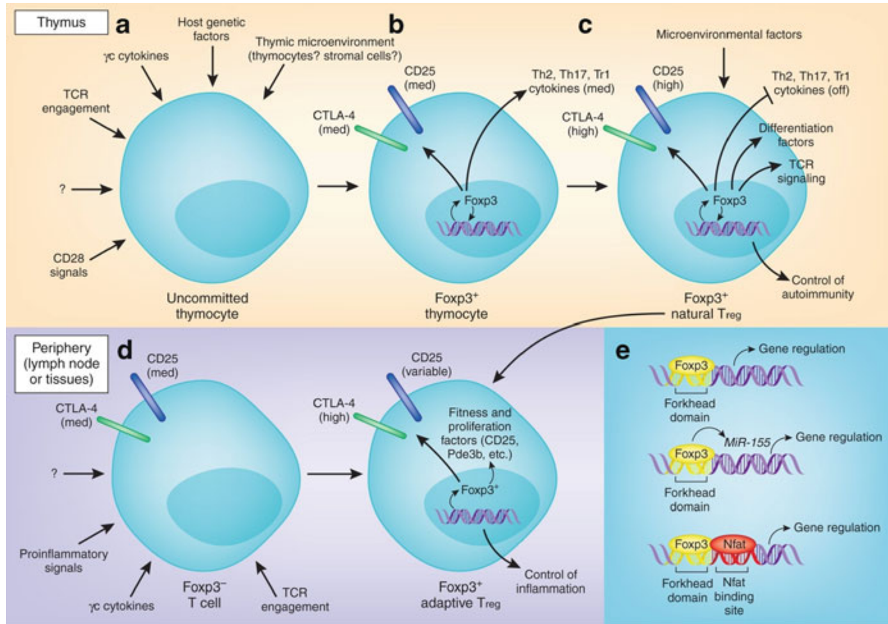

Fig. 9.1 Differentiation and phenotypes of nTregs and iTregs. (**a**) An uncommitted thymocyte in the thymus receives many instructions regarding its differentiation fate including from T cell receptor (TCR) engagement by antigen, cytokines binding the γc chain (such as IL-2), and other signals to be fully determined such as neighboring thymocytes or stromal cells, or host genetic factors. “X” represents signals yet to be discovered. (**b**) Early thymic signals begin a differentiation pathway. If the integration of signals induces Foxp3, cells start to differentiate into the Treg pathway and begin to express phenotypic features such as high-level CD25 and CTLA-4. These cells may still express Th2 cytokines such as IL-4, Th17 cytokines such as IL-17 or Tr1 cytokines such as IL-10 but are not suppressive. These cells resemble the T_{FN} cells or Tregs from FILIG mice. (**c**) Foxp3 expression reinforces its own expression, and in conjunction with continuing and new signals develops the fully developed Treg phenotype with higher CD25 and CTLA-4 expression than in b, suppressive function, reduced Th cytokines, and reduced phosphodiesterase (PDE) 3b. This is now a natural Treg, developing in the thymus and suppressing through contact-dependent mechanisms. It can exit the thymus to circulate or migrate to peripheral lymphoid organs such as lymph nodes and spleen. (**d**) A Foxp3^{-} thymocyte exiting the thymus can encounter local conditions that induce Foxp3 (such as vascular endothelial growth factor in a tumor) and lead to extrathymic Treg development from this Foxp3^{-} cell, producing adaptive Tregs (ITregs) that can suppress through soluble or contact-dependent mechanisms depending on speficics of their generation. The natural Foxp3^{+} Treg (nTreg) exiting the thymus (*upper right* part of *lower panels*) can encounter additional factors that change its properties as an iTreg. (**e**) Foxp3 can regulate gene expression either through binding to the forkhead domain (*top*), induction of regulatory mi-R155 (*middle*) or in cooperation with NFAT (*lower*). Additional levels of regulation occur when induced genes then mediate downstream effects. Reproduced from T. Curiel *Nature Medicine* 13:250–253 2007. Graphic: Katie Ris.

9.2 Properties of Tumor-Associated Tregs

9.2.1 General Properties

Tumor-associated Tregs are a heterogeneous mix of cells that have developed in distinct developmental pathways from cells arising in various anatomic compartments. They mediate disparate functions through diverse mechanisms as a result, which was the subject of an excellent review[31]. No definitive reports thus far document the relative contributions of various developmental pathways (such as iTreg versus nTreg) in the various Treg populations of a given tumor. Nonetheless, in a mouse model for cancer, nTregs and iTregs each contribute to tumor tolerance[32]. The recent generation of iTreg-deficient mice[29] will greatly aid further studies.

Tumor-associated Tregs are not typically distinguishable phenotypically from Tregs in other pathologic conditions. That is, tumor Tregs are $CD3^{+}$ T cells expressing CD4, CD25, GITR, and CTLA-4 among other features common to most Tregs identified to date.

9.2.2 Tumor-Specific Properties of Tregs

Tumor-associated Tregs also have specific and unique characteristics as a consequence of tumor microenvironment influences. For instance, blood $CD4^{+}CD25^{+}FOXP3^{+}$ Tregs in prostate cancer patients are more suppressive than comparable cells in blood from control subjects, despite similar total numbers[33]. Tregs in tumors of some human cancers could be more likely to induce $CD8^{+}$ effector T cell apoptosis through FasL-mediated interactions[34]. Tumor-associated TGF-β production promotes local Treg generation from naïve T cells[35]. A novel population of $CD4^{+}CD25^{-}CD69^{+}$ Tregs suppressing T cell function through membrane-bound TGF-β was reported in mouse cancer models including melanoma, hepatocellular carcinoma, and lung cancer[36]. Lack of expression of CD127 (IL-7 receptor α chain) is a feature of functional Tregs in human blood from normal subjects[37]. CD127 expression in relationship to function is relatively unstudied in tumor Tregs. We found that $CD4^{+}CD25^{+}FOXP3^{+}CD127^{+}$ and $CD4^{+}CD25^{+}FOXP3^{+}CD127^{-}$ T cells in blood and ascites of ovarian carcinoma patients both contained highly suppressive Tregs (manuscript submitted). Neuropilin-1 expression defines functional Tregs in mice[38]. A similarly functionally suppressive Neuropilin-1^{+} population has been described in human cervical cancer[39]. These cells resided preferentially in lymph nodes draining the tumor and were reduced by cytotoxic chemotherapy in direction relationship to reduction of the tumor mass. Additional differences between homeostatic and tumor-associated Tregs are likely to be described.

Apparent malignancies deriving from Tregs appear to occur, which is not altogether surprising, as hematologic neoplasms derived from essentially all

hematopoietic elements have been described. FOXP3 expression is reported in subsets of cutaneous T cell lymphomas. The malignant $FOXP3^+$ T cells have suppressive function in in vitro assays akin to typical $CD4^+Foxp3^+$ Tregs[40]. Mycosis fungoides cells undergoing large cell transformation express FOXP3 along with their clinically aggressive behavior[41]. In a study of lymphoma patients, $FOXP3^+$ and $FOXP3^-$ leukemia/lymphoma cases did not differ by major prognostic factors including tumor stage, patient age, tumor distribution, and concentrations of serum lactate dehydrogenase or serum calcium, and there was no difference in overall survival[42].

Recent observations of tumor Tregs suggest that they can promote metastasis in breast cancer through RANK/RANK ligand signals from RANK ligand produced by Tregs interacting with tumor RANK[43]. Tumor hypoxia can contribute to tumor Treg accumulation through hypoxia-driven CCL28 production[44].

9.3 Issues in Identifying *Bona Fide* Functional Tumor Tregs

9.3.1 Functional Testing Issues

As discussed further below, testing the function of putative Treg populations remains the gold standard to confirm Treg identity in specific settings. Further, it is now recognized that tests of Treg function are still limited. A useful distinguishing feature between Tregs and other activated $CD4^+$ T cells is the relative in vitro anergy of the former, despite significant proliferative potential *in vivo*[45]. The classic Shevach assay[16] tests the ability of a candidate Treg population to suppress proliferation of naïve T cells in vitro. Although useful, the assay is incomplete as it might not fully or accurately reflect the regulatory properties of that particular Treg population in vivo. For example, Tregs also suppress T cell IL-2 production which can be a major in vivo suppressive mechanism[46]. Aside from regulating T cell function, Tregs also affect antigen presenting cell function and the effects of various other immune cells (reviewed in 31, 46).

Even with relatively standardized tests, specific aspects of the functional tests can alter findings, leading to differing results and conclusions. For example, T cell receptor signaling strength partly determines the susceptibility of the responder T cells whose proliferative suppression is used to gauge Treg effects in standard in vitro Treg functional tests. Tregs can also exert effects on a specific T cell subset not tested in vitro, among other considerations. We have used Richard Flavell's FIR mice, in which viable Tregs can be flow cytometrically sorted based on red fluorescence protein expression under *foxp3* promoter control[47]. *Bona fide* Tregs from FIR mice can be transferred into tumor-bearing mice for specific tests of in vivo effects on tumor growth, tumor-specific immunity, and *de novo* Treg generation[48].

Because human samples are generally limiting, some investigators have tested T cell proliferation in mixed cell populations before and after $CD25^+$ cell depletion as a surrogate for specific Treg testing[49]. Testing suppression of T cell activation markers in vitro either with Treg addition or $CD25^+$ T cell depletion can give some information about the existence of functional Treg populations while using relatively small quantities of blood. We have shown that malignant human ascites is a good source of functional tumor-associated Tregs[8].

9.3.2 *Surrogates for Functional Testing*

As numbers of Treg are usually limiting in human tissues, additional techniques that can corroborate Treg identity in human tumors have been investigated. For example, FOXP3 expression identifies functional Tregs in selected human carcinomas[50]. FOXP3 methylation has been suggested as a way to identify functional Tregs when only small specimen quantities are available[51]. CD39 expression might distinguish functional Tregs from other T cells expressing the $CD4^+CD25^{hi}$ phenotype, including in patients with cancer[52]. Additional work is required to determine which nonfunctional surrogate tests are adequate for Treg identification in specific conditions. This issue is especially important following immune-based interventions, because treatments can have unexpected and unstudied effects on T cell phenotype that require additional study. Our lab policy is to confirm the functional identity of a potential Treg population in a setting for which such function has not previously been specifically established. We also continue to perform confirmatory functional testing in each experimental animal or human subject to the extent possible to continue to understand how reliable the phenotypic descriptors of potentially functional cell populations are. For example, we have found that interferon-α increased the prevalence of $Foxp3^+$ T cells in mice and humans with ovarian cancer, although these induced $Foxp3^+$ T cells do not necessarily have Treg function (manuscript submitted).

9.4 Significant Issues in Understanding a Role for Tregs in Tumor Immunopathology

Important questions to address include: (1) What mechanisms induce tumor-associated Tregs? (2) Why are Tregs increased in most cancers? (3) What are the specific roles for specific Treg subsets in tumor immunopathology? (4) What mechanisms do tumor-associated Tregs use to mediate cancer immunopathology? Answering these overarching questions helps understand cancer immunopathology and helps generate tools to develop novel and effective anti-tumor immunotherapies, a goal that has proven relatively elusive thus far.

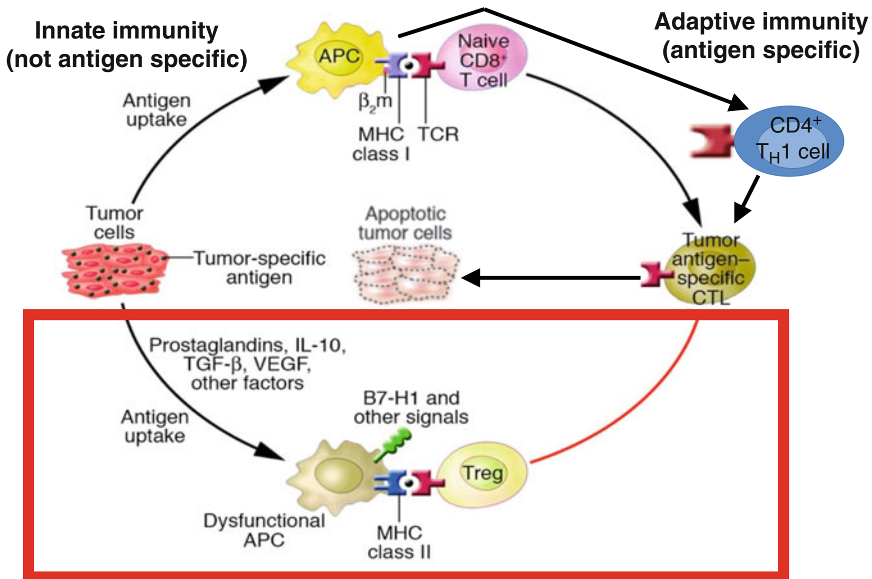

Fig. 9.2 Critical elements of tumor-associated immune dysfunction. Although anti-tumor immunity is elicited as shown in the top half of this figure, active tumor-driven immune dysfunction (*red* box in bottom half of the figure) thwarts immune cancer elimination. Antigen presenting cells, which in the top half can activate tumor-specific immunity, can also elicit dysfunctional immune cells that turn anti-tumor immunity off, or inhibit it through subversion by tumor factors. Factors responsible for this dysfunction can derive from the tumor itself, or from local stroma or immune cells. These agents include immune suppressive vascular endothelial growth factor (VEGF), transforming growth factor (TGF)-β, and interleukin (IL)-10. These molecules can directly inhibit immunity, such as the ability of TGF-β, IL-10, or VEGF to inhibit T cell activation, or can indirectly elicit other dysfunctional cells. In this latter instance, tumor IL-10 or VEGF can promote antigen presenting cells to express B7-H1, an immune molecule that can directly inhibit T cells, or promote generation of regulatory T cells (Tregs) that inhibit anti-tumor immunity. Tumors can attract Tregs through CCL22, CCL28, and other factors. Novel strategies to overcome these complex and potent tumor-driven active defenses against anti-tumor immunity represent major new opportunities to improve the efficacy of anti-tumor immunotherapy. Figure adapted from Curiel, TJ. *Drug Resistance Updates* 2012;15(1–2):106–13.

9.4.1 Origins of Tumor Tregs

Tregs accumulate in tumors and the patients with them for various reasons including: (i) control of autoimmunity, (ii) control of inflammation, (iii) *de novo* local differentiation (which means iTreg generation), (iv) recruitment from distant compartments, (v) local proliferation, and (vi) decreased death. Details of these potential mechanisms will be addressed in turn. Specific mechanisms for Treg actions, however, could nonetheless differ by tumor type and perhaps stage, and also could differ based on the anatomic compartment for any given tumor. Figure 9.2 illustrates how many factors generate Tregs at the same time that anti-tumor immunity is generated.

9.4.1.1 Control of Autoimmunity

The identification of increased Tregs specific for self-antigens is consistent with dysfunctional attempts to control autoimmunity as the basis for their increased numbers. Such normal self-antigen-specific Tregs in cancers have been described for a subset of self-antigens that are also tumor-associated antigens. As an example in humans, Tregs specific for the autoantigens gp100, TRP, NY-ESO-1, and survivin have been described in melanoma[53].

9.4.1.2 Control of Inflammation

Inflammation is a dual-edged sword in cancer. Chronic inflammation contributes to development of some cancers, and cancers generally promote a pro-inflammatory environment[54]. Thus, it is plausible that some tumor-associated Tregs are iTregs generated or attracted to help control the tumor microenvironmental inflammation. Thus far, described normal Tregs including those isolated from the tumor microenvironment can inhibit production of inflammatory cytokines in vitro. Nonetheless, to my knowledge, Treg accumulation specifically to control tumor-associated inflammation has not yet been formally demonstrated. However, consistent with the concept that Treg-mediated reductions in chronic inflammation can help prevent cancer, in a mouse model for chronic inflammation in the colon, Tregs lowered colorectal cancer incidence by inhibiting local inflammation[55]. Further, it is now clear that specific Treg subsets have defects in controlling certain types of inflammation[29,56,57], suggesting that some cancer-driven inflammation could be from reduced iTreg-mediated control, and suggesting possible means for novel therapeutic attack.

9.4.1.3 Enhanced *de Novo* Local Differentiation

Some experimental data support the notion that tumor environmental factors can facilitate Treg differentiation locally. Tumor cells can promote Treg differentiation by direct action on T cells, or indirectly by altering local antigen presenting cells, particularly dendritic cells[31] and likely other cells as well. Soluble as well as contact-dependent tumor mechanisms that promote local Treg generation have been described. Soluble mediators include cyclooxygenase-2, which is associated with (although not proven to mediate) increased Treg numbers in patients with head and neck cancers[58]. TGF-β produced by tumor cells differentiates naïve $CD4^{+}CD25^{-}$ T cells into Tregs (iTregs) in mouse models of renal cell carcinoma and prostate cancer[35]. The human SK-OVCAR3 cell line produces TGF-β that differentiates naïve $CD4^{+}CD25^{-}$ human T cells into Tregs (iTregs) *in vitro*[59]. Indoleamine 2,3-dioxygenase produced by human leukemia cells (and from other sources) induces Tregs in vitro and *in vivo*[60]. B cell CD70 signals in non-Hodgkin

lymphoma can boost FOXP3 expression in naïve human $CD4^{+}CD25^{-}$ T cells *in vitro*[61]. Gal1 from Reed-Sternberg cells in classic Hodgkin lymphoma can facilitate immune suppression directly and also indirectly by helping generate Tregs[62]. We found that tumor B7-H1 signals contribute to iTreg generation in mice with B16 melanoma[48], including sexually dimorphic effects discussed further in Chap. 13. Additional, tumor-associated factors whose identity remains unknown or poorly understood also contribute to tumor Treg generation[63,64].

The tumor can render local cells dysfunctional by promoting generation of iTregs or enhancing their function. For example, plasmacytoid dendritic cells in tumor draining lymph nodes in a mouse cancer model directly activated preexisting Tregs through indoleamine 2,3-dioxygenase production. The suppressive mechanism in this case (B7-H1/PD-1 signaling) is distinct from Tregs activated without indoleamine 2,3-dioxygenase[65]. Ovarian cancer-derived IL-10 and vascular endothelial growth factor induce dendritic cell B7-H1 expression that generates IL-10 producing Tregs in human ovarian cancer[66]. Tumor-conditioned human plasmacytoid dendritic cells also can redirect T cell differentiation to either $FOXP3^{+}$ Tregs or to $IL\text{-}10^{+}$ Tregs[67].

The relative importance of individual mechanisms for local cancer Treg generation remains poorly defined and likely will differ by tumor and by anatomic compartment.

9.4.1.4 Enhanced Recruitment

Different types of tumors produce factors preferentially recruiting local Tregs. The best studied axis is via CCR4 expressed on Tregs and the chemokines CCL17 or CCL22 in the tumor microenvironment (reviewed in 31). Specific examples include Treg attraction in ovarian[8] or gastric[68] carcinoma via CCL17 or CCL22 signaling. $CXCR4^{+}$ Tregs might also be attracted to the tumor microenvironment through local CXCL12 production, as preliminarily suggested in malignant mesothelioma[69]. The cytokine IL-2 is FDA-approved to treat specific cancers including malignant melanoma. However, it is now recognized that therapeutic IL-2 can increase CXCR4 expression on Tregs, increasing their accumulation in ovarian cancer patients[70]. Whether CXCR4/CXCL12 signaling boosts Treg accumulation in ovarian cancer patients not treated with IL-2 remains to be established. Elements in tumor stroma also might help attract Tregs locally to the tumor microenvironment. For example, tumor-associated macrophages in ovarian cancer secrete the chemokine CCL22 that can attract Tregs locally through a CCR4 interaction[8]. Local tumor hypoxia can generate CCL28 that attracts Tregs[44].

9.4.1.5 Enhanced Local Proliferation

TGF-β from certain tumors can impede local, myeloid dendritic cell maturation, contributing to local Treg proliferation[71]. Additional work supports the concept that

tumors produce soluble factors and surface-expressed molecules that can promote local Treg proliferation[32]. Other factors that impede dendritic cell maturation and thus Treg generation, such as vascular endothelial growth factor[72], can induce Tregs, but specific effects on Treg proliferation are not reported.

9.4.1.6 Reduced Local Treg Death

Increased death could be a mechanism to augment Treg accumulation in the tumor microenvironment, but such a mechanism remains to be demonstrated experimentally. There are therapeutic strategies known to increase Treg death locally in the tumor environment[73].

9.4.1.7 Miscellaneous Host Factors

Several models using genetically defined conditions have identified host factors that could alter tumor surveillance or anti-tumor immunity through Treg effects. As an example, IRAK-M$^{-/-}$ mice (lacking IRAK-M, a negative regulator of innate immunity) exhibit increased anti-tumor T cell immunity and reduced Tregs[74]. B7-H1 T cell cosignaling can generate IL-10^{+} Tregs in human ovarian cancer[66] and female B7-H1$^{-/-}$ mice have reduced Treg function compared to wild-type females[75] that is a sexually dimorphic B7-H1 effect on Tregs[48]. We have recently demonstrated that estrogen regulates B7-H1 T cell co-signaling effects in Treg generation (manuscript submitted) that is further discussed in Chap. 13.

9.5 Clinical Relevance of Tumor Treg Content

9.5.1 Treg Content and Prognosis

FOXP3 expression in immune cells in the tumor has been proposed as a prognostic biomarker[76]. Nonetheless, FOXP3 expression can be transient and/or reversible, thus altering the function of T cells in which it is expressed[77,78]. Foxp3^{+} T cell differentiation is now also known to be highly plastic[79]. For example, in human ulcerative colitis and colon cancers developing in that setting, FOXP3^{+} Treg are functionally suppressive, but also produce cytokines not produced by homeostatic Tregs, including TNF-α, IFN-γ, and IL-17[80]. It is thus unlikely that simple analysis of immune cell or tumor cell Foxp3 content alone will be a highly specific predictive tool without additional information including functional data from FOXP3-expressing cells, the content of other local immune cells, and the anatomic relationships of immune cells to each other and to the tumor, among many additional considerations.

Tumor Treg numbers correlate negatively with survival or treatment response in several studies, including in ovarian cancer[8], R0 (fully resected) gastric carcinoma[81] and hepatocellular carcinoma[82,83]. Intratumoral Foxp3$^+$ T cells positively correlated with local recurrence in the vertical phase of melanoma[84].

By contrast, a few studies, notably in hematologic malignancies[85], suggest that increased Tregs are beneficial to survival or prognosis. In colorectal cancer, tumor FOXP3$^+$ cell number was positively correlated with survival whereas FOXP3$^+$ cell number in unaffected tissue in the same patients was negatively associated with survival[86]. In head and neck cancer, tumor Treg positively correlated with regional cancer control[87]. Studies in experimental animal cancer models provide at least one plausible mechanism by which Tregs function could benefit anti-tumor immunity. In a mouse melanoma model, Tregs augmented anti-tumor immunity, potentially by inhibiting complete tumor eradication. Residual tumor could potentially generate sufficient tumor antigen to stimulate anti-tumor immunity without clinical detrimental effects[88]. Functionally suppressive FOXP3$^+$ Treg in ulcerative colitis produce TNF-α, IFN-γ, and IL-17 as noted above[80] that could promote anti-tumor immunity.

Finally, a few studies show that Treg content conveys no prognostic value including the finding that tumor-infiltrating FOXP3$^+$CD4$^+$CD25$^+$ T cells had no predictive power in renal cell carcinoma[89]. In anal cancer, Treg cell content was not prognostic[90], but patients were studied following radio-chemotherapy treatments, potentially confounding results. The differing conclusions and results in these various studies could owe to a number of factors, including issues discussed above, and those to be addressed below. A recent review of Tregs and prognosis is available[76] for additional reading.

9.5.2 Tregs and Treatment Response

Levels of blood Tregs (CD4$^+$CD25^{hi} T cells) in human cancer patients prior to therapy predicted overall survival after treatment with a dendritic cell vaccine in which some patients were also simultaneously treated with activated T cells[91]. Reduction of tumor-infiltrating FOXP3$^+$ cells with simultaneous increase in tumor-infiltrating CD8$^+$ cells correlated positively with pathologic complete response after neoadjuvant therapy in human breast cancer[92]. In prostate cancer patients receiving anti-cancer vaccination plus androgen deprivation, Treg did not predict clinical efficacy[93,94]. In head and neck cancer patients with squamous cell carcinomas and no evident disease after conventional treatment, increased peripherally circulating Tregs were more suppressive than in patients not getting similar treatments[95], suggesting that therapy boosted numbers and function of suppressive Tregs even while affording a net clinical benefit. If results such as these are confirmed, especially by demonstrating the identity of putative Tregs with functional tests, these data could be useful to help develop algorithms predictive of long-term treatment efficacy, help identify individuals most likely to respond, or help screen out individuals unlikely to benefit from treatments.

9.5.3 Additional Sources of Confusion in Studies of the Prognostic Significance of Tumor Tregs

Demonstrating Treg function can be difficult, particularly in human tissues as sample size is usually limiting. Consequently, prognostic studies often use FOXP3 expression as a surrogate for functional Tregs, but without doing confirmatory functional testing. Some studies use immunohistochemistry to detect Foxp3$^+$ cells but do not demonstrate that Foxp3$^+$ cells are CD3$^+$ T cells. Such approaches could lead to confusion because FOXP3 expression is not an absolute or specific marker for functional Tregs[5]. In this regard, it was recently suggested that FOXP3 expression plus cytokine profiling could help distinguish FOXP3$^+$ Tregs from FOXP3$^+$ activated effector cells in certain epithelial carcinomas[50]. Another potential source of conflicting results is that patient populations and factors known to confound survival estimates or treatment response data are not fully defined or identified in some studies.

Absolute numbers and/or functional status of Tregs are prognostic indicators as discussed, but Tregs also appear to have prognostic importance based on their specific anatomic location, or distribution within this anatomic location. For example, in gastric carcinoma, survival was affected by Treg distribution in the tumor but not on total FOXP3$^+$ tumor Treg numbers[96]. The ratio of Tregs to various immune cells, including anti-tumor effector cells also predicts survival in some studies. For example, a low ratio of CD8$^+$ T cells to Tregs predicted a poor prognosis in patients with cervical cancer[97]. The ratio of FOXP3$^+$ cells to granzyme B$^+$ cells predicted survival in Hodgkin lymphoma patients[98]. The timing of these changes can also be important. For example, the simultaneous reduction of FOXP3$^+$ cells with increased infiltration into breast carcinoma tissue of CD8$^+$ cells was the best predictor of pathologic complete response following cytotoxic breast cancer chemotherapy[92]. As we understand specific aspects of tumor-associated Tregs better, it is likely that better algorithms to predict treatment responses (including to surgery, radiation and cytotoxic agents) and survival will be produced. These advances will also be further facilitated as we better understand how to identify tumor Tregs with logistically tractable tests.

9.6 Tregs and Cancer Prevention

Efforts to understand the immunopathologic role of cancer Tregs have focused on their immunopathologic influences on clinically apparent cancer. Nonetheless, because Tregs dampen anti-tumor immunity it is plausible that they could also affect the degeneration of a premalignant lesion to a frank malignancy, or could affect the immunoediting that occurs after malignant degeneration but before the

tumor is clinically apparent (the immune equilibrium phase). A significant finding in this regard is that Tregs mediate loss of concomitant immunity[99], making it biologically plausible that they could inhibit anti-tumor immunity early in preclinical cancer progression. Tregs inhibit tumor immune surveillance in skin and connective tissues in a mouse model for carcinogen-induced sarcoma[100]. It has been proposed that a mechanism for ultraviolet radiation-induced skin cancer could include the induction of dermal Tregs[101] and that Tregs could contribute to malignant progression in cervical cancer[19]. As many cancers, including cervical carcinomas are virus-associated, Tregs could contribute to malignant progression by suppressing immunity to virus-associated antigens in addition to any effects on nonviral antigen immunity[102], also supported by the finding of human papilloma virus antigen-specific Tregs in human cervical cancer[103], a cancer in which human papilloma virus is a key etiologic agent. In a chronic inflammation model for colorectal cancer in mice, Tregs decreased colorectal cancer development by blunting microbe-driven local inflammation[55]. Managing Tregs in cancer prevention remains little explored yet merits additional attention. Significant issues with clinical application of this concept include the many obvious difficulties of their pharmacologic manipulations (and possible side effects) when there is no clinical malignancy.

9.7 Tumor Treg Effects of Anti-Cancer Therapies

Increased attention to the immunopathologic effects of cancer Treg effects has demonstrated some surprising findings regarding Treg effects on treatment outcomes for some cancers. At present, detailed mechanisms of how Tregs affect clinical outcomes in cancer patients remain unknown, with studies generally describing correlations. Future work is likely to shed considerable light on mechanistic details.

9.7.1 Vaccine Effects on Tregs

While it has long been known that active vaccinations generate vaccine antigen-specific effector T and B cells, recent work now establishes that such vaccines can also generate antigen-specific Tregs in mouse cancer models[104]. In humans, a vaccine for cervical cancer-induced $CD4^+CD25^+FOXP3^+$ cells[105], but Treg functional capacity and vaccine antigen specificity, remain to be demonstrated. Conversely, in a human anti-tumor vaccination trial to treat B cell chronic lymphocytic leukemia, vaccination reduced $CD4^+CD25^+FOXP3^+$ T cells suggesting Treg reduction[106], although Treg function of these cells was not tested. A MAGE-A3 peptide vaccine-induced MAGE-A3-specific $CD4^+CD25^+FOXP3^+$ T cells with regulatory properties

detected in peripheral blood of melanoma patients[107] and a dendritic cell vaccine expanded functionally suppressive blood $CD4^{+}CD25^{+}FOXP3^{+}$ Tregs in multiple myeloma patients[108]. Thus, active vaccination in cancer patients clearly appears to have potential to induce vaccine antigen-specific Tregs, along with any beneficial cells that might concurrently be generated.

In recognition of the issue of vaccine-induced Tregs, development of vaccines that foster generation of antigen-specific anti-tumor effector cells over generation of antigen-specific Tregs has been proposed[109]. Approaches include combining a CD40 agonist with Toll-like receptor activation[110] and a DOTAP (N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium methyl-sulfate) vaccine against human papilloma virus E7 antigen that generates $CD8^{+}$ T cells with concurrent $Foxp3^{+}$ T cell reductions in a mouse cancer vaccine model[111]. Challenge of mice with tumors engineered to express ectopic CD137 single chain antibody generates better anti-tumor immunity and simultaneously generates lower numbers of Tregs than challenge with wild-type tumor cells[112]. Dendritic cells from multiple myeloma patients inhibited T cell activation, and tumor cell lysates from multiple myeloma or anti-myeloma idiotype antibodies-induced $CD4^{+}CD25^{+}FOXP3^{+}$ cells *in vitro*[113]. In this study, forced calnexin expression in these dendritic cells with a lentivirus vector boosted tumor antigen-specific effector T cell generation without increasing the generation of $FOXP3^{+}$ T cells.

9.7.2 Cytokine Treatment Effects on Tregs

IL-2 is FDA-approved to treat renal cell carcinoma and malignant melanoma. Its clinical development was based on its activating and proliferation-inducing effects on T cells generally, and anti-tumor effector T cells specifically. However, more recent work demonstrates that a key physiologic function of IL-2 in vivo is maintenance of peripheral tolerance through critical growth and differentiation effects on Tregs[114,115] (see also Chap. 7). In light of this new understanding, therapeutic consequences of IL-2 in relationship to its effects on Tregs were undertaken. IL-2 given systemically in patients with ovarian cancer altered molecules involved in Treg trafficking and boosted numbers of Tregs[70]. Administration of IL-2 (combined with gp100 peptide vaccination) in three phase II melanoma clinical trials had variable effects on circulating $CD4^{+}CD25^{+}FOXP3^{+}$ T cells (suggesting Tregs) in circulation, with increases and decreases that bore no clear relationship to clinical outcomes[116]. Other cytokines can also alter numbers of Tregs, and their differentiation, function or migration. As an example, gene therapy with IL-12 reduced Treg generation in a mouse model for hepatocellular carcinoma[117].

9.7.3 *Additional Agents That Alter Tregs*

9.7.3.1 Drugs That Reduce Treg Numbers or Function

Relatively low doses of the anti-cancer cytotoxic alkylating agent cyclophosphamide (Cytoxan) can reduce Treg numbers in mice and humans[118]. Addition of cyclophosphamide can boost the efficacy of dendritic cell vaccines efficacy in mouse models for melanoma or colon carcinoma, association with reduced phenotypic Treg numbers that is thought to a mechanism[119]. At the high doses used in standard cancer treatment protocols, it is unlikely that Treg depletion alone is a significant immune mechanism for the anti-cancer effects of cyclophosphamide. The aromatase inhibitor letrozole can reduce Tregs in breast cancer[120]. Low dose metronomic temozolomide is reported to reduce phenotypic Tregs in a rat model for glioma, but function of the phenotypic Tregs was not tested[121]. The kinase inhibitor imatinib mesylate (Gleevec) enhances vaccine-induced anti-tumor immunity in mice, thought at least in part by reducing Treg numbers and function[122]. Imatinib mesylate actions on Tregs appear to be partly through reducing T cell receptor signaling, including reduced expression of the ZAP70 component of the T cell receptor signaling complex[122]. Cyclooxygenase-2 inhibitors have been proposed to reduce colorectal cancer risk in part by reducing Treg function, based on suggestive but not definitive evidence[123], including the finding that cyclooxygenase-2 inhibitors can reduce $FOXP3^+$ cell content in human colorectal cancers[124].

9.7.3.2 Drugs That can Increase Treg Numbers or Function

Histone deacetylase inhibitors are another class of drug gaining much research attention for direct effects on tumor cell proliferation, but they can also increase Treg suppressive functions and numbers[125,126]. IL-2 can further boost this effect of histone deacetylase inhibitors on Tregs[127]. Retinoids, including all-trans retinoic acid used in specific acute leukemias can promote generation of Tregs that preferentially home to gut[128]. The mTOR inhibitor rapamycin is in cancer clinical trials as an anti-proliferative agent. It is widely considered to be immunosuppressive and thought to increase Treg numbers based on the finding that mTOR suppression is required for optimal Treg function[129]. In our studies of normal mice given chronic oral rapamycin based on its longevity extension effects[130], we found no increase in Treg numbers or function and no evidence for immune suppression when given for up to 19 consecutive months (manuscript submitted). The thalidomide congeners lenalidomide and pamolidomide reduce Treg numbers and function[131] possibly by reducing Foxp3 expression in $CD4^+$ T cells rather than by altering their production of IL-10 or TGF-β. Trastuzumab (Herceptin), an anti-Her2/neu antibody, effected a decrease in peripheral blood Treg numbers while simultaneously increasing IL-17-producing Th17 T cells in patients being treated for breast cancer, suggesting that the Th17/Treg Th differentiation pathway had been skewed[132]. Additional effects of other drugs for other indications have been identified[133,134].

Given the significant Treg effects of these agents, and additional immune effects of these and other agents that are being discovered, it is worthwhile to reassess the mechanisms of action of certain anti-cancer drugs, including active vaccines, passive adoptive cell transfers, tyrosine kinase and/or growth factor signaling inhibitors, anti-angiogenesis agents, and a variety of others. Such studies might suggest new insights into mechanisms of action or help identify subsets of patients that could benefit from, or be harmed by, certain treatment strategies. The dual effect of IL-2 on promoting proliferation of anti-cancer effector T cells and inducing dysfunctional Tregs in cancer is an excellent example discussed above. The National Cancer Institute's Provocative Questions Program in 2011 addressed the issue of novel uses for well-known agents in its provocative question 5 (http://provocativequestions.nci.nih.gov/rfa).

9.8 Strategies to Manage Tregs

Cancer-associated Tregs appear to reduce the efficacy of endogenous and therapeutically induced anti-cancer immunity in most cancers so studied, reviewed in 31. The concept that reducing cancer Treg function will be therapeutically beneficial is thus a logical supposition[133]. In support of such thinking, experimental Treg depletion improves *de novo* anti-tumor immunity[14], and increases tumor-specific immunity[135] including shared tumor antigens[136]. Experimental Treg depletion also improves the immune and clinical activity of other therapeutic approaches including active vaccination[137,138]. Our increasing understandings of mechanisms governing tumor Treg function and local accumulation[31,139], suggest distinct categories of strategies to approach reducing tumor Treg function: depletion; blocking differentiation, trafficking or effector functions; raising effector cell suppression threshold, or diversion into alternate Th differentiation pathways[133,134]. Outright depletion is the best studied strategy thus far in preclinical cancer models and in human trials[133,140–144]. However, the clinical and immunologic effects of Treg depletion alone are usually limited by Treg regeneration that in some cases yields Treg numbers that exceed pre-depletion levels[145,146]. Thus, *managing* Tregs in conceptually a better approach to frame strategic thinking. The following sections outline Treg management strategies that have been or could be tested.

9.8.1 Nonspecific Treg Depletion

A basic feature of most Tregs studied thus far is the uniformly high expression of IL-2 receptor alpha chain (CD25). Targeting anti-CD25 through antibodies (usually clone PC61) is the most commonly employed approach in preclinical mouse cancer models[1]. We and others have demonstrated that denileukin diftitox (ONTAK), a recombinant fusion protein of the majority of human IL-2 plus the toxin moiety of

diphtheria toxin[147] that targets cells expressing IL-2 receptor[148], has been used to deplete Tregs in renal cell carcinoma[149], melanoma[141,142,150], and ovarian cancer[148]. We have also depleted Tregs with denileukin diftitox in patients with breast, pancreatic, bladder, and lung cancer in addition to melanoma (manuscript submitted). One group failed to demonstrate that denileukin diftitox could deplete Tregs in melanoma[143]. The basis for this discrepant result is unknown at present, but does not appear due to differences in doses or schedule, or prior treatments with IL-2. We recently reported that aged mice have increased numbers of $CD25^{lo}$ but $Foxp3^{+}$ functional Tregs[151]. Denileukin diftitox depleted these Tregs, but consequences for tumor immunotherapy remain to be fully established.

The alkylating agent cyclophosphamide[118] (Cytoxan), improves the clinical efficacy of dendritic cell vaccines in preclinical mouse models for colon carcinoma and melanoma in association with reducing Treg numbers[119]. $ICOS^{+}$ and $TNFR2^{+}$ Tregs are reportedly more suppressive than the total $CD4^{+}CD25^{hi}$ T cell population that includes Tregs; cyclophosphamide appears selectively to deplete numbers of these highly suppressive Tregs[152]. Metronomic low dose schedules of cyclophosphamide can deplete functional Tregs in peripheral blood of cancer patients[118]. Fludarabine can deplete Tregs, although it is toxic to most T cells[153]. Paclitaxel-based chemotherapy in non-small cell lung cancer patients reduced Treg numbers in peripheral blood, which in vitro tests suggested was specifically due to the paclitaxel[154].

Immunity generated against Foxp3-expressing cells (including Tregs) increased tumor immunity in a mouse model for renal cell carcinoma[155]. However, mechanisms of action of this approach could include attack against Foxp3 expression reported in some non-hematologic cancers[156].

LMB-2 is a *Pseudomonas* immunotoxin conjugated to the Fv moiety of an anti-CD25 antibody targeting the toxin to $CD25^{+}$ cells and depletes Tregs in human cancer patients[157]. Although depleting $CD25^{+}$ T cells from hematopoietic stem cell transplant in a mouse cancer model boosted anti-tumor immunity[158], depleting $CD25^{+}$ cells (including, but not exclusively Tregs) in an autologous cell adoptive cell transfer protocol in combination with high-dose IL-2 in vivo did not affect prolonged Treg reduction in a clinical trial[159].

The anti-CD25 monoclonal antibody daclizumab was recently demonstrated to deplete human Tregs in breast cancer patients, and reprogram their Tregs as evidenced by increased IFN-γ production[140].

CpG treatment in melanoma patients can reduce lymph node $FOXP3^{+}$ T cell numbers[160], but functional status of $FOXP3^{+}$ cells was not reported in this study. The small-molecule STAT3 inhibitor JSI-124 augmented activated effector T cell infiltration into tumor and reduced cells with a Treg function in a mouse melanoma model when combined with CpG treatment[161]. Adding cyclophosphamide to OX40 ligation enhances anti-tumor immunity and promotes tumor rejection in a mouse melanoma model, thought in part due to increasing local Treg apoptosis[73]. Many more examples similar to these have also been described. $Foxp3^{DTR}$ mice are engineered for diphtheria toxin expression under control of the Foxp3 promoter[162]. Using these mice, it is definitively established that depleting just Tregs alone can

significantly enhance anti-tumor immunity and clinical tumor rejection[163]. Due to the technical challenges inherent in human Treg work, the clinical and immunologic effects exclusively attributable to depleting Tregs has not been definitively demonstrated in any human cancer. Chesney, et al., showed that denileukin diftitox improved clinical and immune outcomes in advanced-stage human melanoma, including with cerebral metastases, but attributed effects to transient T cell depletion of the immunotoxin[141]. A follow-up study by the same group confirmed the efficacy of the immunotoxin and its ability to deplete human Tregs in metastatic melanoma[142].

9.8.2 Antigen-Specific Treg Targeting

Tumor antigen-specific Tregs have now been described as occurring *de novo*, with no prior vaccinations or other interventions[53,164], although they are also described as increasing in response to active cancer vaccination[104]. Significant pathologic autoimmunity from Treg depletion in human cancer patients has yet to be reported, contrasting with many reports of significant autoimmune complications of anti-CTLA-4 antibody treatment[165] (and see Chap. 8). Lack of significant autoimmunity with current attempts to deplete Tregs no doubt is partly due to the relatively inefficiency of the approaches evaluated, as total Treg depletion can induce significant autoimmunity even in naive mice[162]. Nonetheless, if tumor antigen-specific Tregs could be specifically targeted for destruction, it could be possible to improve anti-tumor immunity with minimal induction of unwanted pathologic autoimmunity from Treg depletion. No technique that manages human antigen-specific Treg function uniquely has yet been described. In some cancers, folate receptor 4-expressing tumor Tregs includes a population of tumor antigen-specific Tregs the reduction of which augments clinically significant anti-tumor immunity in a mouse cancer model[166].

9.8.3 Raising the Effector Cell Suppression Threshold

The anti-CTLA-4 antibody ipilimumab was FDA-approved to treat metastatic melanoma in March 2011. It can increase the proliferation of Tregs in human subjects despite its clinically proven efficacy[167]. It was initially thought perhaps to work by reducing Treg numbers, but that is probably not a significant mechanism, and if anything Treg numbers can increase after anti-CTLA-4[168–170]. Mechanisms for its clinical effects include reducing Treg function and increasing the threshold for Treg-mediated suppression of effector cells by action on the latter. The relative contributions of each mechanism of action is incompletely understood and might

depend on tumor type and anatomic compartment, although increasing the Treg suppression threshold appears generally to be more important. Anti-CTLA-4 antibody treatment reduced $CD3^+CD4^+FOXP3^+$ T cells in blood that phenotypically appeared to be Tregs in a human trial. Nonetheless, $CD8^+$ cytotoxic T cell numbers in blood did not change significantly. Further, $CD3^+CD4^+FOXP3^+$ T cells quickly returned to baseline numbers, and their function as Tregs was not confirmed[171]. Please see Chap. 8 for many additional details on anti-CTLA-4 effects.

In an alternative approach to raising effector T cell suppression threshold, A20 (a zinc finger protein) was genetically silenced in dendritic cells. In a mouse cancer model, tumor-infiltrating T cells previously activated with A20-silenced dendritic cells resisted Treg-mediated suppression[172]. The cytokine IL-7 raises effector T cell threshold for Treg-mediated suppression in mouse models of autoimmunity[173], but IL-7 effects in this regard in tumors have not been reported. Notch signaling in effector T cells appears important in modulating Treg-mediated suppression when the suppression involves membrane-bound TGF-β[174], but Notch effects on resistance to human Tregs have not yet been reported.

9.8.4 Altering Treg Trafficking

Specifically impeding the trafficking of Treg ingress into areas where anti-tumor immunity is primed[8] or executing its effector functions is likely to be a beneficial treatment strategy. Improving effector T cell trafficking over Treg trafficking into tumor could be a useful treatment strategy, as was demonstrated using cyclophosphamide plus anti-OX40 antibody in a mouse model for melanoma[73]. We showed that anti-CL22 antibody prevented Tregs from infiltrating into human ovarian cancer cells xenografted into immunodeficient mice, promoting immune-mediated rejection by adoptively transferred autologous $CD8^+$ T cells (T. Curiel, W. Zou, et al., unpublished data). CCL22 also attracts Tregs in human breast cancer[175]. However, CCL22 may also facilitate trafficking of effector T cells. Therefore, any potential benefit of CCL22 blockade or of interrupting other trafficking signals requires further study as to potential therapeutic utility. Selective in silico studies identified small-molecule chemokine receptor antagonists or monoclonal antibodies able to block in vitro CCL22-mediated recruitment of human Treg and Th2 cells[176] and have gone into phase I clinical trials[177]. Therapeutic utility may be limited by the binding promiscuity of chemokine receptors, the redundancy of chemokine/ligand pairs, or the role of chemokines in normal tissue homeostasis or anti-tumor immunity. These additional effects of chemokine/receptor antagonism must be taken into account in strategies to block chemokines and their receptors.

Therapeutic IL-2 administration can alter Treg trafficking, and increase numbers of circulating Tregs[70,178] even if their functionality could be reduced[178].

9.8.5 Inhibiting Treg Suppressive Functions

Tumor Tregs use a variety of mechanisms to exert suppression of anti-tumor immunity[31]. Mitigating or preventing these mechanisms could be therapeutically beneficial[133,134]. Inoculation of *Escherichia coli* engineered to express the *Listeriolysin-O* gene promoted generation of specific cytotoxic T lymphocytes, but also simultaneously made local Tregs nonfunctional[179]. Inhibiting STAT3 reduces Treg suppressive function[180], and specific ablation of STAT3 signaling reduces the capacity of Tregs to restrain Th17-polarized T cells[56], which was also recently suggested in endogenous Tregs in aged mice[181]. As detailed in Chap. 2, IL-17-producing cells and Th17-polarized immunity have incompletely understood roles in anti-tumor immunity[80,182]. Thus, how these findings will translate into specific anti-cancer therapies remains unclear. Agonizing OX40 expressed on tumor-infiltrating Tregs with a specific antibody blunts their capacity to inhibit anti-tumor effector T cell activity and thus promotes improved immune-mediated tumor rejection[146]. Agonizing GITR signals in vitro with monoclonal antibodies reduces the suppressive capacity of Tregs in mice[183,184], but whether the approach will work with human Tregs remains unclear[185]. Toll-like receptor signaling in virus-based anti-tumor vaccines can reduce Treg-mediated immunosuppression. Because dendritic cell-based vaccines do not induce Toll-like receptor signaling in the absence of some additional manipulations, they could be maximally effective in promoting the efficacy of anti-tumor $CD8^+$ T cells either by reducing Treg function or by coadministration of a Toll-like receptor agonist[186]. There is much interest in reducing Treg function using Toll-like receptor ligation (e.g., TLR9 ligation with CpG oligonucleotides) as an effective way to improve the efficacy of some cancer vaccines. Sendai virus reduced Treg function in a mouse model for colorectal cancer in which virus-induced IL-6 was suggested as a mechanism[187]. Additional considerations to block Treg function include interrupting Foxp3 interactions with NFAT, which is required for Treg suppressive function[188], and blocking Treg effector molecules such as IL-10, TGF-β, or IL-35 among other strategies. Most of these strategies have not yet been assessed in human trials.

9.8.6 Blocking Treg Differentiation

The nuclear transcription factor Foxp3 controls Treg differentiation through mechanisms that are increasingly understood[30,189,190] that could be used in therapeutic applications. For example, the CNS1 region of Foxp3 controls induced Treg generation[29,30] that could be silenced to reduce tumor-driven Treg generation. Tumor environmental products such as vascular endothelial growth factor retard maturation of local dendritic cells[191]. These tumor-associated immature dendritic cells can contribute to defective T cell activation, and to generation of Tregs. Thus, preventing dysfunctional dendritic cell activation of T cells could help reduce Treg

generation in tumors. We showed that interferon-α improves dendritic cell maturation in a mouse model for ovarian cancer associated with reduced Treg generation and function and that adding it to denileukin diftitox improved clinical responses in ovarian cancer in mice and in human patients (manuscript submitted).

9.8.7 Subverting Treg Differentiation

Tumor-associated Tregs include those that are tumor antigen-specific[53,164]. If these antigen-specific T cells could be reprogrammed into a clinically useful pathway, for example, a Th1-polarized or polarizing pathway, these counterproductive tumor-specific Tregs could be induced to become tumor-specific effector T cells. Using the common aryl hydrocarbon receptor to redirect T cells into a Th17 differentiation pathway over Treg differentiation[192] is an example of a means to subverting Treg differentiation, assuming that the resulting Th17 cells are not detrimental and that a safe common aryl hydrocarbon receptor targeting molecule is identified. Th17 immunity is beneficial in some tumors[182,193] (and see Chap. 2 for details). For example inhibiting indoleamine 2,3-dioxygenase in a B16 mouse melanoma model skews Tregs towards the Th17 pathway that could promote anti-tumor immunity[194]. In this same melanoma model, treating dendritic cells ex vivo with an anti-B7-DC antibody facilitated the conversion of antigen-specific Tregs into tumor antigen-specific Th17 effector cells that mediated anti-tumor immunity[195]. A recent report demonstrated that the anti-CD25 monoclonal antibody daclizumab depleted Tregs and also reprogrammed them towards a Th1 phenotype as evidenced by IFN-γ production in a clinical trial of an hTERT vaccine in metastatic breast cancer[140].

9.8.8 Combining Treg Management with Other Treatment Modalities

The timing of treatment modalities for anti-cancer immunotherapy can have significant influences on immunologic and clinical efficacy, including the timing of Treg depletion in relationship to other treatments[196]. Our understanding of how best to combine various Treg management strategies with other treatments remains limited. Transient lymphodepletion to foster homeostatic effector T cell expansion combined with Treg depletion is a testable concept, and could help explain some of the treatment effects of denileukin diftitox[141]. Another approach worth additional exploration is combining radiation, or selected doses of certain cytotoxic[197] or hormonal agents[94] that can increase tumor immunogenicity (such as by generating release of, or improving the immunogenicity of, tumor antigens as an endogenous vaccination) with Treg management. Further, the timing of chemotherapy can slow Treg re-accumulation after other approaches[198].

9.8.9 Additional Treg Management Considerations

Even when Treg management itself is highly effective in reducing functional Tregs, clinical efficacy can nonetheless be hampered by poor intrinsic effector cell function, low effector cell trafficking into the correct compartment or immunoediting[199,200], among many other factors. For example, the efficacy of depleting Tregs in a mouse melanoma model was hampered due to relatively poor effector cell trafficking to appropriate sites. Combining Treg depletion with endothelial damage from external beam irradiation significantly improved appropriate effector cell trafficking and immune and clinical efficacy of Treg depletion[167].

Relatively little has been studied regarding age or gender effects on tumor immunity generally, the effects of Tregs specifically and responses to tumor immunotherapy. Lack of age-specific studies is particularly striking as age is the biggest risk factor for cancer[201]. The effects of aging on Treg function in naïve mice and humans have led to contradictory results[181]. In our BL6 mice, there is little age-associated decline in Treg function in naïve and tumor-bearing hosts[163,181]. Nonetheless, depleting Tregs is ineffective in improving anti-tumor immunity and clinical effects in B16 melanoma in aged mice, whereas it is highly effective in young mice. Lack of efficacy was found due to a compensating increase in myeloid-derived suppressor cells in aged, but not young B16-bearing hosts following Treg depletion. Combining Treg plus myeloid-derived suppressor cell depletion was thus effective in aged mice, whereas adding myeloid cell depletion to young B16-bearing mice provided no additional benefit. By contrast, Treg depletion was effective in aged hosts in a model of MC-38 colorectal cancer because myeloid-derived suppressor cells did not increase after denileukin diftitox-mediated Treg depletion[163].

Regarding gender differences, we showed that estrogen and B7-H1 immune co-improve anti-tumor immunity and clinical responses in females better than males in a mouse B16 melanoma model. Differences owed in part to greater Treg functional reduction with B7-H1 blockade or deficiency in females, whereas Treg depletion was equally efficacious in either sex[48]. Please see Chap. 13 for many additional miscellaneous strategies and details.

9.9 Other Regulatory Cells

In some mouse models for cancer, Tregs might not be the significant mediators of immune dysfunction, and in such cancers, managing Tregs might not be the optimal therapeutic approach. As one example, immune suppression is reportedly mediated principally by $CD4^+$ NKT cells, not $CD4^+$ Tregs in a mouse model for lung metastasis due to CT26 colon cancer[202]. Nonetheless the role of Tregs in lung metastasis, and their role for affecting tumor growth in other anatomic compartments was not specifically reported. In the TRAMP mouse model for prostate cancer, Treg depletion with the anti-CD25 antibody PC61 did not improve

tumor-specific tolerance or increase tumor rejection[203]. In humans, Tregs do not always correlate negatively with clinical responses to treatment or to survival as discussed above in the Section 5. Thus, it is likely that other regulatory cells are also immunopathologically relevant to clinical cancer outcomes.

In this regard, $CD8^+$ T cells, certain myeloid cells and NKT cells have also been reported as mediating dysfunctional immune suppression in cancer[204]. $CD8^+FOXP3^+$ T cells in prostate cancer can suppress in a contact-dependent manner, which effects are blunted by TLR8 agonists[205]. $CD8^+CD25^+Foxp3^+$ suppressive T cells have been reported in peripheral blood and the solid tumor mass in patients with colorectal cancer. These $CD8^+$ Tregs expressed high CTLA-4 and GITR and suppressed the proliferation of, and cytokine secretion from $CD4^+CD25^-$ T cells in vitro, similar to conventional $CD4^+CD25^{hi}Foxp3^+$ Tregs[206]. In a mouse leukemia model of allograft rejection, anti-CD3 antibody treatment induced $CD8^+FOXP3^+$ suppressor T cells mitigating graft versus host, but not graft versus leukemia responses[207]. In a mouse colon cancer model a specific subpopulation of NKT cells and IL-13 were implicated in immune suppression[208]. This immune suppression was specifically suggested not to be Treg-dependent[202]. How these various suppressive and immune dysfunctional mediators contribute to tumor immunopathology, and how their management will contribute to novel anti-cancer immunotherapy strategies remain fully to be defined, and are interesting and important areas for additional investigation. For example, we recently identified how Treg depletion affects myeloid-derived suppressor cells, and how their combined management was superior to management of either alone in aged mice with B16 melanoma[163].

9.10 Summary and Challenges

Recent detailed studies make plain that not all types of malignancies, or at all pathological stages, and not all hosts will benefit from specific Treg management strategies proposed here. For example, Tregs could contribute to immunopathology in specific lymphomas. Nonetheless, steroids that are used in some treatment strategies could blunt clinically meaningful activation of effector T cells. Alternatively, if Tregs are beneficial in some lymphomas as suggested, reducing their function could be detrimental. Understanding which agents are most useful in Treg management, and in what combination and in which order are important details generally not known at present. Treg-specific agents are not currently available for use in humans, but with our rapid understanding of Treg biology, development of a specific management agent in the near term is a realistic expectation.

Practically applicable, accurate immune assays that predict Treg function and clinical outcomes following Treg management interventions must be developed and validated to assess clinical and immune consequences of proposed interventions. As an example, to understand the effects of Treg-mediated T cell suppression, methods to isolate large numbers of viable immune cells at high purity must be developed that

do not compromise patient safety. Clinical trial design must address appropriate proof-of-concept issues that account for the unique, specific challenges inherent in understanding effects of these novel approaches. For example, assays to link Treg management strategies with alterations in tumor-specific immunity will be important to develop, to show conclusively that Treg function (however defined) is accomplished with a specific intervention, and that this reduction is a mechanism for any improved clinical and immune outcomes observed. Developing tests of local tumor microenvironmental changes is challenging in human subjects where patient safety and limiting tissue amounts are major considerations. Thus, developing a blood test or relatively non-invasive test that will convey the relevant data needed to come to meaningful conclusions regarding mechanisms of Treg management strategies would be a significant advance.

Despite our current state of relatively incomplete understandings, there is reason for much optimism given the rapid and useful advances made in the past 5 years alone. Developing clinically useful and logistically tractable Treg management strategies to treat cancer is a reasonable and realistic goal for the near term.

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Chapter 10
Myeloid-Derived Suppressor Cells in Cancer: Mechanisms and Therapeutic Perspectives

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Abstract Malignant cells create a chronic inflammatory microenvironment that facilitates their proliferation, promotes migration and invasion, and blunts any antitumor response by the innate and adaptive immune systems. This state of immunologic tolerance has been well characterized and is in part responsible for impairing the potential therapeutic benefits of immunotherapy approaches such as cancer vaccines and the adoptive transfer of T cells. One major mechanism by which tumor cells induce a chronic inflammatory microenvironment is through the recruitment of myeloid-derived suppressor cells (MDSC). MDSC are potent inhibitors of the immune response through the expression of arginase I which depletes L-arginine from the tumor microenvironment or by the production of various intermediates such as reactive nitrogen species and reactive oxygen species that can suppress T cell function. Here, we review recent concepts on how MDSC can regulate T cell function in cancer and other chronic inflammatory diseases and suggest possible therapeutic interventions to overcome this inhibitory effect.

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10.1 Introduction

Malignant transformation can lead to the expression of viral proteins, mutated oncoproteins, and the overexpression of normal proteins in the cancer cell. This finding supports the possibility that a strong innate immune response or the induction of potent antigen-specific immune responses can prevent the development of cancer or control tumor growth once it is established. In fact, viral vaccines against the human papilloma virus are highly effective at preventing the development of cervical cancer. However, three decades of clinical trials in cancer immunotherapy have also made evident that once established, tumors have sophisticated mechanisms to impair and evade antitumor-specific immune responses. Among the most prominent mechanisms by which tumors impair antitumor immunity is by hijacking the initial immune response converting it instead into a chronic inflammatory process that inhibits tumor-specific T cells. A major cellular component of this chronic inflammatory process is the accumulation of myeloid-derived suppressor cells (MDSC). MDSC have been shown to modulate different T cell functions by depleting the amino acid L-arginine, by nitrosylating the T cell receptor, by impairing recognition of antigens and cytotoxic functions, or by inducing T cell apoptosis through the release of reactive oxygen species. MDSC not only inhibit the antitumor immune response, but also appear to protect tumor cells from the effects of certain chemotherapeutic agents. Therefore, understanding the molecular and cellular mechanisms used by MDSC to suppress antitumor responses has been a matter of extensive research and these have become important to develop therapeutic targets. Here we will review some of the most recent concepts on how MDSC could regulate T cell function in disease and suggest possible therapeutic applications to inhibit their suppressive activity.

10.2 Alterations of the Immune Response in Cancer

A dysfunctional immune response in cancer patients manifested by the loss of delayed type hypersensitivity to bacterial or chemical agents was demonstrated several decades ago[1–4]. Initial explanations included the development of blocking antibodies, the production of suppressor factors, and the generation of suppressor macrophages[5–7]. However, their significance for the progression and outcome of the disease was unknown. Although cancer patients generally do not develop the characteristic opportunistic infections seen in patients receiving high doses of corticosteroids or chemotherapy, they nonetheless show impaired T cell responses against bacterial and/or chemical antigens[1–4] and tumor-associated antigens. In fact, immunotherapy models in mice and immunotherapy clinical trials have demonstrated a decreased therapeutic response in advanced tumors as a result of the loss of T cell responses (reviewed in [8]). In addition, several vaccine trials have demonstrated the progression of tumors in spite of a robust T cell response[9]. Most studies agree that tumor cells trigger the multiple mechanisms that suppress the

antitumor immune responses, including the recruitment of immune cells that act as mediators of this effect. Cellular and molecular models of T cell tolerance have identified various mechanisms by which tumors inhibit T cell function including the upregulation of immunological checkpoints on antigen-presenting cells and/or tumor cells,[10–13] the induction of regulatory T cells[14, 15], and the generation of MDSC[16–19].

MDSC range in morphology from immature myeloid cells to mature macrophages and granulocytes. Their production in the bone marrow is induced by factors such as granulocyte-macrophage colony-stimulating factor (GM-CSF) produced by tumor cells. Young and colleagues first demonstrated that bone marrow-derived suppressor macrophages blocked T cell responses by producing IL-10, TGF-β, and prostaglandin E_2 (PGE_2)[20]. In the early 1990s, Gabrilovich et al.[21] demonstrated that vascular endothelial growth factor produced by the tumor cells arrested the differentiation of dendritic cells, resulting in immature dendritic cells that caused T cell dysfunction. These cells were increased in patients with breast, head and neck, and lung cancer[22, 23]. Since then multiple groups have described the presence of myeloid cells that were able to suppress T cell responses. These cells range from immature myeloid cells to mature macrophages or granulocytes. In 2007 a group of leading investigators proposed the name myeloid-derived suppressor cells (MDSC) to identify this heterogeneous population of myeloid cells[24]. The mechanisms used by these cells to suppress T cell function include the production of reactive oxygen and nitrogen species, and arginase I, an enzyme that depletes the amino acid L-arginine[25]. In addition, although technically not considered to be part of the MDSC mechanisms, immature dendritic cells can also express the enzyme indoleamine 2,3-dioxygenase, which depletes tryptophan causing T cell dysfunction[26, 27].

10.3 Mechanisms of T Cell Anergy in Cancer

Although T cell dysfunctions in patients with cancer had been described since the 1960s, it was not until the early 1990s that investigators described the molecular basis for these functional changes. Several investigators described multiple changes in the expression of signal transduction proteins in the T and NK cells from cancer patients and tumor-bearing mice, including a decreased expression of the T cell receptor ζ chain (CD3ζ), a diminished expression of tyrosine kinases $p56^{lck}$ and $p59^{fyn}$, and an inability to upregulate Jak-3 kinase and to translocate NF-kBp65 into the nucleus[28]. These changes resulted in diminished T cell responses to antigen stimulation as shown by a decreased ability to mobilize Ca^{++}, a diminished proliferation, and a decreased production of interferon (IFN)-γ[28–30]. These initial findings made in tumor-bearing mice were confirmed in patients with renal cell carcinoma, melanoma, Hodgkin disease, ovarian cancer, colon carcinoma, and cervical cancer among others[31–33]. Preliminary studies also showed that patients with melanoma or head and neck tumors had a decreased survival time if expressing

low levels of CD3ζ[33, 34]. However, the mechanism(s) to explain these changes was unknown.

10.4 Mechanisms Leading to a Decreased CD3ζ Chain in Disease

Otsuji et al.[35] and Kono et al.[32, 36] demonstrated that the co-incubation of activated murine peritoneal macrophages with T cells induced the loss of CD3ζ chain. This phenomenon could be blocked by the oxygen radical scavenger catalase and was therefore thought to be mediated by H_2O_2[37]. Similarly, Schmielau et al. described an increased number of activated neutrophils in peripheral blood of patients pancreatic and breast cancer who had a diminished expression of CD3ζ chain[38]. Another mechanism suggested that the loss of CD3ζ chain was a consequence of Fas-FasL induced T cell apoptosis[39, 40]. In addition, Baniyash and colleagues proposed that chronic stimulation of T cells by specific antigens led to the decreased expression of CD3ζ chain leading to the induction of anergy[41–43]. These changes however were not unique to cancer patients and were observed in T cells from patients with chronic or acute inflammatory events. Zea et al.[44, 45] described that patients with lepromatous leprosy or active pulmonary tuberculosis presented similar alterations in T cells. In addition, mice with severe trauma were shown to have a decreased CD3ζ and a diminished T cell function[46]. Additional studies in trauma patients also showed that they underwent a rapid depletion of L-arginine levels within a few hours of the traumatic event, which was paralleled by the loss of T cell function. Furthermore, replenishment of L-arginine reestablished normal T cell function and increased the number of $CD4^+$ T cells[47–49], suggesting that amino acid depletion might play a role in the induction of T cell anergy in these disease models.

10.5 L-Arginine and the Immune Response

The association of L-arginine metabolism and the immune system was initially reported in experiments showing that the thymic involution and decrease in T function seen in trauma models in mice was reversed by the injection of L-arginine[50], which was also essential for the process of wound healing[16, 51]. The depletion of L-arginine was also prominently shown in liver transplant and trauma patients in whom the rapid depletion of plasma levels of L-arginine was accompanied by markedly decreased T cell function[47–49].

Our initial experiments demonstrated that Jurkat T cells cultured in medium containing L-arginine levels <50 μM lost the expression of CD3ζ and had decreased proliferation[52–54]. Experiments using primary T cells showed that L-arginine depletion caused the same functional (decreased proliferation and IFN-γ production) and

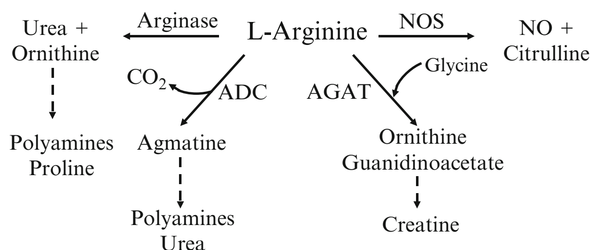

Fig. 10.1 Intracellular metabolism of L-arginine. L-arginine is internalized by MDSC through the cationic amino acid transporter CAT-2B. It is then metabolized by one of the 4 enzymatic pathways shown in this figure.

molecular (decreased CD3ζ, inability to upregulate Jak-3, and decreased translocation of NF-kBp65) alterations found in cancer patients. These changes were selective since other functions, including the production of IL-2 and the upregulation and expression of the IL-2 receptor chains (CD25, CD122, CD132), were similar to cells cultured in medium with L-arginine. These results suggested a potential role for L-arginine depletion as a mechanism for the induction of T cell dysfunction in vivo.

In healthy adults, L-arginine is a nonessential amino acid whose levels are maintained through dietary intake and the *de novo* endogenous synthesis from citrulline produced by epithelial cells of the small intestine (Reviewed in[55]). Normal levels of L-arginine in serum range between 50 and 150 μM. L-Arginine is also classified as a conditional essential amino acid in certain conditions such as trauma and cancer where changes in its systemic levels cause major alterations in the immune response[56]. L-Arginine is the substrate for seven enzymes that exist as multiple isoforms: nitric oxide synthase (NOS1, NOS2, and NOS3), arginase (arginases I and II), arginine:glycine amidinotransferase, and L-arginine decarboxylase (Fig. 10.1). Dietary L-arginine is taken up by intestinal epithelial cells and traverses the plasma membrane via the cationic amino acid transporters[57]. L-Arginine is metabolized in endothelial cells or macrophages by nitric oxide synthase to produce citrulline and nitric oxide, the latter of which plays an important role in cytotoxic mechanisms and vasodilatation[58, 59]. Alternatively, arginase I and arginase II metabolize L-arginine to L-ornithine and urea, the first being the precursor for the production of polyamines essential for cell proliferation and the second an important mechanism for detoxification of protein degradation[60]. Of the remaining two enzymes, L-arginine decarboxylase converts L-arginine to agmatine, which in turn is converted to putrescine and urea by agmatinase. L-Arginine decarboxylase and arginine:glycine amidinotransferase are not prominently expressed in immune cells. Instead L-arginine decarboxylase is highly expressed in the brain[61, 62], and arginine:glycine amidinotransferase is expressed in the brain and the heart[63, 64].

Arginase I and NOS2 play important roles in the immune response. The expression of arginase I and NOS2 in murine macrophages is differentially regulated by Th1 and Th2 cytokines[65, 66]. Stimulation of murine macrophages with IFN-γ upregulates NOS2 exclusively, while IL-4, IL-10, and IL-13 induce arginase I[67, 68]. The

mitochondrial isoform arginase II is not significantly modulated by Th1 or Th2 cytokines[69]. The inhibition of arginase I leads to an increased NOS2 expression and consequently promotes nitric oxide (NO) production[70]. Conversely, upregulation of arginase I inhibits NOS activity and contributes to the pathophysiology of several disease processes, including vascular dysfunction and asthma[71]. The mechanisms of inhibition of NOS2 expression by arginase I appear to be mediated by L-arginine depletion that blocks the induction of NOS2 expression and the subsequent NO production in macrophages[72]. In addition, low levels of NOS induce nitrosylation of cysteine residues of arginase I, which increases the biological activity of arginase I and reduces L-arginine[73].

Activation of peritoneal macrophages with Th1 or Th2 cytokines also has different effects on the extracellular levels of L-arginine. Peritoneal macrophages stimulated with IL-4 plus IL-13 increase the expression of arginase I and the cationic amino acid transporter CAT-2B, which results in a rapid increase in the uptake of extracellular L-arginine with the consequent reduction of L-arginine in the microenvironment. In contrast, macrophages stimulated with IFN-γ preferentially express NOS2, do not increase CAT-2B, and do not deplete L-arginine from the microenvironment[69]. Recent data from arginase I and arginase II knockout mice confirm that only arginase I is able to deplete serum levels of L-arginine[74, 75]. Furthermore, conditional knockdown of arginase I in myeloid cells impaired their ability to deplete extracellular L-arginine (our unpublished data). Both arginase I and nitric oxide play an important role in suppressing T cell function. In vitro coculture experiments (using transwells separating macrophages and T cells by a semipermeable membrane) showed that macrophages producing arginase I, but not macrophages expressing NOS2, were able to cause the prolonged loss of CD3ζ in T cells and inhibit T cell proliferation and IFNγ production. This effect was reversed with the addition of arginase inhibitors N-hydroxy-nor-L-arginine or N-hydroxy-L-arginine or exogenous L-arginine[69]. These results were confirmed with macrophages from arginase I conditional knockout mice (manuscript in preparation).

MDSC can also cause T cell tolerance through cell-cell contact. This mechanism appears to require the co-expression of arginase I and NOS2[16]. The addition of arginine or arginase inhibitors to cocultures of MDSC and activated T cells completely reestablishes T cell function[76]. It is possible that T cell suppression is in part mediated by the production of peroxinitrites. Under limiting amounts of L-arginine, NOS2 produces peroxinitrites ($ONOO_2$), a highly reactive oxidizing agent that nitrosylates proteins and induces T cell apoptosis[77]. This appears to affect the conformational flexibility of the T cell antigen receptor (TCR) and its interaction with major histocompatibility complex (MHC) by inducing nitration of TCR proteins in $CD8^+$ T cells. Thus, MDSC directly disrupt the binding of specific peptides on MHC by $CD8^+$ T cells[78]. In addition, TCR nitration prevents the recognition of target cells by cytotoxic T cells. MDSC co-expressing arginase I and NOS2 primarily impair $CD8^+$ T cell function[77, 79–81] by blocking their ability to secrete IFN-γ when stimulated with specific antigens[82, 83] and inducing apoptosis[77]. This suppression requires the production of IL-13 and IFN-γ[80, 84, 85] and signaling through the STAT1 transcription factor[77]. In addition, MDSC have

been shown to produce high levels of stem cell factor when stimulated by IFN-γ and IL10[86]. Blocking of stem cell factor signaling in MDSC significantly impairs their ability to generate regulatory T cells[87].

10.6 Molecular Effects of L-Arginine Starvation on T Cells

The molecular and functional changes that occur in T cells cultured in the absence of L-arginine are not associated with the induction of apoptosis and are fully reversed by the simple replenishment of L-arginine at physiologic (50–150 μM) concentrations[52–54, 69]. Rodriguez et al. showed that activated T cells cultured in the absence of L-arginine were arrested at the G_0-G_1 phase of the cell cycle, while cells cultured with L-arginine progressed into S and G_2-M phases[88]. Arginine starvation impaired the ability of T cells to upregulate cyclin D3 and cdk4, but not cdk6[88] which are essential for the progression from G_1 into the S phase of the cell cycle[89]. Additional results showed that the decreased expression of cyclin D3 and cdk4 occurred through posttranscriptional and translational mechanisms[88].

In eukaryotes, amino acid deprivation activates mechanisms that inhibit translation. The accumulation of empty aminoacyl tRNAs caused by amino acid starvation activates GCN2 (general control nonrepressed 2) kinase which phosphorylates the translation initiation factor eIF2α. The phosphorylated form of eIF2α binds with high affinity to eIF2B, blocking its ability to exchange GDP for GTP, which inhibits the binding of the eIF2 complex to methionine aminoacyl tRNA which in turn blocks the initiation of protein translation. In T cells cultured in arginine starvation conditions, we have identified some of the similar mechanisms. The absence of L-arginine triggers the activation of GCN2 kinase which in turn leads an to increase in phosphorylated eIF2α, triggering an overall arrest in protein translation (Fig. 10.2). In fact, T cells from GCN2 kinase knockout mice do not undergo cell cycle arrest or decreased proliferation when cultured in medium without L-arginine[88].

The decreased translation is at least in part responsible for the diminished stability of cyclin D3 mRNA. Cyclin D3 mRNA is characterized by a long 3′ untranslated region containing an 11-nucleotide AU-rich element which forms a complex with the DNA-binding protein HuR, resulting in stable mRNA. The global decrease in translation caused by arginine starvation decreases the synthesis of HuR, which in turn decreases the stability of cyclin D3 mRNA[90] (Fig. 10.2).

10.7 Arginase Expression in Tumors

Some tumors, including non-small lung carcinoma and breast carcinoma, have been shown to express arginase[91–93]. This was thought to be a mechanism for the production of polyamines needed to sustain the rapid proliferation of tumor cells.

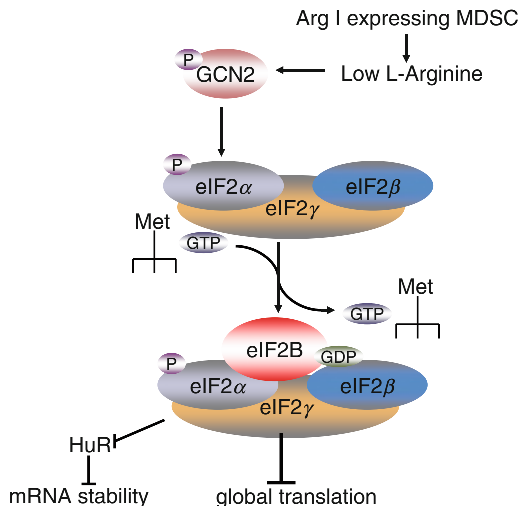

Fig. 10.2 Arginine depletion inhibits protein translation in T cells. Low levels of L-arginine activate GCN2 kinase which phosphorylates eIF2 alpha. In turn p-eIF2 alpha binds the translation initiation complex and inhibits translation of selected proteins including HuR. The absence of HuR results in a decrease of selected mRNA, compounding the detrimental effect of arginine depletion on T cells.

However, most tumors do not express arginase I, but instead attract an influx of arginase I-expressing myeloid cells that infiltrate the tumor or accumulate in the peri- and intra-tumoral area and could represent a mechanism of tumor evasion[79]. Myeloid suppressor cells express the common marker CD11b, but vary in their nuclear morphology and maturation markers (e.g., Gr1). This variability in expressed markers resulted in multiple names being used to describe these cells including tumor-associated macrophages, tumor-infiltrating macrophages, and immature myeloid cells. In an effort to standardize the name used to describe these myeloid suppressor cells, a group of leading investigators agreed in 2007 to use the common term "myeloid-derived suppressor cells" (MDSC)[24]. In mice, MDSC can be divided into two subsets, granulocytic MDSC are $CD11b^{+}LY6G^{+}LY6C^{lo}$, whereas monocytic MDSC are $CD11b^{+}LY6G^{-}$ $LY6C^{hi}$[94]. Granulocytic and monocytic MDSC are present in the bone marrow of healthy mice and accumulate in the spleen and tumors of tumor-bearing mice[80, 83, 95]. Depletion of MDSC using anti-Gr-1 antibodies induced an antitumor effect mediated by $CD8^{+}$ T cells[18, 19, 96]. Similarly, the injection of the arginase I inhibitor N-hydroxy-nor-L-arginine into tumor-bearing mice, or the use of nitro-aspirin which inhibits both arginase and NOS2, prevents the loss of T cell function and results in a dose-dependent immune-mediated antitumor response. The inhibition in tumor growth caused by N-hydroxy-nor-L-arginine does not happen in tumor-bearing *scid* mice (lacking T and B lymphocytes), strongly suggesting that the antitumor effect caused arginase inhibition is dependent on lymphocyte function[79]. These results were

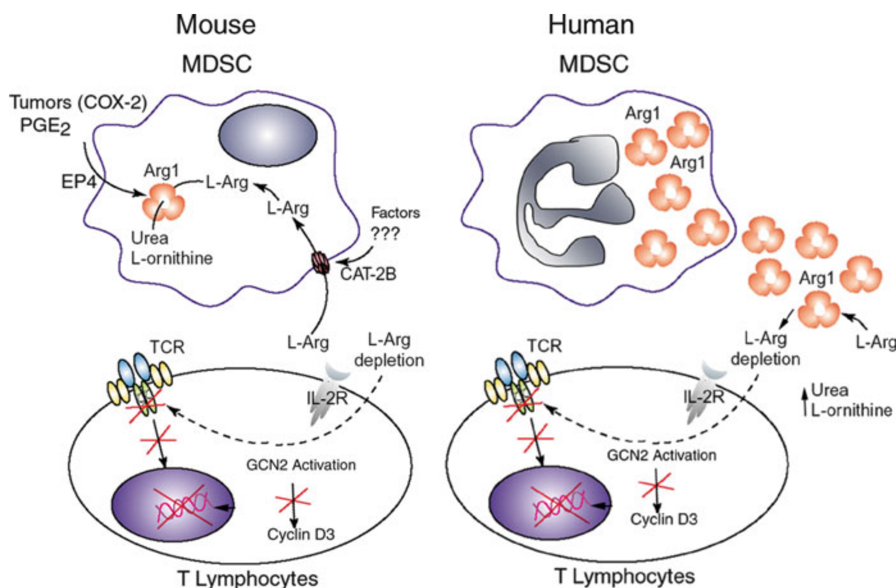

Fig. 10.3 Mechanisms of arginine depletion by murine and human MDSC. Mouse and human MDSC differ in the mechanism for depleting L-arginine. Murine MDSC actively take up L-arginine from the microenvironment, while human MDSC (which are primarily represented by granulocytic cells) degranulate, releasing large quantities of arginase I into the microenvironment. Both mechanisms deplete arginine from the tumor microenvironment.

recently confirmed by data showing a significant inhibition of tumor growth in arginase I conditional knockout mice (unpublished results).

Human MDSC phenotypes vary significantly ranging from immature myeloid cells[97, 98] to activated granulocytes[99]. They have been reported to express a wide range of markers including CD11b, CD14, CD15, CD33, vascular endothelial growth factor receptor, and CD66b (reviewed in[100]). A study of patients with metastatic renal cell carcinoma demonstrated a six- to tenfold increase in arginase activity in their peripheral blood mononuclear cells, as compared to normal controls[101]. Separation of MDSC from peripheral blood showed that the cells containing all arginase activity were activated granulocytes that separated with the peripheral blood mononuclear cells[99]. Patients with high numbers of MDSC had a significantly decreased expression of T cell CD3ζ chain. Depletion of the MDSC from patient samples in vitro reestablished T cell proliferation and IFN-γ production. A similar subpopulation of activated granulocytes had been described by Schmielau and Finn[38] in patients with pancreatic cancer, where they also demonstrated a correlation between these cells and reduced T cell CD3ζ expression and decreased cytokine production[38]. Furthermore, clinical trials with IL-2 in patients with renal cell carcinoma and melanoma have shown an association between a poor clinical response and an increased numbers of granulocytes in peripheral blood of these patients[102].

Although human MDSC also express high levels of arginase I, this does not appear to be upregulated by cytokines or other signals once these cells are in circulation. Furthermore, human MDSC do not take up L-arginine. Instead, arginase I stored in primary[103] or gelatinase granules[104] is released into the microenvironment inducing a significant decrease in local L-arginine levels, which impairs T cell function and CD3ζ chain expression[101, 105, 106] (Fig. 10.3). In fact, the release of arginase I into the blood of renal cell carcinoma patients induced a decrease in plasma of L-arginine levels to <50 μM and an increase in ornithine levels demonstrating that arginase I not only had a metabolic effect (L-arginine depletion), but also had a negative effect on the T cell response[101]. Therefore, MDSC appear to be a major contributor to the induction of T cell dysfunction or tolerance in patients with cancer.

10.8 Regulation and Activation of MDSC in Cancer

MDSC are recruited from the bone marrow by vascular endothelial growth factor and GM-CSF produced by tumors[107]. In fact, serum levels of vascular endothelial growth factor directly correlated with numbers of MDSC in the blood and spleen[108] and have been associated with poor prognosis in cancer patients. Tumor-derived vascular endothelial growth factor has been previously associated with an arrest in dendritic cell maturation[109, 110] through the inhibition in NF-κB signaling. Treatment of MDSC with all-*trans* retinoic acid appears to counter this inhibition and promotes MDSC differentiation into mature antigen-presenting cells[111]. Increased levels of GM-CSF have also been associated with MDSC-dependent immune suppression that was reversed by the use of neutralizing antibodies to GM-CSF[95]. Similar effects on MDSC have been suggested with other growth factors including Fms-like tyrosine kinase 3 (Flt3) ligand[112], stem cell factor (SCF),[87] and S100A9 protein[113]. Furthermore, blocking of MDSC accumulation in tumor-bearing hosts with antibodies against Gr-1, CD11b, or CSF1 (colony-stimulating factor 1), using the inhibitor of CSFR1/c-kit, sunitinib, or using the antimetabolites gemcitabine and 5-fluorouracil, has been shown to restore T cell function partially in tumor-bearing hosts[25, 87, 114–119].

Using the 3LL murine lung carcinoma, we studied which tumor-derived factors might contribute to the expression of arginase I in MDSC. Supernatants from 3LL tumor cells maintained arginase I expression of MDSC isolated from tumors, while MDSC cultured in regular tissue culture media rapidly lost arginase I expression. Cytokines such as IL-4, IL-13, TGF-β, and others were not detected in the supernatants of the 3LL single-cell suspensions. Instead, we found high cyclooxygenase-2 (COX-2) expression and an increased production of prostanoids including prostaglandin E2. The addition of COX-2 inhibitors or silencing of COX-2 blocked the ability of 3LL cells to induce arginase I in MDSC[120]. This effect was mediated through the E-prostanoid receptor (EP4) on MDSC and was associated with increased cAMP levels (Fig. 10.3). Consequently, treatment of

tumor-bearing mice with the COX-2 inhibitor SC-58125 decreased the expression of arginase I in MDSC infiltrating the tumor and induced an immune-mediated antitumor effect. Similar results have been reported in mice bearing the 4T1 breast carcinoma where the treatment with the COX-2 inhibitor SC-58236 reduced the accumulation of MDSC in the spleen in an EP2-dependent manner[121]. In addition, celecoxib, a selective COX-2 inhibitor was able to inhibit the induction of colon tumors in Swiss mice treated with 1,2-dimethylhydrazine diHCl[122]. Other factors could also play a role in the induction of arginase in MDSC including hypoxia-inducible factors 1 and 2 (reviewed in[123]), IL-4, IL-13, and IFN-γ[84]. In conclusion, although the mechanisms of induction of arginase I in MDSC have been partially identified in mice, the factors inducing the activation of MDSC in patients are still unclear. A recent publication by Rotondo et al. suggests the possible role of tumor-derived IL-8 in the release of arginase from human MDSC[124].

10.9 MDSC: Lessons from Other Diseases and Future Applications

MDSC are not unique to cancer. Trauma patients and patients with chronic infections including leprosy and active pulmonary tuberculosis also have increased numbers of MDSC-expressing arginase I that inhibit T cell function. This data suggests that MDSC could be increased by a chronic inflammatory process as a response to tissue damage (danger signal). A demonstration of this mechanism was described by Albina et al.[51] studying the healing of surgical wounds. They found that surgical wounds were initially infiltrated by cells expressing inducible NO synthase which could represent an initial effort to eliminate any agents contaminating the wound. This was followed by infiltration by cells expressing arginase I that would metabolize L-arginine to ornithine and to proline. The latter triggers the activation of fibroblasts and the synthesis of collagen, ultimately leading to the healing of the surgical wound. The local depletion of L-arginine would also prevent T cells from infiltrating healing tissue. In cancer or chronic infections, tissue damage would also trigger a similar response with the proliferation of fibroblasts-producing collagen aimed at isolating and healing the damaged tissue (i.e., the malignant growth). As a matter of fact, many tumors are surrounded by dense fibrous tissue that makes its surgical excision difficult. The major difference between both disease processes (surgical wound versus malignant tumor) would be that the surgical wounds eventually heal, ending the role for arginase-producing MDSC, while malignant tumors do not stop growing and destroying tissue (i.e., do not “heal”) instead promoting a chronic inflammatory process mediated by MDSC. The continuous production of arginase I would ultimately lead to the depletion of L-arginine from the microenvironment and the development of T cell anergy. Therefore, it is our hypothesis that tumors hijack the normal healing process making it instead a vicious cycle that results in the inhibition of a

potentially protective T cell antitumor response. Although this is surely to be an oversimplified version of the complex mechanisms triggered in vivo, it provides a model with which to understand a complex event in the development of cancer and could help with the design of new therapeutic approaches that might interrupt this dysfunctional response.

MDSC are not only present in cancer and trauma; data from hosts infected with tuberculosis also shows the existence of MDSC[125], suggesting that these cells play a central role in chronic inflammation. In addition, MDSC-expressing arginase I have been shown to be central in the maintenance of fetal tolerance and are found in abundance in the placenta[105].

Much has been learned about the role of MDSC in the progression of tumors in the last ten years. Multiple approaches have been taken to block MDSC suppression including the use of all-*trans* retinoic acid[111]; inhibiting nitric oxide function with nitro-aspirin[126]; inhibiting phosphodiesterase-5[127]; blocking arginase activity with specific arginase inhibitors[79]; blocking MDSC accumulation using antibodies against Gr-1, CD11b, or CSF; or using the chemotherapy agents sunitinib, gemcitabine, or 5-fluorouracil[25, 87, 114–119]. It is likely that the appropriate combination of inhibitors blocking MDSC function and stimuli protecting T cells could overcome this powerful tumor-derived mechanism that impairs the promise of cancer immunotherapy.

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Chapter 11
Antibodies as Cancer Immunotherapy

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Abstract Infused antibodies are the most successful immune-based agents to treat certain cancers. However, their modes of action are not always dependent on immune mechanisms aside from antigen-specific targeting. Immune mechanisms include direct and indirect cytotoxicity while nonimmune mechanisms include the targeting of toxins, such as drugs or irradiation sources, directly to the tumor, the subject of Chap. 12. Therapeutic antibodies can be chimeric or humanized (part human and part animal, usually mouse), but fully human antibodies are in trials or have been FDA approved. This chapter will summarize the current FDA-approved antibodies to treat cancer, and highlight promising antibodies in development.

11.1 Introduction

Paul Ehrlich is credited with conceiving the use of therapies that are targeted to specific cells or tissues over 100 years ago. He coined the term "magic bullet" to refer to targeted agents that he envisioned would kill microorganisms specifically or target cancer cells specifically[1,2]. In the past few decades, Dr. Ehrlich's vision is being realized using targeted therapies that are practical, successful and Food and Drug Administration (FDA)-approved to treat cancer[3,4].

The first targeted anti-cancer agents were monoclonal antibodies that selectively targeted and killed cancer cells. Examples of these drugs include rituximab[5], a chimeric, humanized anti-CD20 monoclonal antibody approved by the FDA in 1997 to treat B-cell lymphoma, and alemtuzumab, a humanized anti-CD52 monoclonal antibody approved in 2001 to treat chronic lymphocytic leukemia[6].

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Although naturally-occurring defenses against cancer depend on T lymphocytes[7], and there appears to be little role for humoral immunity in normal defenses against cancer, some of the more successful cancer immunotherapies approved for human use are antibodies. These clinical results owe in part to the various mechanisms of action ascribed to these agents, and also to their inherent ability to direct cytotoxic agents specifically to tumors.

11.2 Overview of Antibodies

Although there are five major classes of antibodies (IgA, IgD, IgE, IgG, and IgM), all successful and FDA-approved agents are of the IgG class. These antibodies, generally derived from animal sources (notably mice), can be chimeric, where significant portions are of nonhuman origin; humanized, where the antigen recognition site (usually from a mouse antibody) has been genetically engineered into a human antibody backbone; or they can be completely human in origin. The more humanized an antibody is, the less likely it will be to generate a host immune response, which would increase side effects and reduce antibody treatment efficacy. See references [8,9] for recent, excellent reviews.

From a treatment perspective, the two major antibody moieties are the antigen recognition site or Fab region and the constant moiety or Fc region. The Fab region defines the targeting specificity of the antibody while the Fc portion affects immune function, such as binding complement or initiating antibody-dependent cell-mediated cytotoxicity (ADCC). The IgG class of antibodies can be subdivided into the $IgG_{1\text{-}4}$ subclasses, the functional relevance being that IgG_1 and IgG_3 antibodies activate complement. The Fc region of IgG activates complement component C1q, which results in production of complement components C3a and C5a, which are chemotactic, activating factors for innate immune cells including granulocytes and macrophages. Cells interact with the Fc region of the antibody through three classes of Fcγ receptors (FcγR), which can be inhibitory or stimulatory. FcγII receptors include those that mediate inhibitory signals, whereas FcγI and FcγIII receptors include those that transduce immune activating signals[10]. The ADCC that results from Fc–FcR binding on immune cells can be beneficial in two ways. First, innate effector cells or molecules will be activated with potential to kill tumor cells directly. Second, antigens released by killed tumor cells can help prime antigen-specific anti-tumor immunity. In this latter regard, monoclonal antibodies appear to improve immune cross presentation[11], presentation of exogenous tumor antigens in the MHC class I pathway that is required to develop $CD8^+$ cytotoxic T lymphocytes, and as such benefit anti-tumor immunity. Finally FcR expressed by lymphatic endothelial cells can increase the serum half life of immunoglobulins through their Fc interactions. There is much interest, therefore, in not only identifying antibodies of specific antigenic binding capacities, but also in the engineering of specific immunoglobulin subclass and Fc features which would improve immune activation, reduce immune inhibition, prevent immune response

activation, and prolong antibody half-life. These issues are discussed extensively in excellent recent reviews[12–16].

Although immunoglobulins have immune modulating properties, most of the successful and FDA-approved anti-cancer antibody treatments target an essential cancer growth or survival pathway. Examples include cetuximab, which targets the epidermal growth factor receptor (EGFR) which is discussed further in Sect. 5.5. Other anti-cancer antibody-based strategies include immunoconjugates, antibodies that carry toxic payloads. Such conjugates can be radioactive, such as 90Y-ibritumomab tiuxetan that targets CD20 in hematologic malignancies, or toxic to specific cellular components, such as brentuximab vedotin[17] which targets a microtubule toxin to CD30-expressing lymphoma cells. Immunoconjugates are detailed in Chap. 12.

In 1986, the FDA-approved the first therapeutic monoclonal antibody OKT3 (muromonab, Orthoclone), a CD3-specific murine IgG2a monoclonal antibody used to deplete T cells in organ allograft recipients[18]. The list of approved monoclonal antibodies now stands at 20, a dozen of which are routinely used in cancer applications, and many more are being tested alone and in a myriad of combinations with other agents. The field is old enough that one previously approved agent (gemtuzumab ogozamicin) has already been withdrawn, and another has had an indication withdrawn (bevacizumab in metastatic breast cancer). Both topics are discussed in the relevant sections below. The field continues to evolve rapidly.

11.3 Development of Monoclonal Antibodies

The initial discovery of monoclonal antibodies in 1975 by Kohler and Milstein, through fusion of murine myeloma cells with murine antibody-secreting lymphocytes, opened the field to the development of therapeutic monoclonal antibodies[19]. Murine myeloma cells are immortalized B lymphocytes capable of secreting homogenous antibodies. These cells lack the enzyme hypoxanthine guanosine phosphoribosyl transferase (HGPRT) and, therefore, are unable to grow in hypoxanthine–aminopterin–thymidine (HAT) cell culture medium. However, when myeloma cells are fused with murine spleen B lymphocytes that possess and express the HGPRT gene, the hybrid cells, or hybridoma, survive in HAT media. The myeloma cell imparts immortality, allowing indefinite cultivation. Subculturing of a particular hybridoma ensures clonal expansion and the continued production of single antibody with identical antigen-binding specificity, hence the term monoclonal. Large-scale production of such monoclonal antibodies is made possible through generation of ascites tumors in mice or *in vitro* mammalian cell culture fermentation using bioreactors and continuous perfusion culture systems. Purification of monoclonal antibodies is accomplished by chromatography, fragmentation, conjugation with chelating agents, ultrafiltration and controlled precipitation[20,21]. More novel methods used to generate antibodies or antibody-like molecules, include phage display libraries, transgenic mice models and genetically engineered plants [22–24].

One major concern with murine monoclonal antibodies use in the clinical setting is the development of a human anti-mouse antibody (HAMA) response in patients given antibodies as treatment. Such a response can lead to the development of allergic or immune complex hypersensitivities, rapid clearance of the antibody and reduced therapeutic efficacy[25,26]. To overcome the clinical limitations of murine monoclonal antibodies recombinant antibodies or chimeras were generated. Chimera generation involves the fusion of rearranged murine variable gene segments of mouse monoclonal antibodies with human constant domains, or production of recombinant Fab fusion proteins by replacing the Fc region of the mouse monoclonal antibodies with an enzyme moiety[27]. Because the Fc region has little influence on the structure of the variable region, the affinity and specificity of these chimeras are effectively unchanged. Murine and chimeric monoclonal antibodies will, therefore, have equal capacity to induce apoptosis *in vitro*, however, chimeric monoclonal antibodies have the capacity to induce ADCC on human cancer cell targets and/or activate the complement cascade[26,28].

Despite chimeric monoclonal antibodies significantly reducing the HAMA response, such antibodies still show immunogenicity in humans due to the murine portion of these constructs. In an effort to reduce further the immunogenicity of murine antibodies, a functional human-like antibody or "humanized" mAb was designed. These were produced by grafting segments encoding the complementarity-determining regions (CDR) in the variable domains of the murine antibody into human variable domains. These humanized monoclonal antibodies demonstrate considerably less HAMA response although limitations still exist in the efficient construction of these antibodies[25,29,30]. Further optimization of these humanized monoclonal antibodies is often required to re-establish the required specificity and affinity of the original murine antibodies or to reduce their immunogenicity. Several novel strategies have been designed for constructing humanized monoclonal antibodies and are discussed elsewhere[31–33].

11.4 Antibody Nomenclature

Antibodies used in humans, or in development for such use, are named based on the animal from which the antigen binding specificity was derived and on whether it is rodent, chimeric, humanized, or fully human. A newer trend is also to add a specification for the tumor type intended. These harmonized generic names are not capitalized. In contrast to the capitalized trade names used by manufacturers, fully human mAbs are identified with "-tumu-". The two FDA-approved agents in this class are ofatumumab (Arzerra, HuMax-CD20) and panitumumab (Vectibix). Investigational agents in this class include, adecatumumab, cixutumumab, conatumumab, daratumumab, drozitumab, figitumumab, ganitumab, glembatumumab vedotin, intetumumab, iratumumab, lexatumumab, lucatumumab, mapatumumab, narnatumab, necitumumab, olaratumab, pritumumab, radretumab, rilotumumab, robatumumab, teprotumumab, votumumab, and zalutumumab. "- Melu-" designates an agent in this class intended for use in melanoma.

Chimeric mouse/human monoclonal antibodies are identified with “-tuxi-”. The two FDA-approved agents in this class are cetuximab (Erbitux) and rituximab (Rituxin). Investigational agents in this class include, amatuximab, bavituximab, brentuximab vedotin, ensituximab, girentuximab, indatuximab ravtansine, siltuximab, and ublituximab. “-Mexi-” refers to an agent in this class meant to treat melanoma (e.g., the investigational agent ecromeximab).

“-Tuzu-” refers to mouse/human humanized antibodies. The four FDA-approved agents in this class are alemtuzumab (Campath), bevacizumab (Avastin; named prior to harmonization of nomenclature), gemtuzumab ozogamicin (Myelotarg) and trastuzumab (Herceptin). Gemtuzumab ozogamicin has since been voluntarily withdrawn by the manufacturer (see Chap. 12 for details). Investigational agents in this class include afutuzumab, bivatuzumab mertansine, cantuzumab mertansine, cantuzumab ravtansine, citatuzumab bogatox, clivatuzumab tetraxetan, dacetuzumab, dalotuzumab, elotuzumab (showing some success in combination regimens[34,35]), etaracizumab, farletuzumab, ficlatuzumab, inotuzumab ozogamicin, labetuzumab, lintuzumab, lorvotuzumab mertansine, matuzumab, milatuzumab, nimotuzumab, ocaratuzumab, onartuzumab, oportuzumab monatox, pertuzumab, sibrotuzumab, tacatuzumab tetraxetan, tigatuzumab, tucotuzumab celmoleukin, and veltuzumab.

Murine monoclonal antibodies are designated with “-tumo-”. The antibody–radioisotope conjugate ibritumomab tiuxetan (Zevalin) and muromonab (Orthoclone) are the two FDA-approved agents in this class. Investigational agents include altumomab pentetate, anatumomab mafenatox, arcitumomab, bectumomab, blinatumomab, detumomab, ibritumomab tiuxetan, minretumomab, mitumomab, moxetumomab pasudotox, naptumomab estafenatox, nofetumomab merpentan, pemtumomab (currently in phase III trials; www.clinicaltrials.gov), pintumomab, racotumomab, satumomab pendetide, taplitumomab paptox, tenatumomab, and tositumomab. Subdesignations include “govo-” (ovarian cancer; *e.g.* abagovomab, igovomab, oregovomab), “-pro-” (prostate cancer; *e.g.* capromab pendetide) and “-colo-” (colorectal cancer; *e.g.* edrecolomab, nacolomab tafenatox). Oregovomab failed its phase III clinical trials and now faces an uncertain future, see Sect. 6.4 for details.

A final note on nomenclature: companies often use a different trade name of the same drug when used for different indications. For example, the anti-CD20 monoclonal antibody rituximab is marketed as Rituxan to treat hematologic malignancies, and as MabThera when used to treat autoimmune diseases.

11.5 Specific Antibody Agents Approved by the FDA and European Agencies

These agents are summarized in Table 11.1.

Table 11.1 Summary of approved therapeutic antibodies used to treat cancer or related medical problems

Generic/trade names	Ig type/class	Target	Mechanism	Indications	References
Rituximab Rituxan Roche	Chimeric mouse/human IgG1	CD20	Complement-dependent cytotoxicity Apoptosis induction?	$CD20^+$ follicular non-Hodgkin lymphoma as first-line, maintenance and salvage therapy, $CD20^+$ chronic lymphocytic leukemia. Other lymphoid malignancies[a]	36–41 43–45 47–54
Ofatumumab Arzerra Glaxo SmithKline	Fully human IgG1 kappa	CD20	Complement dependent cytotoxicity	Chronic lymphocytic leukemia after fludarabine and alemtuzumab failure Non-Hodgkin lymphoma[a] Certain B cell leukemias[a]	55–59
Trastuzumab Herceptin Genentech	Humanized IgG1	HER-2	Inhibit EGFR signaling	$Her2^+$ breast cancer alone or combined with chemotherapy. metastatic gastric or gastroesophageal junction adenocarcinoma as first-line therapy in combination with cisplatinum and a fluoropyrimidine (capecitabine or 5-fluorouracil)	61,67–78
Alemtuzumab Campath Genzyme	Humanized IgG1	CD52	Inhibit cell trafficking	Stage III mycosis fungoides or Sezary syndrome (second line) First-line therapy for stage II–IV chronic lymphocytic leukemia or small	79,80

				lymphocytic lymphoma. It also supports first-line or second-line alemtuzumab treatment alone or in combination with fludarabine or rituximab in specific subsets of patients with chronic lymphocytic leukemia.	
Cetuximab Erbitux Eli Lilly and Bristol Myers Squibb	Mouse/human chimeric IgG1	Epidermal growth factor receptor	Antibody-dependent cell-mediated cytotoxicity	Metastatic colorectal cancer combined with irinotecan, or as a single agent if irinotecan cannot be tolerated Squamous cell carcinoma of the head and neck	81,88–90,92–102,110,113–122
Panitumumab Vectibix Amgen	Fully human IgG2	Epidermal growth factor receptor	Block EGFR signaling	$EGFR^+$ colorectal cancer after failure of first-line therapy	103–106,113,119–122
Bevacizumab Avastin Roche (Genentech)	Humanized IgG1	VEGF	Reducing VEGF and inhibiting angiogenesis	Colorectal cancer Lung cancer Glioblastoma multiforme Renal cell carcinoma Breast cancer[b] Neuroendocrine tumors[a] Epithelial ovarian carcinoma[a]	126–131,135–152
Natalizumab Tysabri Biogen	Humanized IgG4	α4-integrin	Interrupting leukocyte trafficking	Multiple sclerosis and Crohn disease Hematologic malignancies[*]	162,163
Denosumab Prolia, Xgeva Amgen	Fully human IgG2	RANK ligand	Bind to osteoclast RANKL and reduce osteoclast activation	Prevention of skeletal events in cancer patients	164,165

(continued)

Table 11.1 (continued)

Generic/trade names	Ig type/class	Target	Mechanism	Indications	References
				Giant cell tumors[a] Multiple myeloma with bone metastases[a], Hypercalcemia of malignancy[a], Breast cancer[a]	
Pertuzumab Perjeta Genentech, Hoffman Laroche	Recombinant humanized IgG1	EGFR extracellular dimerization domain (subdomain III)	Block ligand dependent blocking ligand-dependent HER2 heterodimerization	Metastatic breast cancer in combination with trastuzumab plus docetaxel	166
Ipilimumab Yervoy Bristol Myers Squibb	Fully human	CTLA-4 on T cells	Improves T cell function Reduces T cell capacity to be inhibited by regulatory T cells Reduces regulatory T cell function?	Metastatic melanoma	See Chap. 8

[a]Not approved in the FDA or in Europe.
[b]FDA approval later revoked.
[*]Not an FDA approved indication.

11.5.1 Rituximab

Rituximab is a monoclonal chimeric mouse/human IgG1 antibody from Roche recognizing the CD20 antigen expressed by most mature B cells, but notably not plasma cells or bone marrow B cell precursor cells. In 1997 rituximab became the first antibody approved by the FDA to treat cancer. It is composed of a human kappa constant region, an IgG1 Fc moiety and a murine variable region that recognizes CD20[36]. It is used to treat $CD20^+$ follicular non-Hodgkin lymphoma as first-line, maintenance and salvage therapy, and to treat $CD20^+$ chronic lymphocytic leukemia. Its European indications are similar. Rituximab is under investigation for, and also used in off-label treatments for a variety of hematologic malignancies.

Owing to its IgG1 antibody properties, the clinical activity of this antibody appears dependent, in part, on its capacity to induce cellular cytotoxicity[37] as evidenced by its loss of activity in C1q-null mice[38] a finding disputed for $CD20^+$ cell depletion in another study[39]. The requirement for complement-mediated cytotoxicity in humans is suggested indirectly by the finding that *C1QA* (the gene coding for C1q in humans) genetic polymorphisms predict clinical activity in follicular lymphoma patients receiving rituximab treatments[40]. Clinical activity also requires complement for optimal effects as rituximab efficacy in mice xenografted with human B cell lymphoma was reduced by complement depletion[41]. A human requirement for complement in rituximab effects was shown indirectly by the observation that patients with chronic lymphocytic leukemia undergoing rituximab treatment had rapid and significant reductions in serum complement component levels with treatment[42]. Strikingly, polymorphisms in FcγR in patients with non-Hodgkin lymphoma also predicted rituximab clinical efficacy[43,44].

Rituximab has additional potential mechanisms of action that remain to be fully established. For example, the onset of clinical efficacy in rituximab therapy can be delayed until several months after treatment is initiated. This delay is consistent with activation of tumor-specific immunity as a mechanism of action[45] as has also been noted with other tumor antigen-specific antibody therapies[46]. Anti-idiotype antibody generation reacting against the tumor has also been suggested as a mechanism of action[47]. In patients with chronic lymphocytic leukemia, rituximab induces tumor cell caspase activation and apoptosis[48]. This could be an additional mechanism of action, or could be a result of direct tumor toxicity or the indirect consequences of immune mechanisms.

Aside from stand-alone efficacy, rituximab can improve the clinical activity of standard CHOP treatment for patients with diffuse large B cell lymphoma[49]. It may also improve clinical response when combined with fludarabine, cyclophosphamide and mitoxantrone therapy, compared to these cytotoxic agents alone, in patients with relapsed and refractory follicular lymphoma or mantle cell lymphoma[50]. Rituximab has also demonstrated efficacy in AIDS-related malignancies[51].

Because rituximab has mechanisms of clinical action that involve direct effects on tumor cells and indirect effects on immune defenses, it is reasonable to expect that tumor cell intrinsic and extrinsic effects could contribute to rituximab

resistance[52]. Mechanisms for resistance are not fully understood, but include downregulation of surface expressed CD20 on malignant B cells[52]. In a phase II trial of patients with rituximab-refractory indolent non-Hodgkin lymphoma, adding IL-2 increased the number of FcR^+ cells *in vivo,* which improved their antibody-dependent cell-mediated cytotoxicity tested *in vitro*[53]. A recent report suggested that preferential depletion of non-B regulatory cells (Bregs) by rituximab left a majority of dysfunctional Bregs in circulation after treatment that could contribute to treatment resistance[54].

11.5.2 Ofatumumab

Ofatumumab (Arzerra, Glaxo SmithKline) is a fully human anti-CD20 IgG1 kappa monoclonal antibody that binds to an epitope of CD20 distinct from that targeted by rituximab[55]. The original phase 1/2 trial of ofatumumab demonstrated clinical activity in non-Hodgkin lymphoma (CD20 positive follicular lymphoma)[56]. Notably in this trial, there was a 64% clinical response rate in patients previously treated with rituximab. Ofatumumab has since evinced clinical activity in patients with chronic lymphocytic leukemia and in patients with many types of non-Hodgkin lymphoma. It was approved by the FDA in October 2009 to treat patients with chronic lymphocytic leukemia who failed therapy with standard fludarabine and alemtuzumab. Its European indications are similar. Ofatumumab has demonstrated clinical tolerability and efficacy in a phase 1/2 clinical trial of B cell leukemia[57], but is not yet approved for that indication.

Like rituximab, its mechanisms of action appear to include complement-dependent cytotoxicity and antibody-dependent cell-mediated cytotoxicity. Because of differences in cell binding of ofatumumab vs. rituximab (the former can bind CD20 closer to the cell membrane) ofatumumab might induce complement dependent cytotoxicity to a greater extent than rituximab[58], although the clinical significance of this is uncertain. Unlike rituximab, ofatumumab did not induce apoptosis in cultured lymphoma cell lines *in vitro*[59], although the clinical significance is again uncertain.

A number of additional anti-CD20 antibodies are in various stages of clinical development, not only for treating B-cell malignancies but also to treat certain autoimmune disorders, some showing evidence for clinical efficacy. These anti-CD20 antibodies under clinical development include PRO131921 (Genentech) and others[60].

11.5.3 Trastuzumab

Trastuzumab is a recombinant, humanized IgG1 anti–HER-2 monoclonal antibody from Genentech that exhibits high-affinity binding to the extracellular domain of HER-2. It is the first FDA-approved agent to target this molecule[61]. For FDA-approved indications, tumors typically have to have confirmed HER2 gene

amplification (over-expression, not simple positivity) by an approved, validated method (fluorescence *in situ* hybridization or immunohistochemistry) performed in a certified laboratory. Its European indications are similar.

The human epidermal growth factor receptor (HER) family of proteins consisting of EGFR, HER2, HER3, and HER4 are type I transmembrane growth factor receptors that activate intracellular signaling pathways in response to extracellular signals. These receptors activate numerous downstream pathways in response to extracellular ligands, and regulate diverse processes including differentiation, migration, proliferation, and survival[62,63] .

A distinguishing characteristic of family members is their complementary function and dependence on each other. HER2 overexpression results in increased formation of HER2-containing heterodimers and interaction with the HER family of receptors. Increased HER2-EGFR heterodimers drive proliferative and invasive functions. Increased HER2 homodimers disrupt cell polarity. Of these, HER2 and HER3 are particularly interdependent, as on their own they are functionally incomplete receptors. HER2 has an extracellular domain, but appears to lack ligand-binding activity, while HER3 has a nonfunctional kinase domain meaning it has no catalytic activity[64,65]. Together the HER2–HER3 heterodimer is a highly functional signaling unit and constitutes the most active signaling dimer in this family, exemplifying the role of complementary functions in this complex receptor family[66].

11.5.3.1 Breast Cancers

Trastuzumab was originally approved by the FDA in September 1998 for treatment of HER2 overexpressing metastatic breast cancer. Follow-up studies demonstrated that trastuzumab improved overall survival in metastatic breast cancer when combined with cytotoxic chemotherapy[67]. In November 2006 the FDA granted expanded approval for trastuzumab in HER2 overexpressing breast cancer for use in early stage patients with involvement of breast or regional lymph nodes to be used after surgical treatment (lumpectomy or mastectomy), of which 20–25 % of patients with invasive breast cancers could be eligible[68]. Trastuzumab yielded an estimated 3 year disease-free survival rate of 87 % in combination with chemotherapy vs. 75 % with chemotherapy alone in this setting. A recent long-term follow up study confirmed the disease-free survival benefit and showed a clear overall survival benefit of trastuzumab plus chemotherapy over chemotherapy alone in locally invasive (and non-metastatic) breast cancers[68].

Trastuzumab can mediate significant cardiotoxicity especially if combined with anthracycline-containing or cyclophosphamide-containing chemotherapy regimens[69]. Nonetheless, it can be used with these agents for clinical benefit with appropriate patient selection and careful monitoring of cardiac function[67]. As with most therapies, breast cancer patients with an initial positive treatment response to trastuzumab can become resistant. Potential mechanisms of resistance include reduced HER2 expression, altered downstream HER2 signals, and preferential or new activation of non-HER2 growth factor pathways[70]. The relative importance of

these potential mechanisms remains to be established clinically. Addition of other agents to trastuzumab to block HER2 signaling for additional clinical benefit is an area of active investigation[71]. For example, adding the HER-2-targeted monoclonal antibody pertuzumab to trastuzumab[72] or using multiple anti-EGFR agents has been assessed with some potentially beneficial results[71]. Pertuzumab was FDA approved in combination with trastuzumab plus docetaxel for breast cancer on June 8, 2012, detailed below in Sect. 5.10. European breast cancer indications for trastuzumab are similar to FDA indications.

11.5.3.2 Gastrointestinal Cancers

In October 2010, the FDA granted approval for trastuzumab to treat HER2-overexpressing metastatic gastric or gastroesophageal junction adenocarcinoma as first-line therapy in combination with cisplatinum and a fluoropyrimidine (capecitabine or 5-fluorouracil). The approval was based on the ToGA trial of 594 patients with HER2-overexpressing metastatic adenocarcinoma of the stomach or gastroesophageal junction, (3 % of patients had locally advanced disease)[73,74]. Median survival was increased to 13.5 months vs. 11.0 months by addition of trastuzumab. Survival improvement was maintained at a later follow-up.

In both breast and gastric cancers, HER2 overexpression and gene amplification should be determined using tests done in certified laboratories. Interpretation of the test results depends on tumor histopathology and the expression levels of HER-2 in these tumors (breast, gastric or gastroesophageal junction cancer)[75]. European gastrointestinal tumor indications for trastuzumab are similar to FDA indications.

11.5.4 Alemtuzumab

Alemtuzumab (Campath, MabCampath, Campath-1H; Genzyme Corporation) is a humanized IgG1 monoclonal antibody (originating from a rat anti-CD52 antibody) targeting the adhesion molecule CD52. Initial studies of this antibody were done in the pathology department at Cambridge University, England leading to "Campath" as its name. The CD52 binding domain was later engineered into a human antibody backbone[76] providing the basis for alemtuzumab.

It received initial FDA approval in 2001 for treatment of chronic lymphocytic leukemia failing treatment with an alkylating agent (chlorambucil or melphalan) or fludarabine. The FDA expanded the label and granted regular approval on September 19, 2007, to use alemtuzumab as a single agent to treat B cell chronic lymphocytic leukemia[77]. In the pivotal study[78] supporting this new indication, 297 patients were randomized to receive alemtuzumab alone or combined with chlorambucil. Adding alemtuzumab increased progression-free survival, reduced progression or death and increased the development of minimal residual disease or disease-negative remissions compared with chlorambucil, without undue increases in toxicity. Its European indications are similar.

Alemtuzumab has been tested in cutaneous T cell leukemia/lymphoma with minimal clinical efficacy and significant toxicity[79]. In another phase II study, alemtuzumab was tested in 22 patients with advanced mycosis fungoides/Sezary syndrome where it demonstrated potential efficacy with manageable toxicities in the subset of patients who had not been heavily pretreated[80]. Current practice guidelines (The National Comprehensive Cancer Network Drug and Biologics Compendium, 2010) supports alemtuzumab as a second-line therapy for selected patients with stage III mycosis fungoides or Sezary syndrome. This Compendium also supports alemtuzumab as first-line therapy for stage II–IV chronic lymphocytic leukemia or small lymphocytic lymphoma. It also supports first-line or second-line alemtuzumab treatment alone or in combination with fludarabine or rituximab in specific subsets of patients with chronic lymphocytic leukemia. Alemtuzumab is currently being tested in multiple sclerosis under the trade name Lemtrada.

11.5.5 Cetuximab

Cetuximab (Erbitux; Eli Lilly and Bristol Myers Squibb) is a mouse/human chimeric IgG1 antibody that targets the epidermal growth factor receptor (EGFR), an important regulator of cancer cell growth and survival[81]. It was approved by the FDA for use in metastatic colorectal cancer on February 12, 2004, when combined with irinotecan, or as a single agent if irinotecan cannot be tolerated. It received FDA approval to treat squamous cell carcinoma of the head and neck in March 2006, and European Commission approval in November 2008.

The EGFR or ErbB1 is a member of the HER family of receptors as discussed in Section 5.3. At least six different ligands, known as EGF-like ligands, bind to the EGFR. EGF, transforming growth factor alpha (TGF-α), and amphiregulin bind only to EGFR while heparin binding EGF (HB-EGF), betacellulin, and epiregulin bind both EGFR and HER4/ErbB4[82,83]. EGF-like ligands induce formation of EGFR homodimers and EGFR/HER-2 heterodimers, with EGFR/HER-3 and EGFR/HER-4 heterodimers detected occasionally. Following receptor dimerization, intrinsic protein tyrosine kinase activation and tyrosine autophosphorylation occur. These downstream signaling events lead to recruitment and phosphorylation of several important intracellular substrates. Ras-Raf-MAP kinase and phosphatidylinositol-3 kinase (PI3K)-Akt-mTOR are major signaling cascades activated which lead to mitogenic signaling and cellular activities. The discovery of the EGFR by Cohen and colleagues[84], and the observation that EGFR is markedly overexpressed in a large variety of epithelial cancers, led to the production and characterization of anti-EGFR monoclonal antibodies to deprive tumor cells of EGF-mediated growth signaling[85–87].

11.5.5.1 Colorectal Cancer

For several decades, 5-fluorouracil plus leucovorin (5-FU/LV) was the only effective treatment regimen for patients with advanced colorectal cancer (CRC), associated with a median overall survival of approximately 12 months. The introduction of the cytotoxic agents irinotecan and oxaliplatin further improved median overall survival. More recently, a new class of targeted agents, namely the monoclonal antibodies that bind to the ligand or the extracellular domain of the EGFR (cetuximab or panitumumab) or VEGF (bevacizumab) has provided further benefit when combined with irinotecan/5FU/LV (FOLFIRI) or oxaliplatin/5FU/LV (FOLFOX) with median overall survival now approaching 2 years.

The two anti-EGFR monoclonal antibodies are currently available for the treatment of metastatic colorectal cancer. Cetuximab is a chimeric immunoglobulin G1 antibody, and panitumumab is a fully human immunoglobulin G2 antibody. It was soon clear however that not all advanced colorectal cancer patients respond to anti-EGFR therapy similarly. The response to anti-EGFR antibody therapy in patients has been reported to be independent of EGFR expression in tumor tissue. Factors that have been reported to be associated with resistance to anti-EGFR therapy include increased EGFR ligand expression, alterations in downstream signaling pathways, and cross-talk between different members of the HER or ErbB receptor family[88]. Data from the OPUS study that combined cetuximab plus FOLFIRI, and the CRYSTAL study that combined cetuximab plus FOLFOX demonstrated that response to cetuximab is limited to patients without a mutation in the *KRAS* oncogene[89,90]. *KRAS* mutations are observed in ~38 % of colorectal tumors[91]. A mutation in *KRAS* results in constitutive activation of the Ras/Raf/MAPK signaling pathway, which is independent of EGFR activation by ligand binding[92]. Within the subgroup of patients with *KRAS* wild type tumors, the efficacy of anti-EGFR therapy appears to be further limited to patients with *BRAF* wild type tumors, although it is still not clear if presence of *BRAF* mutations is a prognostic rather than a predictive marker[93].

In the CRYSTAL clinical study, the most common grade 3 or 4 adverse events reported in the cetuximab plus FOLFIRI compared to FOLFIRI alone cohorts included skin reactions (19.7 % vs. 0.2 %), infusion-related reactions (2.5 % vs. 0 %), and diarrhea (15.7 % vs. 10.5 %)[89]. In the OPUS study the most common grade 3 or 4 adverse events were neutropenia (30 % vs. 34 %), rash (11 % vs. 0.6 %), and diarrhea (8 % vs. 7 %) respectively, in the cetuximab plus FOLFOX vs. the FOLFOX alone group[90].

Efforts to incorporate cetuximab treatment into earlier-stage colorectal cancer have not yet yielded conclusive evidence for efficacy over standard chemotherapy regimens[94]. Combination of small molecule EGFR inhibitors, such as erlotinib, show promising efficacy when combined with cetuximab in preclinical and early clinical studies[95] but additional clinical studies are required to confirm its efficacy.

11.5.5.2 Head and Neck Cancers

Cetuximab is the first targeted therapy to show a significant benefit in head and neck cancer and received FDA regulatory approval for use in patients with head and neck cancers. Approval of this agent for locally advanced squamous cell carcinoma of the head and neck was based on a landmark trial, that showed the combination of cetuximab and radiotherapy compared to radiotherapy alone led to a significant improvement in median overall survival (49 vs. 29.3 months; hazard ratio for death 0.74, $p = 0.03$) and locoregional control (24.4 vs. 14.9 months; hazard ratio for locoregional progression or death 0.68, $p = 0.005$). In addition to demonstrating the efficacy of cetuximab in this setting, the trial confirmed that the administration of cetuximab does not compromise the delivery of scheduled doses of radiotherapy and the combination of cetuximab-radiotherapy was well tolerated[96].

Burtness *et al.* provided the first evidence for the activity of cetuximab in the first-line treatment of recurrent and/or metastatic squamous cell carcinoma of the head and neck whereby 117 patients with previously untreated disease were randomized to receive either cisplatin plus cetuximab or cisplatin plus placebo. There was a significant increase in response rate in the cetuximab arm compared with the placebo arm (26 % vs. 10 %, $p = 0.03$). Although the study was not adequately powered to detect significant differences in survival a trend towards an increase in median progression-free survival (4.2 vs. 2.7 months) and median overall survival (9.2 vs. 8.0 months) was shown in the combination therapy [97]. Based on this early clinical evidence of activity, the rationale was that cetiximab was more effective with combination chemotherapy regimen (cisplatin or carboplatin plus 5-FU) rather than single agent cisplatin led to the EXTEME study where patients with head and neck cancer were randomized to one of two arms: cetuximab plus either carboplatin (AUC 5, day 1) or cisplatin (100 mg/m^2, day 1) plus 5-FU (1,000 mg/m^2, days 1–4; $n = 222$); or carboplatin or cisplatin plus 5-FU alone ($n = 220$). A maximum of six 3-weekly chemotherapy cycles were administered, after which patients received either cetuximab or no treatment. Among the 442 patients, overall survival was significantly prolonged in the cetuximab arm, with a median overall survival of 10.1 vs. 7.4 months. The hazard ratio for survival was 0.797 in favor of cetuximab ($p = 0.036$)[98]. EXTREME is notable as the first Phase III trial in three decades to demonstrate a survival benefit in recurrent or metastatic squamous cell head and neck cancer, but also underscored inadequacies in predicting treatment benefit based on tumor EGFR status in head and neck cancers[98,99].

The cetuximab serum half-life is ~114 h. Infusions are relatively well-tolerated but infusion reactions can occur. Photosensitivity and pulmonary and cardiac toxicity also occur, but are not common. Hypomagnesemia can occur in 15–20 % of patients[100]. As is true with other receptor tyrosine kinase inhibitors, skin rash is relatively common with continual use. It resembles acne, is generally well-tolerated and is usually reversible with discontinuation treatment[101,102].

11.5.6 Panitumumab

Panitumumab (ABX-EGF, Vectibix, Amgen) is a fully human IgG2 monoclonal antibody with high affinity for EGFR and a mean half-life of ~7.5 days in humans (range 3.6–10.9 days), therefore, administered every 2 weeks, compared to the weekly cetuximab dosing[103]. It was approved in September 2006 by the FDA for use in patients with metastatic colorectal that express the epidermal growth factor receptor and who have progressed after receiving standard therapies. It was approved by the European Medicines Agency in 2007, and by Health Canada in 2008 for similar indications in metastatic colon cancer with the further stipulation that tumors had to express wild type KRAS. Following suit, the FDA updated the panitumumab indication to include a statement in the package insert that use of panitumumab was not recommended in patients whose tumors demonstrated KRAS mutations, and included a similar revision and package insert update for the other anti-extracellular growth factor receptor antibody, cetuximab.

There are no trials directly comparing panitumumab vs. cetuximab. When compared across clinical studies, cetuximab and panitumumab appear to have comparable efficacy when used for single agents for salvage therapy in patients with chemotherapy-refractory metastatic colorectal cancer, and when used for first-line and second-line therapy of metastatic colorectal cancer in conjunction with a chemotherapy regimen[104,105]. For unclear reasons, panitumumab adds benefit to a first-line oxaliplatin-based regimen based on the PRIME clinical study, while results are mixed on the benefit of adding cetuximab to first-line oxaliplatin-based therapy[106].

Similar to the anti-epidermal growth factor receptor antibody cetuximab, panitumumab also binds the epidermal growth factor receptor extracellular domain, which reduces receptor-mediated activation of downstream signaling events. Because IgG2 subclass antibodies do not activate complement, antibody-dependent cellular cytotoxicity might not be a mechanism of action as seen in the anti-extracellular growth factor receptor antibody cetuximab. From a pharmacologic standpoint, cetuximab is a chimeric monoclonal antibody, while panitumumab is a completely human monoclonal antibody, and thus the incidence of hypersensitivity reactions with panitumumab is much lower, eliminating the need for routine premedication before treatments.

11.5.6.1 Resistance to EGFR Monoclonal Antibodies

In 2004, a series of landmark papers identified *EGFR* mutations in the tyrosine kinase domain in non-small cell lung cancer patients that predicted response to the EGFR tyrosine kinase inhibitors erlotinib and gefitinib[107–109]. These mutations included in-frame deletion of amino acids 746–750 in exon 19, and a point mutation in exon 21 (L858R). Notably, these mutations led to gain-of-function and conferred dependence of the tumor cell on the mutated kinase. These mutations in *EGFR*

rendered tumors dramatically more sensitive to the effects of erlotinib and gefitinib than tumors without these mutations. However, no mutations in *EGFR* have been identified to date that are reliably predictive for response to antibody-based anti-EGFR therapies. This finding suggests that other molecular mechanisms exist that modulate intrinsic (primary) or acquired (secondary) resistance to EGFR antibody-based therapies.

One of the prevalent biological themes underlying intrinsic or acquired resistance involves oncogenic shift, which occurs when other membrane-bound receptor tyrosine kinase signaling pathways are involved in resistance. Chronic therapy with cetuximab results in dysregulation of EGFR processing and subsequent activation of HER2, HER3, cMET, MAPK, and AKT[88]. Acquired resistance to cetuximab therefore could result from the activation of receptor tyrosine kinases, such as HER2 and HER3, which share overlapping proliferative and survival signaling pathways.

As discussed in the section above, one of the most reliable predictive biomarkers to emerge in the clinic has been that of *KRAS* mutation status in metastatic colorectal cancer. Based on extensive clinical data, it is now recommended that all patients with metastatic colorectal cancer who are candidates for anti-EGFR antibody therapy should have their tumor tested for *KRAS* mutations in a CLIA-accredited laboratory. If codon 12 or 13 of *KRAS* is mutated then patients with metastatic colorectal cancer should not receive anti-EGFR antibody therapy as part of their treatment, as they have a high likelihood of *de novo* resistance[110]. Reported rates of *KRAS* mutations in non-small cell lung cancer and squamous cell carcinomas of the head and neck range between 8–20 % and 3–7 % respectively. The role of *KRAS* mutations in these tumor types has yet to be determined[111,112]. Other molecular determinants of antibody resistance include mutations in PI3K. Mutations in the catalytic subunit of PI3K, p110alpha, have been reported in metastatic colorectal cancer and are significantly associated with resistance to cetuximab and panitumumab therapy[113]. It also has been reported that the overexpression of EGFR ligands amphiregulin and epiregulin in KRAS wild type metastatic colorectal cancer tumors correlates with enhanced response to cetuximab therapy, and, therefore, could serve as biomarkers for selection of in the future may patients for cetuximab therapy although this concept needs to be further validated[114].

Cetuximab has also been shown to have antitumor effects through mediating antibody-dependent cell-mediated cytotoxicity in which antibody Fc portion interacts with Fc-receptors expressed by immune cells, with the antibody-dependent cell-mediated cytotoxicity response influenced by Fc-RIIa-H131R and Fc-RIIIa-V158F polymorphisms[115]. Results show that polymorphisms in the FC-receptor IIa and FC receptor IIIa correlate with the lack of clinical response in metastatic renal cell carcinoma patients treated with cetuximab.

Preclinical studies have shown tumors that are resistant to EGFR therapy have a dramatic increase in phosphorylated MAPK, and an increase in protein expression of COX-2 and VEGF, suggesting that EGFR inhibitors can alter VEGF production and highlights neoangiogenesis as a potential mechanism of EGFR inhibitor

escape[116]. This is further supported by Bianco *et al*. reporting that VEGFR1 was overexpressed in cells with resistance to cetuximab. Experiments silencing VEGFR1 in cetuximab-resistant cells restored sensitivity to cetuximab, whereas exogenous overexpression of VEGFR1 in cetuximab-sensitive cells conferred resistance to cetuximab[117]. It therefore would seem reasonable to combine EGFR and VEGFR inhibitors, although further clinical studies assessing efficacy and safety of the combination are still required.

Although no point mutations are known to be associated with resistance to cetuximab or panitumumab, preclinical models analyzing the EGFR variant III (EGFRvIII), which lacks the ligand-binding domain, expressed in 42 % of squamous cell head and neck tumors correlated with increased proliferation *in vitro* and increased tumor growth *in vivo*. Furthermore, head and neck squamous cell carcinoma cells engineered to overexpress EGFRvIII showed decreased proliferation in response to cetuximab compared to vector-only controls, implicating that a percentage of these tumors could express EGFRvIII contributing to cetuximab resistance[118]. These reviews provide a further insight into the resistance to EGFR monoclonal antibody[119–122].

11.5.7 Bevacizumab

Vascular endothelial growth factor (VEGF) is the most potent angiogenic factor identified to date, and consist of 6 VEGF family members, of which VEGF-A has been the most extensively studied. The production of VEGF-A is stimulated by hypoxia-inducible factor-1α (HIF-1α) and epidermal growth factor (EGF) [123]. Members of the VEGF family exert their effect through binding to one of three VEGF receptors (VEGFR), which are localized predominantly on endothelial cells and angioblasts. Binding of VEGF to its receptor leads to activation of several intracellular signal transduction pathways[124]. In neoangiogenesis, the release of VEGF-A and other pro-angiogenic factors promotes degradation of the extracellular matrix allowing proliferation and migration of endothelial cells. The dependency of malignant tumors on neoangiogenesis suggests VEGF and the VEGF receptor as potential therapeutic targets. These agents may function to normalize the tumor vasculature, which in turn may improve delivery of concurrently administered chemotherapeutic drugs likely enhancing the sensitivity of the tumor to cytotoxic therapies through improved oxygen delivery[125]. Other possible mechanisms of action include inhibition of essential tumor blood supply or disruption of the perivascular-tumor stem cell niche.

Bevacizumab (Avastin, Genentech/Roche) is a humanized IgG1 anti-VEGF antibody whose presumed mechanism of anti-cancer action is thought to be by inhibiting blood vessel formation in growing cancers. By binding to VEGF, it prevents this ligand from binding to the VEGF receptor, preventing subsequent stimulation of downstream intracellular signal transduction pathways. Bevacizumab is approved, in combination with intravenous 5-FU chemotherapy,

for first- or second-line treatment of patients with metastatic carcinoma of the colon or rectum; in combination with carboplatin and paclitaxel for the first-line treatment of patients with unresectable, locally advanced, recurrent or metastatic non-squamous non-small cell lung cancer; and to treat metastatic renal cell carcinoma when combined with interferon-α.

11.5.7.1 Colorectal Cancer

Hurwitz, et al., conducted the first Phase III study of the effect of adding bevacizumab (5 mg/kg every 2 weeks) to irinotecan (125 mg/m^2) plus 5-FU bolus (500 mg/m^2) plus luecovorin (20 mg/m^2) s first-line treatment for metastatic colorectal cancer. The primary end point was median overall survival. The addition of bevacizumab was associated with an absolute benefit on median overall survival of 4.7 months compared with no added bevacizumab (20.3 vs. 15.6 months, respectively; $p < 0.001$)[126]. This was the pivotal trial that led to the FDA approval of bevacizumab in 2004 for use in combination with chemotherapy as first-line treatment for metastatic colorectal cancer. Since then, benefit for adding bevacizumab to a variety of irinotecan and oxaliplatin-containing regimens used for first and second-line therapy for metastatic colorectal cancer has been shown in large clinical studies[127–129]. The most common grade 3–4 adverse events reported with the use of bevacizumab in the clinical studies include hypertension (~11–16.4 %), bleeding (~2–5 %), gastrointestinal perforation (~1–2 %), venous thromboembolic events (~4–19.4 %) and rarely proteinuria (~1 %).

In the US, based on the significant difference in outcome favoring FOLFOX over bolus irinotecan/5-FU/leucovorin reported shortly after approval of bevacizumab by the US Intergroup N9741 trial[130], many US oncologists choose FOLFOX as the chemotherapy backbone for the addition of bevacizumab. Bevacizumab also appears to provide a survival benefit even in the absence of an objective response to therapy. In the pivotal trial by Hurwitz, *et al*., the benefit of the addition of bevacizumab to irinotecan/5-FU/leucovorin had a more profound impact on progression-free survival and overall survival compared to its effect on response rates. Further analysis of data from clinical studies that utilize bevacizumab show that patients who were thought to be non-responders benefited as much from the addition of bevacizumab as did those who had an objective response[131]. This phenomenon has been shown across other clinical studies in different tumor types that utilize agents that inhibit the VEGF pathway and it serves to underscore the relative lack of importance of objective response rate as a predictor of treatment benefit in patients undergoing palliative treatment in particular with VEGF-targeted agents[132–134].

11.5.7.2 Lung Cancer

In non-small cell lung cancer, high levels of VEGF expression are associated with a poor prognosis, suggesting that treatment targeted toward this pathway might be

useful therapeutically. In 2006, the FDA-approved bevacizumab for use in first-line advanced non-squamous non-small cell lung cancer in combination with carboplatin plus paclitaxel. A driving factor was results of a phase III trial demonstrating that median survival was significantly increased to 12.3 months in patients treated with standard carboplatin plus paclitaxel chemotherapy combined with bevacizumab at 15 mg/kg dose every 3 weeks vs. 10.3 months with chemotherapy alone (death hazard ratio, 0.79; $p = 0.003$). Bevacizumab was associated with significantly more bleeding (4.4 % vs. 0.7 % with chemotherapy alone; $p < 0.001$)[135]. Similarly, the AVAiL trial assessed bevacizumab at 7.5 mg/kg vs. 15 mg/kg every 3 weeks vs. placebo in combination with cisplatin and gemcitabine[136]. Progression-free survival or response rates were better with both doses of bevacizumab compared to placebo, but there was no demonstration of improved overall survival for the use of bevacizumab in combination with the chemotherapy. Both trials were limited to patients with non-squamous non-small cell lung cancer, with ECOG performance status zero or one, and excluded patients with brain metastases or a history of hemoptysis. In regards to the exclusion of brain metastasis patients, recent data have demonstrated the safety of utilizing bevacizumab in patients with previously treated brain metastases[137], although its cautious use in the elderly patients must be considered in view of the increase toxicity noted in this population[138].

11.5.7.3 Breast Cancer

The first large clinical study to show the benefit of bevacizumab in breast cancer was the E2100 study that enrolled 722 women (mostly HER2-negative) without prior treatment for metastatic breast cancer to receive bevacizumab (10 mg/kg on days 1 and 15) and paclitaxel (90 mg/m^2 days 1, 8, and 15 of every 28-day cycle) or paclitaxel alone[139]. The combination of bevacizumab and paclitaxel significantly increased the response rate (37 vs. 21%) and progression-free survival (the primary endpoint, 11.8 vs. 5.9 months), but there was no significant increase in median overall survival (26.7 vs. 25.2 months). Likewise, progression-free survival was increased when bevacizumab was combined with docetaxel (AVADO trial) and investigator-selected chemotherapy (either capecitabine, or a taxane-based, or an anthracycline-based chemotherapy) in the RIBBON-1 (first-line) and RIBBON-2 (second-line) trials, without improvement in overall survival[140–142]. As regards to safety, a pooled analysis of phase 3 studies showed that utilization of bevacizumab in metastatic breast cancer patients led to an increase the risk of left ventricular dysfunction and hemorrhagic events. The addition of bevacizumab to chemotherapy in patients with metastatic breast cancer however was not associated with a significant increase in grade $\geq$3 arterial or venous thromboembolic events, gastrointestinal perforation, or fatal events[143].

Preliminary FDA approval was granted for the combination of first line paclitaxel and bevacizumab in metastatic breast cancer based on the E2100 trial. However, in November 2011, the FDA revoked this approval because no study

has shown an improved overall survival advantage when bevacizumab was combined with chemotherapy. Despite this FDA decision, there is still evidence to support the use of the combination of bevacizumab and weekly paclitaxel in HER2-negative metastatic breast cancer given the improvement in progression-free survival and a trend towards improved overall survival benefit. Currently there are no predictive markers for response to bevacizumab, although potential predictive role of proteins involved in the angiogenic pathway such for high plasma VEGF-A levels are currently being explored[144] .

11.5.7.4 Glioblastoma Multiforme

In May 2009 the FDA granted approval for bevacizumab to treat recurrent glioblastoma multiforme under its accelerated approval authority. It shows activity over conventional treatments vs. historical controls and could mitigate clinical effects of radiation necrosis, possibly through inhibiting blood vessel formation and hence consequent edema formation. A phase I clinical trial suggested that direct intra-arterial bevacizumab injection after selective blood brain barrier disruption with mannitol followed by systemic bevacizumab could be superior to systemic administration alone or with chemotherapy in 14 patients with glioblastoma multiforme recurring following temozolamide plus cranial irradiation[145]. In a further non-comparative phase II study of 167 patients with recurrent glioblastoma multiforme that were randomly assigned to bevacizumab (10 mg/kg), either as a single agent or at this dose in conjunction with irinotecan. Treatment cycles were repeated every 2 weeks. All patients had received prior chemotherapy with temozolomide which was standard of care. The objective response rates with bevacizumab alone or in combination with irinotecan were 28% and 38%, respectively, and the 6-month progression-free survival rates and overall survival were 43% and 50%, and 9.2 months and 8.7 months, respectively[146]. Treatment with bevacizumab or bevacizumab plus irinotecan was generally well tolerated, and toxicity was limited to that expected with these agents. A more comprehensive review of the clinical use of bevacizumab in glioblastoma multiforme was recently reviewed[147].

11.5.7.5 Renal Cell Carcinoma

The European Union approved bevacizumab to treat renal cell carcinoma in 2007. In 2009, bevacizumab received an FDA indication for treatment of metastatic renal cell carcinoma based on its ability to extend progression-free survival. Approval was based on two large phase 3 studies. In the AVOREN trial, 649 previously untreated patients were randomly assigned to interferon-α, nine million units three times per week for 1 year plus either bevacizumab (10 mg/kg every 2 weeks) or placebo. The bevacizumab or placebo was continued until there was evidence of progressive disease[148]. In this study the combination therapy resulted in a

significant prolongation of median progression-free survival (10.2 vs. 5.5 months), a significantly increased objective response rate (31 vs. 13%) and a statistically nonsignificant trend toward improved survival (median survival 23.3 vs. 21.3 months). Approximately 60% of patients went on to receive second-line therapy, potentially obscuring a survival difference between the two treatment arms. In the Cancer and Leukemia Group B (CALGB) trial 90206, 732 previously untreated patients with metastatic renal cell carcinoma were randomly assigned to either interferon-α plus bevacizumab or interferon-α plus placebo on schedules similar to that used in the AVOREN trial[149]. Again the median progression-free survival was significantly increased in patients treated with the bevacizumab plus interferon-α regimen (8.5 vs. 5.2 months), and again as the AVOREN study a trend toward improved overall survival (median 18.3 vs. 17.4 months) which would have been significant and difficult to analyze fully by the fact that more than one-half of patients on both arms received second-line therapy, including VEGF-targeted therapy in 46% of those originally treated with interferon-α alone.

11.5.7.6 Investigational and Other Potential Uses

Bevacizumab is among the most studied immunologic agents for cancer. It has shown potential clinical efficacy in selected neuroendocrine tumors[150] and advanced epithelial ovarian cancers[151] but not in pancreatic cancer[152]. A large variety of other drug combinations in other settings have also been tested, with mixed results. Since VEGF can hinder dendritic cell maturation and promote regulatory T cell generation, anti-VEGF agents, including have been tested as immune modulators in cancer, discussed in more detail in Chaps. 9 and 13.

11.5.7.7 Resistance to Anti-VEGF Therapy

Despite the initial benefit seen in some patients treated with VEGF pathway inhibitors, complete responses have not generally been documented and most patients will progress and succumb to their disease. The initial or eventual failure of VEGF-targeted therapy suggests that mechanisms of *inherent* and *acquired* resistance play a role in the progression of disease in patients treated with these agents. There is some activity with the VEGF tyrosine kinase inhibitors as a monotherapy in renal cell carcinoma and hepatocellular carcinoma where resistance is thought to be primarily related to the VEGF pathway. In colorectal cancer, non-small cell lung cancer, and breast cancer, VEGF-targeted therapy with bevacizumab where administration is with various cytotoxic regimens, resistance is likely to be more complex.

Resistance to bevacizumab can include use utilization of alternative or compensatory angiogenesis mechanisms. The fibroblast growth factor family of ligands was the first resistance mechanism identified. Hanahan and colleagues showed that treatment with an anti-VEGF R2 monoclonal antibody was associated with a

decrease in vascular density after 10 days of therapy. However, these investigators noted an angiogenic rebound in tumors at 4 weeks that was associated with an increase in expression of redundant angiogenic factors, including members of the fibroblast growth factor family. By using a fibroblast growth factor-trap, these investigators showed that blocking fibroblast growth factor signaling minimized the acquired resistance to VEGF-targeted therapy and decreased tumor burden[153]. These studies were supported clinically showing an increase in the circulating levels of basic fibroblast growth factor when tumors progressed on VEGF-targeted therapy[154].

More recently, the cell membrane bound notch ligand/receptor system has been proposed as a resistance pathway for anti-VEGF therapy[155]. The activation of notch signaling by one of the five notch ligands can complement VEGF signaling by contributing to a more mature tumor vasculature network. Inhibition of notch signaling with an anti-DLL4 antibody can block notch signaling, which paradoxically leads to an increase in vessel count. However as the function of these newly developed vessels is poor, the overall result is a paradoxical decrease in tumor tissue perfusion.

In both preclinical studies and clinical trials, placenta growth factor is increased in plasma following blockade of VEGF signaling. Inhibition of placenta growth factor using monoclonal antibodies showed that both VEGF-sensitive and VEGF-resistant tumors respond to placenta growth factor neutralization[156]. One interesting aspect of this study was the fact that antibodies to placenta growth factor can inhibit recruitment of macrophages that are thought to play an important role in contributing to the angiogenesis or possibly have an indirect effect in the tumor microenvironment. Anti-placenta growth factor therapy also results in a reduced hypoxic response with less induction of compensatory angiogenic mediators.

Pericytes play an important role in providing survival signals to endothelial cells and mediate blood flow and endothelial cell permeability[157]. In addition, pericytes secrete paracrine factors that mediate endothelial cell survival signaling via Akt activation[158]. The migration and proliferation of pericytes is predominantly mediated by platelet derived growth factor (PDGF)-BB secreted primarily by endothelial cells, interacting with the PDGF-β receptor on pericytes[158]. Targeting both pericytes and endothelial cells (e.g., with PDGF-R and VEGFR inhibitors) leads to greater efficacy than either agent alone[159]. Due to the homology between VEGFRs and PDGFRs, many kinase inhibitors targeting VEGFRs also inhibit PDGFR signaling. Finally, it has been recently shown that tumor-derived PDGF can stimulate secretion of fibroblast growth factors from cancer-associated fibroblasts, leading to further angiogenic support, again highlighting the importance of the microenvironment in regulating tumor angiogenesis, and providing additional targets in attempts to abrogate inherent or acquired resistance[160]. The clinical benefit of multiple targeting of the vasculature (PDGFR, VEGFR, or fibroblast growth factor inhibition) remains to be determined. Some investigators are attempting to use anti-angiogenesis agents that target multiple angiogenic pathways simultaneously[161]. It is too early to know how effective such strategies will be.

11.5.8 Natalizumab

Natalizumab (Tysabri, Biogen IDEC and Elan) is a humanized monoclonal IgG4 α4-integrin antibody. This integrin is important in lymphocyte trafficking, and a purported mechanism of action is prevention of activated immune cells from reaching target organs in autoimmune diseases. Its mechanisms of action in cancer are less clear. It was approved in 2004 by the FDA to treat multiple sclerosis and Crohn's disease. It was withdrawn by the manufacturer following reports that when combined with interferon-β for multiple sclerosis it could cause progressive multifocal leukoencephalopathy, a usually exceedingly rare neurological disorder that can be fatal. It returned to the market in the United States in 2006 for treatment of autoimmune disorders following additional safety evaluations and the determination that the clinical benefits in some cases outweighed the risk for progressive multifocal leukoencephalopathy. In the European Union, natalizumab is approved for as monotherapy for multiple sclerosis. It has preclinical efficacy in some hematologic malignancies or their management[162,163], and is currently in clinical trials by Biogen as treatment for certain T cell leukemias/lymphomas.

11.5.9 Denosumab

Denosumab (Prolia, Xgeva; Amgen) is a fully human IgG2 anti-RANK ligand monoclonal antibody.

It received FDA approval in June 2010 to reduce post-menopausal osteoporosis risk (under the trade name Prolia) and thus became the first FDA-approved anti-RANL ligand agent. It received FDA approval to reduce the risk of skeleton-related events (fractures and pain) in patients with solid cancers and bone metastases in November 2010 under the trade name Xgeva.

Danosumab binds to RANK (receptor activator of nuclear factor-kappa B) ligand on osteoblasts in bone, and prevents their maturation and activation from RANK interactions on local osteoblasts. Reduced osteoclast activation reduces their capacity to degrade bone and helps preserve bone integrity, by tipping bone remodeling in favor of net new bone deposition[164,165]. It is currently in clinical trials to treat giant cell tumors, multiple myeloma with bone metastases, and hypercalcemia of malignancy. It could have anti-cancer activity in breast cancer[164,165].

11.5.10 Pertuzumab

The concept of combining two HER2-targeted agent to overcome possible resistance to trastuzumab led to a trial in patients with HER2-positive metastatic breast

cancer who had never gotten anti-HER2 therapy or chemotherapy previously. Pertuzumab (Perjeta, Hoffmann-La Roche/Genentech) is a recombinant humanized monoclonal antibody targeting the extracellular dimerization domain of HER2, thereby blocking ligand-dependent HER2 heterodimerization with other HER family members. manized that can prolong survival in metastatic breast cancer when combined with trastuzumab plus docetaxel[166]. 808 patients were treated with either pertuzumab or placebo in combination with trastuzumab plus docetaxel. Pertuzumab significantly increased median progression-free survival vs. placebo by 6.1 months (hazard ratio 0.62, $p < 0.0001$). Median progression-free and overall survival was also increased ($p = 0.0053$ for overall survival). Common side effects of pertuzumab in combination with trastuzumab plus docetaxel were diarrhea, alopecia, neutropenia, nausea, fatigue, rash, and peripheral neuropathy. Data were presented at the 2012 American Society for Clinical Oncology Meeting followed by FDA approval on June 8, 2012.

11.5.11 Ipilimumab

This anti-CTLA-4 blocking monoclonal antibody, and other antibodies directed against immune co-signaling molecules are discussed in great detail in chap. 8.

11.6 Promising Antibodies in Clinical Trials

The following are examples of promising approaches to antibody-mediated strategies to treat cancer that have progressed to human clinical trials. These agents are summarized in Table 11.2.

11.6.1 Anti-Chemokine Receptor and Anti-Chemokine Antibodies

11.6.1.1 Mogamulizumab (Anti-CCR4)

We previously demonstrated that regulatory T cells can migrate to the tumor microenvironment in ovarian cancer through their CCR4 expression in conjunction with microenvironmental CCL22[167]. Because some T cell leukemia/lymphoma cells behave like regulatory T cells and express CCR4, an anti-CCR4 monoclonal antibody was developed that could potentially directly kill $CCR4^+$ malignant cells, and reduce $CCR4^+$ regulatory T cell trafficking in T cell malignancies[168]. The novel, humanized anti-CCR4 IgG1 antibody KW-0761 is defucosylated to augment its potential to mediate antibody-dependent cellular cytotoxicity. In a phase I study of KW-0761 in 16 patients with relapsed $CCR4^+$ adult T-cell leukemia-lymphoma or

Table 11.2 Summary of therapeutic antibodies and related molecules now in clinical trials for cancer, including those agents already approved for non-cancer indications

Generic name Trade name	Class	Target	Trial phase	References
Mogamulizumab (KW-0761)	Defucosylated, humanized IgG1 monoclonal	CCR4 (chemokine receptor 4)	Several phase II trials, with further development planned.	168–171
Oregovomab	Murine IgG1 monoclonal	CA-125	Failed its pivotal phase III trial with uncertain future.	177–179
Farletuzumab	Humanized monoclonal	Folate receptor	Two phase III trials in ovarian cancer are in progress.	180,181
AS1402 (formerly R1550)	Humanized IgG1 monoclonal	PDTR sequences in the MUC1 core tandem repeat	Several phase I and one failed phase II trial (combined with letrozole) for breast cancer.	182,183,185,186
Volociximab	Chimeric IgG4 monoclonal	AAB1 (a component of α5β1 integrin)	Phase III trial for ovarian cancer in progress.	187–190
Mapatumumab. (HGS-ETR1, TRM-1)	Fully human monoclonal	TNF-related apoptosis-inducing ligand receptor-1	Several phase II completed with promising results in hematologic malignancies. Further studies planned.	191–194
Etaracizumab Abegrin	IgG1 humanized monoclonal	αvβ3 integrin	Ongoing phase I and phase II trials.	195,196
3H1	Monoclonal anti-idiotype	CEA (carcinoembryonic antigen) idiotype	Promising phase III trial in colorectal cancer plus 5-FU/leucovorin completed. Further studies planned.	197–200
Adecatumumab.	Human IgG1 monoclonal	EpCAM (epithelial cellular adhesion molecule)	Phase IB trial completed in breast cancer.	201
MORAb-009	Chimeric monoclonal	Mesothelin	Phase II trial ongoing.	202,203
Sibrotuzumab	Humanized monoclonal	Human fibroblast activation protein	Phase I trial completed.	204,205
Infliximab[a] Remicade	Chimeric IgG1 monoclonal	TNF-α	In phase I for various solid tumors alone and in phase III trials combined with celecoxib and anti-IL-6 for cancer cachexia	206 208–210
Anakinra[a] Kineret	Recombinant protein	IL-1β	In phase I trials for various solid tumors	207
Anti-IL-6	Monoclonal	IL-6	In phase I trials in prostate cancer and ovarian cancer	206
Daclizumab[a] Zenapax	Humanized IgG1 monoclonal	CD25 (IL-2 receptor α chain)	Phase I trial in breast cancer plus vaccine finished.	211
Etanercept[a] Enbrel	Soluble TNF receptor 2 fusion protein	TNF-α	In phase I trials in cancer	212

[a]FDA-approved for a non-cancer indication.
Monoclonal monoclonal antibody; *TNF* tumor necrosis factor.

peripheral T cell lymphoma, it was found to be well-tolerated, and a phase II dose was identified[169]. Initial clinical efficacy was subsequently demonstrated in early human clinical trials, and development was furthered by Amgen where KW-0761 was renamed AMG-761 or mogamulizumab[170]. In a phase II trial of 28 patients with relapsed $CCR4^+$ adult T cell leukemia, KW-0761 produced objective responses in 13 of 26 evaluable patients, including eight complete responses, an overall response rate of 50 %. The toxicity profile was acceptable and has led to further clinical development in adult T cell leukemia and other T cell neoplasms[171].

11.6.1.2 Anti-CCL2 (preclinical)

The chemokine CCL2 is also under active investigation in novel anti-cancer treatment strategies. Anti-CCL2 or anti-CCR2 (CCL2 receptor) antibodies have been studied in preclinical models in prostate cancer. In one study, anti-CCL2 antibody treatments reduced tumor burden, bone loss, and vascular endothelial growth factor concentration[172]. In another preclinical prostate cancer model, anti-CCL2 antibody treatments improved the efficacy of docetaxel chemotherapy to reduce bone metastases[173]. CCL2 produced by breast cancer cells and associated stroma recruits CCR2-expressing monocytes that facilitate the metastatic process. Neutralizing CCL2 with anti-CC2 antibody reduced metastases and prolonged survival in a preclinical model of breast cancer metastases[174]. Based on supporting data such as these, anti-CCL2 antibodies have entered human clinical trials in prostate cancer and ovarian cancer[175].

11.6.1.3 Anti-CCL22 (preclinical)

We identified the chemokine CCL22 as important to attraction of regulatory T cells into the tumor microenvironment[167] and demonstrated that anti-CCR4 antibody (blocking the CCL22 receptor) reduced regulatory T cell trafficking into human ovarian cancer xenografts, improving adoptively T cell transfer therapy (W. Zou, T. Curiel, unpublished data). Further studies are needed in this regard. Please see Chap. 12 for additional anti-chemokine/chemokine receptor strategies.

11.6.2 Anti-CA-125

The tumor-associated antigen CA-125 is used to monitor treatment responses in patients with ovarian cancer. CA-125 was targeted in vivo by the murine monoclonal antibody oregovomab. This antigen–antibody complex could prime dendritic cells[176] thereby activating T cells[177] and underwent a phase III clinical trial[178]. In a pivotal phase III study of 373 ovarian cancer patients, oregovomab maintenance was used after front-line therapy to test if time to progression could be extended,

where no difference in clinical outcome was identified, although treatment was well tolerated. The future for this monoclonal antibody is uncertain, but because of its potential to induce anti-tumor immunity, it could be tested in an immune-boosting strategy in combination with other agents[179].

11.6.3 Farletuzumab

Folate receptor-α is a membrane marker for ovarian cancer and is frequently overexpressed in other major epithelial cancers. It is not well expressed in normal tissue, making it an attractive drug development target in cancer. Farletuzumab (MORAb-003) is a humanized monoclonal anti-folate receptor antibody in clinical trials[180]. A phase I trial demonstrated pharmacokinetics and tolerability. Another phase I trial showed that farletuzumab plus carboplatin was safe and tolerable in ovarian cancer patients with platinum-sensitive disease in first or second relapse. A phase II study of farletuzumab combined with carboplatin plus a taxane to treat ovarian cancer patients with platinum-sensitive disease in first relapse demonstrated improved clinical responses and time to progression vs. historical controls. Two randomized, double-blind, placebo-controlled phase III trials are testing farletuzumab plus either paclitaxel in one trial or carboplatin plus a taxane in the other, both in patients with platinum-sensitive ovarian cancer[181]. Results are pending.

11.6.4 AS1402 (Formerly R1550)

MUC1 is a cell-surface glycoprotein. A 20-amino-acid tandem repeat in the MUC1 core protein is overexpressed and abnormally glycosylated in most epithelial malignancies. These alterations in its glycosylation can affect cell growth, differentiation, transformation, adhesion, invasion, and immune surveillance and are associated with development of cancer[182,183]. In a phase III trial of yttrium-90-labeled murine HMFG1 ((90)Y-muHMFG1) to treat ovarian cancer, development of IgG anti-MUC1 antibodies correlated with improved survival, suggesting that immunotherapy against MUC1 could be useful to treat epithelial ovarian cancer and perhaps other cancers[184].

AS1402 is a humanized IgG1 monoclonal antibody that targets PDTR sequences in the MUC1 core tandem repeat not generally expressed in normal cells. AS1402 induces antibody-dependent cellular cytotoxicity that is specific for MUC1-expressing tumors. In a phase I trial of 26 women with locally advanced or metastatic $MUC1^+$ breast cancer that failed first-line therapy, AS1402 was generally well tolerated and led to an ongoing phase II trial[182].

MUC1 is overexpressed in ~90 % of breast tumors and modulates estrogen receptor activity that contributes to the estrogen-mediated growth and survival of breast cancer cells in vitro. Aromatase inhibitors can enhance antibody-dependent

cell-mediated cytotoxicity in in vitro studies. A phase II trial tested 110 patients with locally advanced or metastatic breast cancer with hormone receptor-positive tumors randomized for treatment with the hormonal agent letrozole alone or combined with AS1402 treatment. The trial was halted early due to evidence for worse responses and faster progression with AS1402 plus letrozole. The final trial data analysis showed no significant efficacy differences, so additional trials could be possible[185].

In a trial of 31 early stage breast cancer patients, oxidized mannan-MUC1 was tested in stage II breast cancer patients without evident disease. They were treated with subcutaneous injections of either placebo or oxidized mannan-MUC1 to immunize against MUC1 and prevent cancer reoccurrence, including metastases. In 5.5 years or more of follow up, recurrence in the placebo group was 27 % vs. 0 of 16 in the treated group ($P = 0.03$). In treated patients, 9 of 13 patients developed anti-MUC1 antibodies and 4 of 10 developed MUC1-specific T cell responses vs. none for either measure in the placebo group. The authors concluded that a phase III should be undertaken based on these results[186].

11.6.5 Volociximab

Volociximab is a chimeric anti-AAB1 (a component of α5β1 integrin) IgG4 monoclonal antibody under development by PDL Biopharma, Inc., and Biogen Idec Inc. Volociximab inhibits tumor neoangiogenesis in mouse cancer models by interrupting α5β1/fibronectin interactions and has proven relatively safe and tolerable in phase I trials.

A phase II trial tested single-agent efficacy in 16 patients with platinum-resistant, advanced stage epithelial ovarian cancers and primary peritoneal cancinomatosis. Clinical efficacy was not established, but tolerability was confirmed[187]. Despite uncertainties regarding efficacy, a phase III trial has begun[188]. Currently active trials are ongoing to test efficacy alone or combined with cytotoxic chemotherapy in different solid tumors, where earlier phase trials found promising activity[189].

Incidentally, volociximab is also undergoing phase II clinical trials for age-related macular degeneration[190].

11.6.6 Mapatumumab

Mapatumumab (HGS-ETR1, TRM-1) is a fully human anti-tumor necrosis factor-related apoptosis-inducing ligand receptor-1 (TRAIL-R1) agonist monoclonal antibody. In a phase I trial, 49 patients with advanced solid malignancies were treated in whom TRAIL-R1 expression was documented in 68 % of tumors. Nineteen patients experienced stable disease, and two of these lasted 9 months[191].

Another phase I study tested mapatumumab combined with paclitaxel plus carboplatin in 21 patients with advanced stage solid cancers. Mapatumumab was well-tolerated with no apparent pharmacokinetic effects on cytotoxic drugs. Anti-cancer effects were seen in most patients[192].

A phase 1b/2 trial evaluated mapatumumab in 40 patients with relapsed non-Hodgkin lymphoma. Treatment was well tolerated. Three patients with follicular lymphoma had clinical responses, including two with complete responses. Strong tumor staining for (TRAIL-R1) (the mapatumumab target) did not correlate with clinical activity[193].

A phase II trial evaluated single-agent mapatumumab in 38 colorectal cancer patients failing standard cytotoxic chemotherapy. No meaningful clinical responses were noted, but 12 patients experienced stable disease (median 2.6 months). Despite lack of demonstrable clinical activity, further evaluations were considered useful to be tested in combination with other agents[194].

11.6.7 Etaracizumab

αvβ3 integrin helps regulate intracellular signaling, cell proliferation, migration, differentiation, and tumor-mediated angiogenesis[195]. Etaracizumab (Abegrin) is an IgG1 humanized anti-αvβ3 integrin monoclonal antibody. In a phase I trial, 16 patients with advanced solid malignancies received escalating etaracizumab doses. There were no objective clinical tumor reductions, but five patients had disease stabilization lasting over 6 months. Etaracizumab was well-tolerated with no evidence for immunogenicity[196].

Etaracizumab was tested in 112 patients with previously untreated metastatic melanoma alone or with the cytotoxic agent dacarbazine. Survival was similar in both treatment arms suggesting that further pursuit of this combination was not likely to be beneficial[195]. Additional phase I and phase II trials are ongoing[196].

11.6.8 Anti-CEA (Carcinoembryonic Antigen)

3 H1 is a monoclonal anti-idiotype antibody whose antigen recognition site mimics carcinoembryonic antigen. In a phase III trial of 630 patients with previously untreated metastatic colorectal cancer, standard 5-flourouracil plus leucovorin was tested alone or in combination with 3H1. Anti-CEA antibodies were generated in 70 % of patients receiving 3H1. Treatment was well tolerated, and appeared to extend life in responders (median survival 8.3 months in non-responders vs. median survival not reached in responders; $P < 0.001$). Further studies of this antibody are planned[197]. Several phase I trials have tested radiolabeled anti-CEA antibodies[198,199]. Lametuzumab is an anti-CEA antibody conjugated to a toxin[200].

11.6.9 Adecatumumab

Adecatumumab is a human IgG1 anti-EpCAM (an epithelial cell adhesion molecule) antibody. It was tested in an open-label phase IB dose-escalation trial in breast cancer patients in combination with docetaxel. In 31 evaluable patients, clinical responses were seen in 44 % overall and in 63 % of patients whose tumors expressed high-level EpCAM, suggesting potential utility[201].

11.6.10 Anti-Mesothelin

Mesothelin is a tumor differentiation antigen over-expressed in a variety of cancers including those of pancreas, ovary and mesothelium[202]. MORAb-009 is a chimeric anti-mesothelin monoclonal antibody used in a phase I trial of 24 patients with mesothelin-expressing tumors of pancreas, mesothelium or ovaries. Eleven subjects experienced stable disease prompting an ongoing phase II trial[203].

11.6.11 Sibrotuzumab

Fibroblast activation protein is an immunopathogenic protein in cancer[204]. Sibrotuzumab is a humanized anti-against human fibroblast activation protein. A Phase I trial tested 26 patients (20 with colorectal carcinoma and 6 patients with non-small cell lung cancer), all likely to be FAP positive. There were no objective tumor responses but two patients had disease stabilization[205].

11.6.12 Antibodies Developed for Other Indications

Infliximab is an anti-TNF-α antibody FDA-approved to treat certain autoimmune disorders. It has been used in clinical trials in cancer where it stabilized disease in some patients with advanced cancers progressing on conventional treatments. An anti-IL-6 antibody was tested in prostate cancer and ovarian cancer without clear evidence for clinical efficacy although immune modulation was observed (reviewed in 206). Anakinra is an IL-1 receptor antagonist. It reduced disease progression in smoldering multiple myeloma[207]. A number of anti-inflammatory approaches, including celecoxib, anti-TNFα and anti-IL-6 have been used to try to treat cancer cachexia, but with limited success thus far[208], including in two recently-completed phase III clinical trials[209,210]. Daclizumab is an anti-CD25 antibody FDA-approved in organ transplantation. It was used in a clinical of a breast cancer vaccine to deplete regulatory T cells[211]. Clinical efficacy was not

reported, but it did reduce and alter the phenotype of the regulatory T cells. Etanercept is a soluble TNFR2 fusion protein that neutralizes TNF-α by direct binding to it. It stabilized disease in a minority of patients in an early stage clinical trial (reviewed in 212).

11.7 Summary

Therapeutic antibodies are the most successful class of anti-cancer immune-based therapeutic agents to date. These agents as a class are generally well tolerated with a good side effect and safety profile. A very large variety of additional agents are in phase I trials against a range of targets, including those expressed by the tumor, its stroma and immune cells. The technology has advanced to the point where a number of highly humanized or fully human antibodies are being produced and tested, reducing issues related to unwanted immunogenicity. Some antibodies work through inducing complement or antibody-dependent tumor cytotoxicity. Current technology allows engineering this functionality into some antibodies. Antibodies are also useful in immunoconjugates to deliver toxins or for diagnostic purposes (discussed in Chap. 12).

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Chapter 12
Targeted Toxins in Cancer Immunotherapy

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Abstract Many anticancer agents are toxic to normal tissues. Thus, the potential to target treatments specifically to tumors would minimize the effects on normal tissues and afford a better safety profile compared to nontargeted treatments. Harnessing immune specificity has allowed the successful development of targeted anticancer agents. Successful targeting strategies include antibodies and derivatives, cytokines, peptides, and recombinant viruses. This chapter will summarize the current agents and strategies that exploit immune principles and reagents to develop targeting specificity to treat cancers with maximum sparing of normal tissues.

12.1 Therapeutic Immunoconjugates

12.1.1 Introduction

An optimal therapeutic agent will target a specific molecule expressed only on the surface of cancer cells. This unique expression rarely exists, and thus, there must be a trade-off in specificity of binding versus agent efficacy. Therapeutic immuno-conjugates deliver toxic cargoes specifically to tumor cells via their targeting moiety. The prototype for such an agent is an antibody conjugated to a moiety toxic to the

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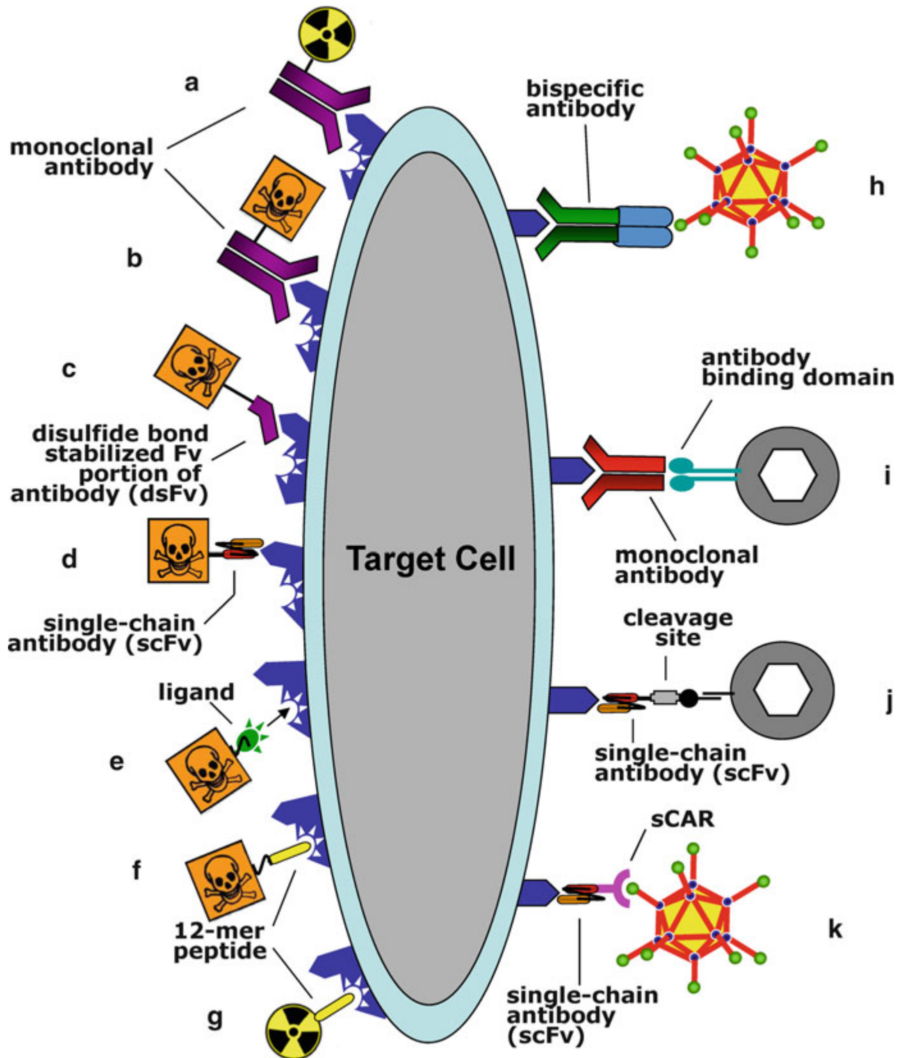

Fig. 12.1 Schematic showing novel targeting mechanisms of immunoconjugates and ligand-receptor based agents. Antibody and ligand-based agents are shown on the left side while viral delivery vehicles are shown on the right side of the figure. Ligands, receptors, and antibodies with identical color schemes indicate that they could theoretically be derived from the same molecule. Black lines represent linker regions.

target cell (Fig. 12.1a). Such toxic moieties include recombinant cytotoxic proteins[1–4], cytotoxic drugs,[5–8], ribonucleases[9], small inhibitory RNAs[10, 11], or radionuclides[12] (Fig. 12.1a, b).

Some immunoconjugates employ whole antibody or single-chain antibody fragments rather than the intact antibody. One immunoconjugate class of this type employs the disulfide-bond-stabilized Fv moiety of an antibody conjugated

to the toxic cargo[1] (Fig. 12.1c). A variation of this approach uses an unmodified single-chain antibody fragment[13] (scFv), that is, a linear configuration of the variable antibody heavy and light chains fused by a short peptide linker, conjugated to the toxic cargo (Fig. 12.1d).

Many immunoconjugates have been designed using rationally selected alternatives to antibodies or antibody derivatives. These newer molecules target tissues using a cell surface receptor-specific binding ligand (Fig. 12.1e). Such binding ligands use immune system molecules with known binding specificities such as interleukins but, in addition, employ novel binding agents identified experimentally through screening of phage display libraries[14] (Fig. 12.1f, g). Virus and virus-based delivery vehicles have also been tested that use immunoconjugate-based concepts to target toxic agents to specific cells or tissues[15] (Fig. 12.1h–k).

12.2 Targeting Toxins to Cancer Cells via Specific Cell Surface Antigens

12.2.1 Immunoconjugates Targeting CD22

One of the earliest and successful uses of immunoconjugates was in the treatment of non-Hodgkin lymphoma. A CD22-targeted monoclonal antibody chemically fused to deglycosylated ricin toxin A[16] induced cell death by ricin-mediated ribosomal RNA cleavage and inhibition of protein synthesis[17, 18]. This approach was clinically effective with complete responses in 2 and partial responses in 10 of 41 patients.[19, 20] However, toxicity of vascular leak limited further clinical development. To attempt to reduce vascular leak, the ricin toxin moiety was modified by converting asparagine-97 to alanine in its A-chain. This modification significantly reduced toxicity but also reduced antitumor activity[21].

The next approach using anti-CD22 immunoconjugates is exemplified by BL22. BL22 is a genetic fusion of the disulfide-bond-stabilized Fv (dsFv) moiety of anti-CD22 antibody and the catalytic and translocation domains of *Pseudomonas aeruginosa* exotoxin A. This conjugate effectively redirected the cytotoxic moiety to CD22-expressing cells. The exotoxin ADP-ribosylates histidine-699 on elongation factor-2, leading to cell death[22]. Individual plasmids expressing the two distinct dsFv chains, one of which is fused to the exotoxin, are co-expressed in *E. coli*. They are then purified and covalently conjugated to BL22 to form the full immunoconjugate (see Fig. 12.1c). BL22 was shown to be efficacious in patients with hematologic malignancies (leukemias and lymphomas), effecting complete responses in 19 of 46 patients and partial responses in 7 additional patients[23–25].

Using peptides identified from screening an antibody phage display library, BL22 was further modified to produce an immunoconjugate with CD22 affinity that was ten times more potent than the parent compound[26]. This modified BL22 immunoconjugate is currently undergoing a phase I clinical trial, while the original drug is the subject of a phase II clinical trial[25]. Their immunoconjugates using

distinct CD22-targeting epitopes have been conjugated to saponin, a toxin that inactivates ribosomes[27], but these agents have not been clinically developed.

Inotuzumab ozogamicin is a humanized anti-CD22 monoclonal antibody (clone CMC-544) covalently conjugated to the DNA-damaging agent calicheamicin. This immunoconjugate has shown significant potential for treatment of acute lymphoblastic leukemia and specific B cell lymphomas[28, 29] and has been tested in combination with the anti-CD20 antibody rituximab[30]. A recently completed phase II clinical trial continues to demonstrate the clinical efficacy of this immunoconjugate in refractory and relapsed acute lymphocytic leukemia[31].

Epratuzumab-SN-28, a humanized anti-CD22 antibody, has successfully been conjugated with the topoisomerase I inhibitor SN-28. This immunoconjugate employs a novel linker that allows 50 % of the IgG-bound SN-38 to dissociate in serum every 24 h and has shown encouraging preclinical results[32].

12.2.2 Immunoconjugates Targeting CD19

Many phase I clinical trials using CD19-targeting agents for CD19-expressing tumors have achieved modest to poor clinical efficacy. It is difficult to judge the efficacy of anti-CD19 agents as these studies used different monoclonal antibodies conjugated to distinct toxic moieties. The B4 anti-CD19 antibody clone chemically conjugated to a blocked ricin toxin, where the ricin oligosaccharide-binding sites were modified with glycopeptides comprised of specific N-linked oligosaccharides[33], effected no clinical response in 16 patients in a phase II trial for non-Hodgkin lymphoma[34]. The HD37 anti-CD19 antibody clone was chemically conjugated to blocked ricin and tested in a phase I clinical trial. There were only 2 partial responses in 22 subjects, with vascular leak, seen with previous related agents, noted[19, 20, 35]. The B43 anti-CD19 antibody clone was chemically conjugated to pokeweed antiviral protein, a toxin that inactivates ribosomes. This immunoconjugate failed to elicit significant clinical response[36]. Similarly, in severe combined immunodeficient mice xenografted with human B cell precursor acute lymphoblastic leukemia cells, this immunoconjugate failed as a single agent, but showed efficacy when combined with vincristine, methylprednisolone, and L-asparaginase[36], cytotoxic drugs used to treat this malignancy.

12.2.3 Immunoconjugates Targeting CD30

Brentuximab vedotin (SGN-35; Adcetris) is a chimeric IgG1 anti-CD30 antibody conjugated to the antimicrotubule agent, monomethyl auristatin E[37]. In phase I studies, this immunoconjugate demonstrated significant clinical activity in patients with relapsed or refractory CD30-expressing lymphomas with tolerable toxicities[38]. In a phase II study of 102 patients with relapsed or refractory Hodgkin lymphoma who failed prior treatments, the overall response rate was 75 %. Impressively, 34 % of these patients, considered to be highly refractory, achieved complete remission[39].

Based on such results, brentuximab vedotin was approved by the FDA in August 2011. This compound is now used for the treatment of Hodgkin lymphoma in patients with failed autologous stem cell transplant, at least two failed multiagent chemotherapy treatments, for transplant-ineligible patients and in the treatment of systemic anaplastic large cell lymphoma patients that failed multiagent chemotherapy[40, 41].

In a phase I clinical trial, anti-CD30 monoclonal antibody conjugated to blocked ricin elicited a partial clinical response in 1 of 15 patients and was not pursued further[42]. A different anti-CD30 monoclonal antibody clone, Ber-H2, conjugated to saponin was tested but was also found to be ineffective[43].

12.2.4 The Case of the Anti-CD33 Immunoconjugate Gemtuzumab Ozogamicin

Gemtuzumab ozogamicin was produced by chemically conjugating the DNA-damaging agent calicheamicin to a humanized anti-CD33 antibody[44, 45]. This immunoconjugate (Mylotarg; Wyeth) received approval through the FDA accelerated approval program on May 17, 2000, for treatment of older patients with acute myeloid leukemia in relapse and those unsuitable for chemotherapy[8]. This was the first antibody-toxin immunoconjugate to gain FDA approval. In June 2010, Wyeth voluntarily withdrew this agent from the market following analysis of results from SWOG S0106, a phase III clinical trial that demonstrated increased treatment-related deaths with no clear benefit over conventional treatments[46, 47]. This withdrawal caused a reevaluation at postmarketing evaluations of new drugs that continues to the present.

A different anti-CD33 monoclonal antibody chemically conjugated to blocked ricin toxin did not elicit significant clinical efficacy[48]. Further preclinical and clinical investigations into novel immunoconjugates to CD33 are therefore required in an effort to develop meaningful antitumor efficacy.

12.3 Antimicrotubule Agents and Immunoconjugates

12.3.1 Auristatins

Auristatins are cytotoxic agents that induce apoptosis by promoting G2-M cell cycle arrest and microtubule disruption. Auristatin, or its analogues, has been chemically conjugated to monoclonal antibodies against a variety of antigens including CD20[49], CD30[50], CD70[51], prostate-specific membrane antigen[52], p97[53], E-selectin[54], glycoprotein NMB[55], and Lewis Y antigen[56]. Aside from the great success of brentuximab vedotin[41] (see section 12.2.3), efficacy of the treatments is still unclear as most agents are still in early clinical development.

12.3.2 Maytansine and Derivatives

CanAg is a novel carbohydrate epitope of the tumor antigen Muc1. It is overexpressed in cancers of the pancreas, biliary system, and colon. huC242 is a humanized anti-CanAg monoclonal antibody that has been chemically conjugated to DM1 [$N^{2'}$-deacetyl-$N^{2'}$-(3-mercapto-1-oxopropyl) maytansine], a potent antimicrotubule agent. The resultant immunoconjugate, cantuzumab mertansine, has produced clinical responses in patients with a variety of cancers including pancreatic, colorectal, and non-small cell lung cancer[57], as well as solid tumors.

Maytansine derivatives have also been tested in other immunoconjugates, trastuzumab-DM1 (anti-HER2/neu)[58, 59], AVE9633 (anti-CD33)[58, 60], HuN901-DM1(anti-CD56)[61], and B-B4-DM1 (anti-CD138)[62], currently in clinical trials. Results of the phase 3 EMILIA clinical study of trastuzumab-DM1 in women with trastuzumab-refractory, advanced HER-2 positive breast cancer were recently reported at the 2012 American Society for Clinical Oncology. Median progression-free survival was 9.6 months in the trastuzumab-DM1 arm versus 6.4 months in the capecitabine plus lapatinib (small molecule inhibitor of HER-2) arm.[63] Furthermore, grade 3 or higher severe adverse events were less frequent in the trastuzumab-DM1 group (40.8 %) versus capecitabine/lapatinib (57.0 %), as was the rate of adverse events necessitating treatment discontinuation (5.9 % versus 10.7 %). The EMILIA study provides convincing evidence that an immunoconjugate-targeting HER2 has potent antitumor activity, and provides some realization of Paul Ehrlich's concept of a "magic bullet" developed over 100 years ago. Its merits are continuing to be evaluated[64].

12.3.3 Early-Stage Antibody-Based Immunoconjugates Targeting Solid Tumors

In the interest of completeness, the following agents have been included and briefly discussed. However, many have failed in clinical trials or are still too early in their development to make judgments on their efficacy.

An anti-HER2/neu single-chain antibody genetically conjugated to PE38 (Erb-38; scFv(FRP5)-ETA) failed a phase I clinical trial due to liver toxicity and poor clinical efficacy in breast cancer patients[65]. However, in other studies, this drug showed potential in 11 patients with metastatic breast cancer, colorectal cancers, and melanoma. Patients receiving injections into dermal tumors showed partial and in some cases complete tumor regression[66]. However, in 18 breast cancer patients treated in a follow-up trial, there were no objective responses[67]. It is likely that further studies are required before any conclusions can be drawn on the clinical efficacy of this approach.

Mesothelin is a tumor differentiation antigen overexpressed in a variety of cancers including those of pancreas, ovary, and mesothelium[68]. An immunoconjugate-targeting mesothelin, SS1(dsFv)PE38, has been developed. SS1(dsFv)PE38 is

comprised of a modified scFv genetically fused to PE38 and has since been designated SS1P. One phase I clinical trial was initiated in 2007[1] involving combined therapy for mesothelioma patients using SS1P, pemetrexed, and cisplatin was undertaken. A second phase I trial is ongoing, involving treatment of unresectable non-small cell lung adenocarcinoma with SS1P, paclitaxel, carboplatin, and bevacizumab.

A phase I trial of SS1P tested continuous infusion in 24 patients with mesothelin-expressing mesothelioma, ovarian, or pancreatic cancer. SS1P was given by continuous infusion for 10 days, and cycles could be repeated at 4-week intervals in the absence of neutralizing antibodies or progressive disease. Immunogenicity and modest clinical activity (e.g., reduced ascites) were noted in some subjects, but results were not better than results of bolus infusions, which are the subject of ongoing studies in conjunction with chemotherapy[69]. Results of early and pre-clinical developments of mesothelin-targeted treatment strategies were recently reviewed[70].

A phase I/II clinical trial testing an anticarcinoembryonic antigen monoclonal antibody chemically conjugated to blocked ricin toxin in colorectal cancer patients failed to show clinical efficacy[71]. In a phase I clinical trial of colon cancer patients, a 72-kDa glycoprotein expressed in colon cancer cells was targeted by chemically conjugating a specific monoclonal antibody to recombinant ricin toxin A-chain. Data from this trial showed that there was no significant clinical response[72, 73]. In a phase I clinical trial of breast cancer patients, an immunoconjugate composed of a specific monoclonal antibody directed against a 55-kDa antigen expressed in breast cancers chemically conjugated to recombinant ricin toxin was used. This trial was halted due to neuropathic toxicities[74, 75]. A trial using murine monoclonal antibody chemically conjugated to blocked ricin in patients with small cell lung carcinoma was halted due to toxicities[76, 77]. A murine monoclonal antibody targeting the OVB3 epitope expressed in ovarian cancers was chemically conjugated to full length *Pseudomonas aeruginosa* exotoxin A. This immunoconjugate was toxic to the central nervous system[78].

A variety of immunoconjugates have been designed in which distinct anti-Lewis Y antibodies, or fragments of the antibody, are conjugated to truncated *Pseudomonas* exotoxin. These immunoconjugates have generally shown little or no efficacy in patients with adenocarcinomas[79, 80].

12.3.4 Radioimmunoconjugates

Radionuclides used in therapy emit shorter-penetrating energy compared to those used in diagnostics where energy emitted must be sufficient for detection by external devices.

Short-range therapeutic radionuclides, for example, ^{211}At ($t_{½} = 7.2$ h; 6.0 MeV) and ^{213}Bi ($t_{½} = 46$ min; 6.0 MeV), emit alpha particles which typically penetrate only several cell diameters[81]. Such short-length emitters can be used in

radioimmunoconjugates, useful for the treatment of localized cancers or small malignant cell clusters. FDA-approved radioimmunoconjugates for treatment of B cell lymphomas include ibritumomab tiuxetan (Zevalin), a murine anti-CD20 monoclonal antibody fused to the ^{90}Y-labeled chelator tiuxetan, and [^{131}I]-tositumomab (Bexxar), a murine [^{131}I]-anti-CD20 monoclonal antibody[82]. [^{90}Y]-epratuzumab, a humanized [^{90}Y]-anti-CD22 antibody, is in human phase III trials for non-Hodgkin lymphoma with encouraging results[83], while ibritumomab tiuxetan shows promise in primary central nervous system lymphomas[84], a disease with a poor prognosis.

A disadvantage for the use of radiolabeled antibodies is their distribution to the liver, spleen, and bone marrow[58]. Pretreatment with unlabeled (cold) immunoconjugates can help mitigate such off-target toxicities. An example of this is the use of tositumomab for the treatment of non-Hodgkin lymphoma. Prior to treatment, rituximab is used to deplete CD20$^+$ B lymphocytes[85]. This pretreatment enhances the clinical efficacy of tositumomab while simultaneously reducing injury to noncancerous tissue[58].

12.3.5 Cytokine-Based Immunoconjugates

An alternative to antibody-mediated targeting are immunoconjugates containing cytokines (Fig. 12.1e) or peptides that target cytotoxins (Fig. 12.1f) or radionuclides (Fig. 12.1g) to specific cancer cells.

12.3.5.1 Denileukin Diftitox and the Treatment of Hematological Malignancies

Denileukin diftitox (DAB_{389}IL-2, ONTAK; Eisai, Fig. 12.2a) is an immunoconjugate that employs the catalytic and translocation domains of diphtheria toxin ($DT_{1\text{-}389}$) genetically fused to human interleukin (IL)-2[86]. Genetic deletion of 146 amino acids at the C-terminus of the diphtheria toxin ($DT_{1\text{-}389}$) removes the wild-type binding domain which is replaced with the entire secreted form of human IL-2 (Fig. 12.2a). The IL-2 moiety preserves IL-2 binding affinity and redirects the toxin to IL-2 receptor (IL-2R)-expressing cells[87], which includes malignant T cells of human cutaneous T cell leukemia/lymphoma[88, 89]. Following binding to IL-2R, and cellular internalization by receptor-mediated endocytosis, the $DT_{1\text{-}389}$ toxin catalyzes ADP-ribosylation of elongation factor-2 at his-699 and inhibits protein translation to induce cell death[90].

After its clinical efficacy was established in several clinical trials, denileukin diftitox (Fig. 12.2a) was approved by the FDA in 1999 to treat CD25$^+$ cutaneous T cell leukemia/lymphoma[91, 92]. It was later found to have clinical activity in other IL-2R-expressing hematological malignancies of both T lymphocytes and B lymphocytes[91], including panniculitic lymphoma[93], chronic lymphocytic leukemia[94], and B cell non-Hodgkin lymphomas[95]. Denileukin diftitox efficacy in hematological malignancies was augmented by coadministration of arginine butyrate[96]

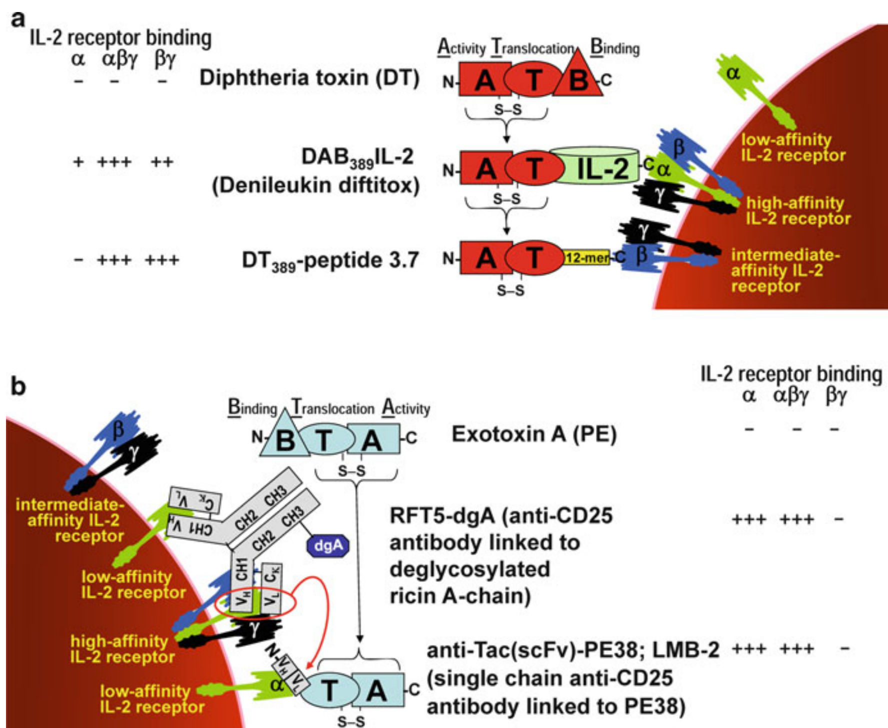

Fig. 12.2 Examples of cytotoxins targeting IL-2R, with emphasis on the modular organization of each molecule. Two types of targeted cytotoxins are shown, one based on diphtheria toxin (DT; **panel a**) and the other based on *Pseudomonas aeruginosa* Exotoxin A (PE; **panel b**). The variable heavy (V_H), light (V_L) and constant kappa chain (C_κ) chains of the anti-CD25 antibody linked to deglycosylated ricin A-chain (dgA) are also shown in **panel b** for comparison. Disulfide bonds critical to the internalization of particular cytotoxins are also shown (S—S). The relative contribution of the CD25 (α-chain), CD122 (β-chain), and/or CD132 (γ-chain) of IL-2R to the binding affinity of each targeted toxin is indicated by the number of "+" signs. The presence of a "−" sign indicates no contribution by a particular subunit.

or RXR rexinoids[97, 98], which was considered to cause increased expression of the IL-2R alpha (CD25, IL-2Rα) and beta subunits (CD122, IL-2Rβ)[99, 100]. These data suggest that immunoconjugate efficacy can be enhanced by altering cognate receptor expression, a strategy which has not yet been used to maximum advantage.

12.3.5.2 Denileukin Diftitox to Deplete Nonmalignant Regulatory T Cells as Immunotherapy

Regulatory T cells (Tregs) are a subset of $CD4^+$ T cells which express CD25, the nuclear transcription factor Foxp3, and other molecules, reviewed in detail in Chap. 9. Tregs are elevated in patients with a variety of cancers and are significant contributors to cancer-driven immune dysfunction[101, 102]. Animal models have shown that Treg depletion enhances endogenous antitumor immunity and increases efficacy of cancer

immunotherapy in several distinct models[102]. Since Tregs express CD25, the α subunit of IL-2R, it was hypothesized, and later confirmed in clinical trials, that denileukin diftitox would target and kill these cells in cancer patients, which has been demonstrated in melanoma, renal cell carcinoma, and ovarian cancer[103–105] (and cancers of bladder, breast, lung, and pancreas; manuscript submitted). Anti-CD25 scFv coupled to *Pseudomonas aeruginosa* exotoxin A also depletes Tregs in vitro[106] and in vivo in melanoma patients[107].

12.3.5.3 Additional IL-2R-Targeting Strategies

RFT5-dgA is an immunoconjugate comprised of the Fc portion of the anti-CD25 antibody RFT5, which is specific for the IL-2R α subunit, and conjugated to a deglycosylated ricin A-chain that targets and binds $CD25^+$ cells (Fig. 12.2b). This immunoconjugate demonstrated some clinical efficacy in Hodgkin disease in a phase II clinical trial[108]. It produced some meaningful clinical responses in $CD25^+$ lymphomas[109] and leukemias[110] and can penetrate into solid tumors[111]; fever and liver damage were important toxicities observed[109, 110, 112]. Efforts to engineer out moieties that cause these side effects without compromising clinical efficacy are in development[113].

12.3.5.4 IL-2R Targeting Using Conjugated Peptides

The immense diversity of T and B cell receptors is due to random recombination of genetic material in their antigen recognition receptor sites by specific recombinase and related gene products[114, 115]. Because the process is random, an almost infinite number of receptors with almost infinite recognition capabilities is generated. This strategy has been exploited in vitro to develop libraries of binding molecules capable of binding a vast array of ligands. The naturally occurring shuffling strategy has been applied to making libraries of single-chain antibody fragments, combinatorial chemicals, and peptides from phage. As an example of the immense diversity of these strategies, commercial phage display libraries have a diversity of up to a theoretical limit of 4×10^{15} unique 12-mer peptides[14].

We have used such a phage display strategy to identify small 12-mer peptides to target and deliver immunogenic antigens to dendritic cells in a mouse model[116]. A similar strategy was applied in an attempt to identify a Treg-binding motif. IL-2R is highly expressed on Tregs. The α chain of the IL-2R (CD25) has been exploited in denileukin diftitox and *Pseudomonas aeruginosa* exotoxin targeting strategies to deplete Tregs (discussed above), but these strategies lack cell specificity. CD122 (IL-2Rβ) was tested as an alternative Treg-targeting molecule. CD122 is less broadly expressed by non-Treg immune cells compared to CD25 and is expressed by some epithelial carcinomas. We identified CD122-binding peptides that performed well in vitro (Fig. 12.1f, g). Three of these candidate peptides were genetically fused to $DT_{1\text{-}389}$, but we failed to confirm significant Treg-depleting capacity or treatment efficacy in vivo in mouse cancer models.

12.3.5.5 Additional Cytokine-Based Immunoconjugates

Human granulocyte-macrophage colony-stimulating factor (GM-CSF) was conjugated to a truncated diphtheria toxin moiety (DT_{388}) to treat acute myeloid leukemia expressing the GM-CSF receptor[117]. This strategy was limited by hepatotoxicity and further clinical study was not pursued[118]. GM-CSF fused to a single-chain human antibody fragment targeting the extracellular domain of fibronectin B, a tumor angiogenesis marker, has been studied as a vascular disrupting agent[119]. An alternative vascular disrupting strategy was recently reported by incorporating TNF-α and vandetanib, a tyrosine kinase inhibitor that inhibits angiogenesis, into the immunoconjugate in a xenograft model for esophageal carcinoma[120].

IL-3 has been fused to the C-terminus of the truncated diphtheria toxin moiety DT_{388} to target IL-3R-expressing hematologic malignancies[121]. A phase I clinical trial to treat acute myeloid leukemia was planned but, to our knowledge, has not been reported yet. IL-4 was conjugated to the *Pseudomonas aeruginosa* toxin moiety (PE38) to treat IL-4R-expressing glioblastoma, but liver toxicity halted clinical development[122]. IL-13 targeting in cancer, especially brain tumors, has received much recent attention[123]. An immunoconjugate using IL-13 conjugated to PE38 demonstrated some clinical efficacy in glioma, and side effects have been tolerable thus far[124], leading to an ongoing phase III clinical trial[125]. A more recent finding is that local tumor-associated hypoxia reduces IL-13 immunoconjugate efficacy[126], and thus, efforts to attenuate local hypoxia could augment therapeutic benefits.

An immunoconjugate fusing TGF-β to PE38 was tested in a trial of intravesical instillation in superficial bladder cancer. Eight out of 43 patients experienced a positive clinical response in a phase I clinical trial[127]. *Pseudomonas aeruginosa* exotoxin was conjugated to an anti-epithelial cell adhesion molecule single-chain antibody fragment and is now in phase II and III clinical trials to treat head and neck squamous cell carcinomas by direct tumor injection, as in phase II clinical trials to treat superficial transitional cell bladder carcinomas by intravesical instillation[128]. The same immunoconjugate was used in a trial of intracerebral injection for recurrent glioblastoma. Three of 15 patients in a phase I clinical trial experienced radiographic responses, including one with complete remission who was cancer free for 83 weeks at the time clinical trial results were reported[129]. Further studies of this immunoconjugate have not been reported to our knowledge.

Several recent reviews have authoritatively covered the developing literature on immunoconjugates utilizing cytokine moieties in detail[4, 130].

12.3.6 Targeting Tumor-Associated Antigens to Dendritic Cells

The murine monoclonal anti-CA-125 antibody oregovomab is thought to complex CA-125 antigen, deliver this antigen to dendritic cells[131], and thereby activate T cells[132]. It failed a pivotal phase III study in 373 ovarian cancer patients[133]. Please see Chap. 11 for additional oregovomab details and Chap. 6 for many additional dendritic cell targeting strategies.

12.3.7 Viral Immunoconjugates

Frequently, viral tropism of given vectors for target cells is not optimal. A viral-based immunoconjugate could help retarget a given viral vector for clinical use using a variety of strategies including bispecific antibodies, monoclonal antibodies, single-chain antibodies, peptides, cytokines, and other techniques.

Bispecific antibodies are comprised of two distinct antibody recognition sites conjugated together, one typically targeted to the vector and the other targeting the cell of interest (Fig. 12.1h). Bispecific antibodies allow using vectors without having to make a genetic targeting modification to that vector itself, greatly facilitating and speeding the use of available vectors. An example is a bispecific antibody targeting the adenovirus vector to pulmonary endothelium, a tissue ordinarily refractory to adenovirus infection. An example of a bispecific viral immunoconjugate is a monoclonal antibody targeting membrane-bound angiotensin-converting enzyme (highly expressed on pulmonary endothelium) conjugated to the Fab moiety of an antiadenovirus vector-specific monoclonal antibody. This immunoconjugate produces 20-fold greater lung transduction compared to an unmodified control vector. Liver transduction, a common problem with adenovirus vectors[134, 135], was simultaneously reduced consistent with the altered natural adenovirus vector tropism and potentially allowing increased treatment efficacy and reduced hepatic toxicity.

A distinct strategy to conjugate antibody to vector is by genetic vector modification. An example is to engineer an immunoglobulin (Ig)-binding domain into the vector (Fig. 12.1i). Unlike in the bispecific antibody approach, the viral vector has now been genetically modified specifically to recognize the Fc domain of the antibody. The *Staphylococcus aureus* protein A Z-domain, which efficiently binds Fc, has been inserted into viral attachment proteins of several distinct vectors successfully[136–139]. Introducing an immunoglobulin binding domain into a lentiviral envelope protein allows conjugation to an anti-P-glycoprotein monoclonal antibody, targeting the P-glycoprotein expressed on several cancers, including melanoma. This strategy allowed targeting to human melanoma cells xenografted into immunodeficient SCID mice[140]. An advantage to this approach is the wide variety of monoclonal antibodies available, allowing relatively easy and rapid screening of a variety of targeting moieties. The disadvantage is that normally circulating antibodies could compete with coupled antibody in vivo and reduce efficacy.

Engineering a single-chain antibody fragment into a viral vector can be useful in a variety of strategies (Fig. 12.1j). For example, a single-chain antibody fragment against the tumor antigen CEA was genetically conjugated with a matrix metalloproteinase cleavage site and the envelope gene of the viral vector. This immunoconjugate facilitated vector tumor binding via the single-chain antibody fragment, followed by cleavage of the tumor-targeting single-chain fragment by matrix metalloproteinases in the tumor[141]. Removing the single-chain moiety from the vector envelope prior to target cell transduction is important because (a) the single-chain on the envelope can change its conformation that could then reduce transduction efficiency and (b) removal of the single-chain moiety exposes the

receptor envelope binding domain, enhancing interaction with its Pit-2 receptor on the tumor cell. This strategy demonstrated in vivo tumor transduction selectivity[141].

Removal of an attachment to virus envelope is not required for adenovirus vectors, as adenovirus entry into target cells does not require these complex conformational changes for appropriate interaction of target cell and adenovirus attachment protein. Nonetheless, adenovirus vectors present different hurdles to be overcome to fuse single-chain antibody moieties to their capsids. Adenovirus proteins are synthesized in cell cytosol, whereas single-chain antibody fragments require the formation of important disulfide bridges that is accomplished in the rough endoplasmic reticulum. To address this issue, cytosolically stabilized single-chain fragments (intrabodies) were employed that could be coupled to an artificial adenovirus fiber protein[142]. Further placing a pair of cysteine residues in specific positions within the V_L and V_H domains produced a disulfide-bond-stabilized single-chain moiety that exhibited greatly increased half-life and stability[143].

In an alternate approach, antibodies are conjugated to adenovirus through the natural adenovirus receptor (coxsackie and adenovirus receptor, Fig. 12.1k). The ectodomain of this natural receptor can be conjugated to single-chain antibody fragments. An immunoconjugate of anti-CEA antibody effected specific hepatic tumor graft transduction with simultaneous reduction of liver tropism, demonstrating potential for augmented efficacy with reduced toxicity[144].

Many other strategies for virus vector targeting, including strategies aside from immunoconjugates, have been reviewed[15, 145–147] (and see Chap. 6).

12.4 Diagnostic Immunoconjugates

Diagnostic immunoconjugates target detection agents to specific cells. Detection agents typically are radionuclides, chemicals, or fluorophores. Radionuclides are selected to ensure that their energy emissions can reach the detector during radioimmunoscintigraphy. High-energy beta-particle emitters such as ^{90}Y ($t_{½} = 64$ h; 2.3 MeV) or ^{188}Re ($t_{½} = 17$ h; 2.1 MeV) are often conjugated to antibodies in diagnostic immunoconjugates, although they can also be used to treat tumors with large (>11 mm) diameters[148]. An example of an FDA-approved radioimmunoscintigraphic imaging agent is capromab pendetide, a prostate-specific membrane antigen-specific monoclonal antibody conjugated to ^{111}In used in prostate cancer patients[149]. By contrast, therapeutic radiolabeled antibodies tend to emit particles with shorter ranges, to maximize energy delivered to the tumor mass and minimize potentially harmful energy delivery to surrounding normal cells and tissues as discussed above in the antibody section.

Techniques for nonradioactive diagnostic tumor imaging using immunoconjugates have evolved quickly in recent years. As an example, antibodies conjugated to fluorophores emitting in the near-infrared spectrum[150] enhance visualization of tumor margins with essentially no interfering background emission from autofluorescence that is generally not in this spectral region. Diagnostic

immunoconjugates are also now employed in ultrasonography using intravenous injection of microbubble-conjugated antibodies[151] that can be detected by an ultrasonographic detector. For example, microbubble-conjugated antivascular endothelial growth factor-specific antibodies can identify tumor margins by ultrasonography[151] as vascular endothelial growth factor is produced at high levels in many tumors.

12.5 Conclusions

Immunoconjugates capitalize on immune system specificity to target anticancer drugs to the tumor while helping minimize damage to bystander normal tissue. Technological advances have helped develop many novel classes of immunoconjugates, some now showing promising clinical results but few have received FDA approvals. Toxins conjugated to antibodies have been the most successful class to date, and hematologic malignancies have been the diseases most successfully treated to date, but the landscape continues to evolve rapidly. A significant current limitation of most immunoconjugates remains suboptimal tumor tissue penetration. The immunogenicity of some immunoconjugates, notably virus-based conjugates, can limit efficacy with repetitive dosing. Engineering out removes immunodominant vector epitopes or using vectors of distinct antigenicity in serial dosing can help with these issues. Strategies to improve targeting and selectivity of agents continue to evolve, helping improve specificity and reduce undesirable side effects, including antigenicity.

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Part IV
Additional Strategies and Considerations

Chapter 13
Miscellaneous Approaches and Considerations: TLR Agonists and Other Inflammatory Agents, Anti-Chemokine Agents, Infectious Agents, Tumor Stroma Targeting, Age and Sex Effects, and Miscellaneous Small Molecules

Robert S. Svatek and Tyler J. Curiel

Abstract The field of tumor immunotherapy is evolving rapidly. Many promising areas are discussed in depth in various chapters in this book. This chapter provides a broad overview of additional approaches not covered in specific book chapters. Some areas are nonetheless quite advanced, such as the use of TLR agonists in clinical trials, and some have received FDA approvals such as BCG to treat bladder cancer and the IMiD lenalidomide to treat multiple myeloma. Other areas are of great interest but lack sufficient information to require a dedicated chapter. These miscellaneous areas hold great promise to further the development of effective cancer immunotherapies.

13.1 Strategies to Mitigate Immune Dysfunction and the Influence of Age on Anti-Tumor Immunity

Age is the leading factor risk for development of cancer[1] and thus most cancer patients are elderly. For example, in the United States, 60% of cancers occur in individuals aged 65 years or more where they account for 70% of cancer-related mortality[2]. Nonetheless, most preclinical evaluations of tumor immunology and responses to immunotherapy are done in young subjects, often to save on animal purchase and care costs and due to reduced availability of older study animals. Although anti-tumor immunity and other immune effector functions can decline with age[3, 4], the functional capacity of T cells and other immune effector arms can

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sometimes be therapeutically improved in aged hosts[5]. Thus, if any tumor-associated immune dysfunctions in aged hosts can be mitigated, it could be possible to generate clinically important anti-tumor immunity.

Age-dependent immune changes include reduced effector T cell function that could reduce anti-tumor immunity. Naive T cells in aged hosts exhibit functional defects including reduced capacity to proliferate, secrete cytokines, and undergo effector T cell differentiation[6–8]. Thus, effective immunotherapies in young hosts could be less effective in aged hosts. For example, CD134 (OX40)-dependent tumor immune rejection in a mouse model decreases with age[8]. Despite these potential functional reductions, clinically efficacious cancer immunotherapy for aged hosts is a potentially realistic goal given that some age-associated immune functional decrements can be reduced or reversed. As an example, in a mouse model for cancer, tumor immunity was improved with specific and sufficient immune co-signaling[9], and age-associated reductions in T cell priming can be improved by activating the CD137 (4-1BB) immune co-signaling pathway[10].

Regulatory T cells are key mediators of tumor immune dysfunction, and their management is under investigation as a rational approach to boosting the efficacy of cancer immunotherapy[11–14]. (See also Chap. 9). Studies of regulatory T cells in relationship to age-related reductions in anti-tumor immunity, or immune responses in nontumor settings are contradictory, including studies demonstrating increases in the prevalence and/or function of regulatory T cells with age in mice and humans[6, 7, 15, 16] and other studies demonstrating either no effects or reduced numbers or effects of regulatory T cells with advancing age[17, 18]. There are few studies of the effects of regulatory T cell management as cancer therapy in aged hosts[19, 20].

Suppressive and potentially dysfunctional myeloid derived suppressor cells also increase in tumors[21–24] where they suppress anti-tumor immunity[25]. Myeloid derived suppressor cells appear to mediate increased immunopathology in aged versus young cancer-bearing hosts[26, 27]. See also Chap. 10.

We recently studied regulatory T cell and myeloid derived suppressor cell effects in cancer immunotherapy in aged mice, and identified a strategy for generating effective anti-cancer immunotherapy in a B16 melanoma model that worked well in aged, but not young hosts, by identifying and then mitigating age-related tumor-associated immune dysfunction[28]. We showed that regulatory T cells in young and aged mice exhibited equivalent in vitro T cell suppression and were increased equivalently after tumor challenge, but regulatory T cell depletion using the fusion toxin denileukin diftitox (see Sect. 9.1) improved tumor-specific immunity and clinically controlled tumor growth only in young mice. Aged mice had higher basal and post-tumor challenge numbers of CD11b^{+}Gr-1^{+} myeloid derived suppressor cells that were also more suppressive than those in young mice. Myeloid derived suppressor cell depletion thus improved tumor-specific immunity and reduced tumor growth significantly better in aged mice. Surprisingly, we found that denileukin diftitox treatment to reduce regulatory T cell numbers also simultaneously and significantly increased numbers of myeloid derived suppressor cells in aged but not young hosts.

Based on this finding, we tested the combination of anti-Gr-1 antibody to deplete myeloid derived suppressor cells plus denileukin diftitox to deplete regulatory T cells in aged mice. Denileukin diftitox plus anti-Gr-1 antibody produced superior immune and clinical benefits over anti-Gr-1 antibody alone in aged B16-bearing mice, with clinical efficacy comparable to depleting regulatory T cells in young hosts, who did not benefit from additional anti-Gr-1 antibody treatment. By contrast, in an MC-38 colon carcinoma model, denileukin diftitox treatment generated increased numbers of myeloid derived suppressor cells in young and aged mice, although effects were greater in the aged hosts, but nonetheless effected immune benefits (including boosted tumor-specific immunity) and improved clinical responses in both young and aged hosts. These studies demonstrate the intrinsic competence of aged anti-tumor immune effector cells. By identifying age-specific immune dysfunction and then targeting immunotherapy against these age-related tumor-associated immune dysfunctions, cancer immunotherapy for aged hosts can be improved significantly. These studies also demonstrate that effects of various immunotherapy approaches can differ by cancer histology.

13.2 Strategies Using Infectious Agents

13.2.1 Background

The idea that an infection could alter the natural history of cancer was introduced in 1866 by Wilhelm Busch in Germany who observed tumor regressions in sarcoma patients surviving postoperative wound erysipelas, a streptococcal bacterial infection. William Coley in Manhattan likewise observed spontaneous tumor regressions after postsurgical wound erysipelas between 1892 and 1896 and used deliberate infections to attempt to treat cancer. Please see Chap. 1 for additional historical details. These bacterial infections worked by generating cytokines that are discussed in Chap. 7 and by ligating Toll-like receptors discussed in Sect. 4 below. The principal infectious agent used in cancer immunotherapy today and newer approaches are discussed below.

13.2.2 Bacille Calmette–Guérin

13.2.2.1 Bladder Cancer

Mycobacteria have long been known as potent stimulators of the immune response. Bacille Calmette–Guérin (BCG, also known as Bacillus Calmette–Guérin) is an attenuated strain of *Mycobacterium bovis* that activates macrophages, natural killer cells, B cells, and $CD4^+$, $CD8^+$, and $\gamma\delta$ T cells[29, 30]. Intravesical instillation of BCG

was FDA-approved on August 4, 1998 for the primary or secondary treatment of carcinoma in situ of the bladder. On August 21, 1998 the FDA expanded the indication for the adjunct treatment of stage Ta or T1 papillary tumors following resection. The efficacy of intravesical BCG was highlighted in a meta-analysis that included over 20 randomized trials, demonstrating a 56% reduction in the tumor recurrence hazard attributable to BCG with tumor resection compared to tumor resection alone[31]. Although its precise mechanisms of action in bladder cancer remains incompletely understood, it is thought to work through cell-mediated immunity. BCG enters bladder cancer cells, the *Mycobacterium* is broken down and its peptides are combined with histocompatibility antigens and displayed on the cell surface. Thus, infected cells could be killed through anti-BCG immunity[30]. Recent work suggests that BCG immunotherapy induces IL-17^{+} T cells that recruit helpful granulocytes in bladder cancer that contribute to anti-tumor activity[32]. Another recent report demonstrated that BCG vaccination in an animal model improved the anti-cancer effect of intravesical BCG, presumably by increasing anti-tumor T cells in the tumor microenvironment[33]. Immune correlates of BCG failure and poor survival are becoming better understood and include local expression of dysfunctional immune co-signaling molecules including PD-1 and B7-H1[34–36]. These recent insights into the mechanisms of BCG activity and immune correlates of BCG failure suggest means to improve its activity in bladder cancer and provide guidance for how it could be applied to additional tumors.

13.2.2.2 Melanoma

Early BCG immunotherapy for melanoma consistently suggested trends for better clinical outcomes over best supportive care. The Malignant Melanoma Active Immunotherapy trials in patients with stage III and stage IV disease (tested in separate trials) showed that overall survival in the BCG treatment group was better than expected versus the placebo group in both stage III and stage IV patients, suggesting that adjuvant BCG could improve surgical results. Additional studies and trials are ongoing[37].

Melanoma enjoys a reputation as a tumor potentially responsive to various types of immunotherapy, and has the additional advantage that many of its tumors and metastases are accessible for direct intratumoral injections. Thus, a large variety of additional attempts at melanoma immunotherapy have tested agents that include microbial products other than BCG, cytokines, cytotoxic agents, adoptive immunotherapy with cells or antibodies and other approaches. Some successes have been seen with in-transit and cutaneous metastases whereas subcutaneous metastases have proven more refractory to treatments. These approaches were all recently reviewed[38].

13.2.3 Viral Vectors

13.2.3.1 Poxviruses

Poxviral vaccines used to prevent smallpox have been given to over one billion individuals with relative safety. Engineered, recombinant poxviruses are now in late stages of clinical trials testing their efficacy as cancer immunotherapy for cancer. The back bone of the vectors in trials is TRI-COM, which is a poxvirus engineered to express the immune co-signaling molecules B7-1 (CD80), ICAM-1, and LFA-3. As most tumors lack these co-signaling molecules, the virus can improve antigen presentation by the tumor and infected cells. TRI-COM can improve T cell immunity to tumor-associated antigens better than is achieved with any one or two of these individual co-signaling molecules[39]. To enhance efficacy further, TRI-COM vectors have been modified to express specific tumor-associated antigens. The most clinically advanced of these derivative vectors is a TRI-COM expressing prostate specific antigens (PROSTVAC-VF-TRI-COM, National Cancer Institute/BN Immuno Therapeutics, Inc.). It is now undergoing a phase III randomized, placebo-controlled clinical trial to treat metastatic castration-resistant prostate cancer[40]. Optimal means to combine poxvirus vaccines with radiation therapy, cytotoxic chemotherapy, targeted small molecules, hormonal therapy, and other immune-based strategies are under investigation due to its promise.

13.2.3.2 Adenovirus

Adenovirus gene therapy and its use to target dendritic cells is discussed in Chap. 6 and in immunoconjugates in Chap. 12. Several adenovirus-based approaches based on immune principles are also being tested. For example, adenovirus expressing IL-24 (Ad.mda-7 (INGN-241)) was recently shown to elicit a 40% clinical response rate in phase I studies[41]. The mechanisms of action could include induced cell cycle arrest, and IL-24 could also be a radiosensitizing agent.

An adenoviral vector encoding human interferon-α (Ad.IFN-alpha2b) was tested in a human clinical trial of malignant mesothelioma. Disease stability or tumor regression was seen in several patients, including one partial tumor regression at sites non-contiguous with vector injection[42].

13.3 Strategies Aimed at Reducing Tumor Inflammation

Numerous cells contribute to tumor-associated inflammation, notably tumor-associated macrophages[43, 44], cells that alter tumor macrophage properties such as neutrophils[45] and fibroblasts[46], and other cells (recently reviewed[44]). These data suggest that altering macrophage properties or the properties of other

tumor-associated cells could improve anti-tumor immunity. Inflammation in cancer was recently reviewed in depth[47, 48].

Altering macrophage phenotype with an agonist anti-CD40 antibody showed some evidence for efficacy in humans with pancreatic cancer[49]. In this study, the CD40 agonist antibody CP-870,893 was combined with the cytotoxic agent gemcitabine. Greater than 50% reduction of tumor size was noted in 4 of 21 treated patients. In two patients, tumor biopsies showed infiltration of cell populations lacking lymphocytes. In a corroborative mouse model, macrophages, not T cells, were found to be important targets of the agonist anti-CD40 antibody.

Tumor microenvironmental cytokines, notably IL-1β, IL-6, and TNF-α can contribute to immune dysfunction and angiogenesis[50, 51]. These findings suggest that inhibiting the concentrations or functions of these cytokines could be beneficial as cancer treatment. In the case of TNF-α, human clinical trials bear out this thesis using infliximab (an anti-TNF-α antibody) to stabilize disease in patients with advanced cancers progressing on conventional treatments in one trial[52], and to demonstrate partial response or disease stability in two phase II trials in renal cell carcinoma using standard and high-dose infliximab[53]. In a trial in ovarian cancer, etanercept (a soluble TNFR2 fusion protein that neutralizes TNF-α by direct binding to it) stabilized disease in a minority of patients (reviewed in 50). An anti-IL-6 antibody was tested in prostate cancer and ovarian cancer without clear evidence for clinical efficacy although immune modulation was observed (reviewed in 54). Anakinra (an IL-1 receptor antagonist) reduced disease progression in smoldering multiple myeloma[55]. Colony-stimulating factor 1 receptor (CSF-1R) appears to regulate myeloid cell proliferation and pro-inflammatory cytokine secretion. Agents to inhibit myeloid cell CSF-1R are now in clinical trials to reduce cancer-related inflammation[56].

Anti-inflammatory agents could also be used to prevent cancer, as evidenced by a recent review of a collective cohort of 25,570 patients on long-term aspirin in several separate clinical trials to prevent cardiovascular events[57]. Aspirin reduced cancer death in the entire cohort (pooled odds ratio 0.79, $p = 0.003$) with effects that lasted throughout the 20-year observation period, and applied to a variety of cancers. A number of anti-inflammatory approaches, including celecoxib, anti-TNFα, and anti-IL-6 have been used to try to treat cancer cachexia, but with limited success thus far[58], including in two recently completed phase III clinical trials[59, 60].

13.4 Strategies Targeting Toll-Like Receptor Agonists

Toll-like receptors (TLRs) are single, membrane-spanning, receptors that recognize molecules derived from microbes. The TLRs activate key immune responses after engaging these microbes which have breached physical barriers such as the gastro-intestinal tract mucosa or skin. TLRs have significant immune modulating effects, and have demonstrated utility in some cancer immunotherapy[61].

13.4.1 Imidazoquinolines Including Imiquimod

Imiquimod (3-(2-methylpropyl)-3,5,8-triazatricyclo[$7.4.0.0^{2,6}$]trideca-1(9),2(6), 4,7,10,12-hexaen-7-amine, Aldara, Zyclara, Beselna; Medicis Pharmaceutical Corporation) is an imidazoquinoline TLR-7/8 agonist that is FDA-approved to treat superficial basal cell carcinoma (and actinic keratosis and external genital warts). It also has activity against other cutaneous neoplasms including dermal metastases of epithelial carcinomas. Imiquimod, its congerers, and other TLR7/8 agonists activate NF-κB that induces pro-inflammatory cytokine and chemokine production among other immune mediators. These effects are primarily thought to be through activation of dendritic cells. Additional mechanisms include inducing tumor cell apoptosis through bcl-2 and caspase-dependent mechanisms (reviewed in 62–64).

852A is an immune response modifier structurally related to the imidazoquinolines that include imiquimod. In a phase II trial of cancer patients it generated immune activation and some disease stabilizations but was associated with significant cardiac toxicity[65].

13.4.2 CpG Oligodeoxynucleotides and Other TLR9 Agonists

Synthetic short single-stranded CpG oligodeoxynucleotides (CpG ODN) mediate strong immunostimulating activity through agonizing TLR9 which is constitutively expressed in B cells and plasmacytoid dendritic cells in humans. These molecules contain a cytosine "C" and a guanine "G" and a phosphodiester "p" backbone although some have a modified phosphorothioate backbone. CpG ODNs can generate Th1 polarized immunity dominated by interferon-γ production considered beneficial to anti-tumor immunity. These CpG ODNs have demonstrated impressive efficacy in preclinical models, although efficacy is best following direct injection into tumors.

A phase 1 trial of CpG-28, a phosphorothioate modified CpG oligodeoxynucleotide, in glioblastoma multiforme demonstrated tolerability and modest clinical effects in several of the 24 patients treated[66]. A phase I trial of CPG 7909 (PF-3512676) in 23 patients with non-Hodgkin lymphoma demonstrated tolerability and some immunomodulation as evidenced by increased natural killer cell activity and increased antibody-dependent cellular cytotoxicity[67]. In a second trial of 23 patients with melanoma, PF-3512676 demonstrated tolerability, altered inflammation, and favorable changes in dendritic cell subsets[68].

In another trial, 15 patients with low-grade B-cell lymphoma received intralesional injections of PF-3512676 that generated tumor-reactive memory $CD8^+$ T cells. Of the 15 patients from the trial, 1 experienced a complete clinical response, 3 experienced partial responses, and 2 patients had prolonged stable disease with therapy. The utility of this approach is that it does not require patient-specific custom

vaccine products or ex vivo cell manipulations[69]. A whole tumor cell vaccine produced by incubating lymphoma tumor cells with a CpG ODN TLR9 ligand effected significant clinical and immune responses in adoptive T cellular vaccination in a mouse lymphoma model and is now in a clinical trial of adoptive immunotherapy for mantle-cell lymphoma[70]. In a phase 2 trial in 184 patients with unresectable stage IIIB/C or stage IV malignant melanoma, PF-3512676 was tested alone or in combination with the cytotoxic agent dacarbazine. Objective responses were modest and did not warrant a follow up trial[71].

The TLR-9 agonist 1018 ISS was combined with rituximab in 23 patients with relapsed/refractory follicular lymphoma. No significant adverse events from treatment were noted and the clinical response rate was 48%. Immune changes included increased antibody-dependent cellular cytotoxicity and blood $CD3^+$ T cells in approximately one-third of patients. Treatment also induced increased tumor-infiltrating dendritic cells, $CD8^+$ T cells and macrophages, demonstrating that immune events in the tumor microenvironment could be improved[72].

13.4.3 Other TLR Agonists

The TLR2 agonists PAM_2CSK_4, PAM_3CSK_4, and FSL-1 reduced Treg suppressive functions in vitro. Proposed mechanisms for this effect include down-regulation of p27Kip1 and increased Akt phosphorylation[73]. TLR2 agonists can also make anti-tumor effector T cells more resistant to regulatory T cell-mediated suppression by augmenting their proliferation and IL-2 secretion[74]. TLR1/2 agonists mediate tumor regression through reducing Treg suppression and boosting $CD8^+$ cytotoxic T lymphocyte function in murine tumors models of melanoma, lung carcinoma, and leukemia[75]. Regulatory T cell-mediated suppression of T cell function can be inhibited by TLR8-derived signals[76] that could include functions of the imidazoquinoline drug class.

In two phase I studies, patients with advanced epithelial malignancies received a vaccine using human chorionic gonadotropin β-chain combined with granulocyte-macrophage colony-stimulating factor and the TLR3 agonist polyinosinic–polycytidylic acid plus the TLR7/8 agonist resiquimod to increase antigen presenting cell activation. Evidenced for enhanced immunogenicity of the dual TLR agonist combination was noted[77]. Other strategies to increase antigen presenting cell performance with TLR agonists have also been assessed in human calls[78].

Additional mechanisms of action of TLR agonists could include control of ubiquitination and micro RNA regulation. The field of TLR agonists and antagonists was recently comprehensively reviewed[79].

13.5 Strategies Targeting Chemokines

As chemokines instruct cell trafficking, including important roles in cancer cell metastasis[80], and can alter their functional properties, there is considerable interest in using chemokine agonists or antagonists as general immune modulators, and specifically as novel anti-cancer therapies[81]. The therapeutic utility of chemokine-targeted treatment approaches could ultimately be affected by the binding promiscuity of chemokine receptors, the chemokine/ligand pair redundancies, or chemokine effects in normal tissue homeostasis or anti-tumor immunity[82]. For example, CCR7 expression on tumor cells can augment lymph node metastases[83], but a recent report demonstrates that CCR7 expression on tumor-infiltrating $CD8^+$ T cells correlates with improved prognosis in advanced colorectal cancers[84].

13.5.1 CXCR4 Antagonists

CXCR4 is the most studied chemokine receptor in cancer as it is among the most widely expressed. Hypoxia and inflammatory mediators including TNF-α increase CXCR4 expression, which likely contributes to high CXCR4 expression in many cancers. Aside from trafficking effects, CXCR4 affects tumor and immune cell proliferation and survival, and contributes to tumor metastases[85, 86]. Many strategies have been developed to inhibit CXCR4 signaling. The best studied of these is the small molecule bicyclam antagonist AMD3100 (plerixafor, Mozobil). Plerixafor, manufactured by Genzyme Corporation, is the only FDA-approved anti-chemokine agent. It is used in combination with granulocyte colony-stimulating factor to mobilize hematopoietic stem cells for autologous bone marrow stem cell transplantation to support high-dose chemotherapy for non-Hodgkin lymphoma or multiple myeloma[87]. Plerixafor and other small molecule CXCR4 antagonists are also being investigated to reduce or prevent metastases in animal models of melanoma, osteosarcoma, and breast and prostate tumors[88, 89]. We showed that the CXCR4 axis helps control regulatory T cell trafficking from bone marrow[90] (in conjunction with granulocyte colony-stimulating factor) suggesting that CXCR4 antagonists could be used to reduce immune dysregulation in cancers. In this regard, CXCL12/CXCR4 signal interruption improved several aspects of tumor immunity in a mouse ovarian cancer model and improved survival[91].

Tumor–stromal cell interactions can reduce the efficacy of chemotherapy. Blocking the chemokine receptor CXCR4 in tumor stroma can improve the clinical efficacy of some cancer treatments as demonstrated in multiple myeloma[92] and other cancers. We recently demonstrated that the small molecule CXCR4 antagonist plerixafor reduces breast cancer metastases and improves endocrine therapy for breast cancer in a mouse xenograft model[93].

13.5.2 CCR4 Antagonists

The novel, humanized anti-CCR4 IgG1 antibody KW-0761 (AMG-761, mogamulizumab; Amgen) is defucosylated to augment its potential to mediate antibody-dependent cellular cytotoxicity[94, 95]. In a phase II trial of 28 patients with relapsed CCR4^{+} adult T cell leukemia, KW-0761 produced objective responses in 13 of 26 evaluable patients, including 8 complete responses[96]. Additional details are in Chap. 11.

13.5.3 CCL2 Antagonists

Anti-CCL2 or anti-CCR2 (CCL2 receptor) antibodies have been studied in preclinical models in prostate cancer with encouraging results[97–99] that have led to human clinical trials of anti-CCL2 antibodies in prostate cancer and ovarian cancer[100]. Additional details are in Chap. 11.

13.5.4 CCL22 Antagonists

We identified the chemokine CCL22 as important to attraction of regulatory T cells into the tumor microenvironment[13]. In silico studies found small-molecule chemokine receptor antagonists that could block CCL22-dependent human regulatory T cell recruitment in vitro (and Th2 cell recruitment)[101]. Some of these agents have entered phase I clinical trials[94]. CCL22 also regulates effector T cell trafficking that could reduce the utility of anti-CCL22 to treat cancer. Further studies are needed in this regard.

13.5.5 CXCR7 Antagonists

CXCR7 is the other chemokine receptor along with CXCR4 known to bind the chemokine CXCL12. Its expression is associated with pathogenesis and cancer progression in several important cancers including bladder, pancreatic, hepatocellular, breast and others, prompting much research into its use in cancer treatments[102]. A number of small molecule CXCR7 antagonists have been generated[103], but clinical trials of these agents have not yet been reported.

13.6 Vaccines

Viruses could account for ~12% of human cancers. Of these most are due to to infections by human papillomavirus, hepatitis B virus, hepatitis C virus, Epstein–Barr virus, and Kaposi sarcoma-associated herpesvirus[104]. Thus immune strategies (especially vaccines) to prevent infections could be used to reduce cancer rates. Vaccines against two agents (hepatitis B virus and human papilloma virus) are already approved and appear to reduce cancer rates, whereas vaccines for the other infections are in development.

13.6.1 Hepatitis B Virus

Chronic hepatitis B virus infection causes ~55% of hepatocellular carcinoma worldwide and of these ~80% occur in the Asia Pacific and sub-Saharan African regions where hepatitis B virus infection rates are high. Immunization of infants against hepatitis B virus has markedly reduced chronic infection rates which has been associated with a ~70% reduction in hepatocellular carcinoma cases in immunized individuals[105].

13.6.2 Human Papilloma Virus

There are two quadrivalent human papilloma virus vaccines expressing antigens of the four most important human papilloma virus types related to cervical cancer (types 6, 11, 16, 18) and marketed under the trade names Gardasil and Silgard. The vaccines are composed of virus-like particles made from recombinant human papilloma virus capsid proteins. These virus-like particles are highly immunogenic and generate high titer type-specific neutralizing antibodies that prevent infection, and can also generate some degree of cross-protective antibodies against other human papilloma virus types. Unlike hepatitis B virus vaccines which can be given to infants, it is not recommended to vaccinate children against human papilloma virus before 9 years of age. Also, unlike hepatitis B virus vaccine, antibody levels do not correlate with protection from infections. Economic modeling suggests that these vaccines will be a cost-effective means to reduce the health care burden of human papilloma virus-related precancerous lesions (such as anogenital warts) and cancers, particularly cervical cancer[106, 107]. It is not clear if these vaccines will protect against human papilloma virus-related squamous cell carcinomas of the head and neck, but this is an area of investigation.

13.6.3 Other Oncogenic Viruses

Vaccines for hepatitis C virus and Epstein–Barr virus infection are in active development, but few have undergone clinical trials, and none have been found sufficiently effective to merit follow up trials. Kaposi sarcoma herpes virus vaccines have lagged[104].

13.7 Strategies to Alter the Microbiome

The Human Microbiome Project has identified many aspects of the human microbiome that could help understand their contributions to health and disease[108]. It is now recognized that the microbiome can contribute to cancer risk[109] and that microbiota, especially colonic flora, can greatly affect immunity and inflammation[110, 111] and can specifically be involved in inflammation-associated cancers, notably colon cancers[112, 113]. Specific contributions of colonic microbial effects on colon cancer rates are under investigation[114, 115]. Important findings include the observation that enterotoxigenic *Bacteroides fragilis,* a major human commensal bacterium, can induce colon cancer in mouse models and has been implicated in human colon cancer[116]. Understanding which bacteria drive inflammation or immune dysregulation and how that contributes to carcinogenesis could help develop novel cancer prevention and treatment approaches.

13.8 Altering Metabolic Effects in Tumors and Immune Cells

Attempts to capitalize on unique metabolic features have figured into novel anticancer strategies for decades[117]. Recently, efforts have been made to capitalize on metabolic features of the local tumor environment or immune cells themselves to develop novel anti-tumor immunotherapies. For example, tumor lactic acid induces vascular endothelial growth factor in a HIF1α-dependent manner in tumor cells and induces dysfunctional M2 macrophage polarization in bone marrow derived macrophages[118], similar to the phenotype of tumor-associated macrophages. This lactate production, along with other acids conspires to lower tumor microenvironmental pH. Low pH was recently demonstrated to induce T cell anergy[119] suggesting that strategies to increase tumor pH could improve effector T cell functions and anti-tumor immunity.

Indoleamine-2′3′-dioxygenase is well known to alter tryptophan metabolism, thereby diverting T cell differentiation into the dysfunctional regulatory T cell pathway in cancers, but now also appears to have effects on glucose metabolism that affect T cell differentiation[120]. Glut-1 levels in renal cell carcinoma tissue inversely correlated with $CD8^+$ T cell infiltration[121], suggesting a link between tumor metabolic effects and anti-tumor immunity. Regulatory T cells and effector

T cells differ metabolically in that the former preferentially use fats and the latter use glucose as their primary energy source[122]. As tumors also preferentially use fats over glucose[117], means to target tumors and dysfunctional regulatory T cells with the same metabolic inhibitors could be possible.

CD73 is an ecto-enzyme that regulates adenosine metabolism and can thereby hinder T cell activation. We showed that CD73 expressed by both tumor and immune cells hinders anti-tumor immunity. We blocked CD73 enzymatic activity and improved immune and clinical outcomes in models for ovarian cancer and other tumors[123, 124].

13.9 Immune Modulating Effects of Drugs Initially Developed for Other Indications

In the last several years, it has become better appreciated that many agents developed for other purposes, including many anti-cancer agents have immune modulating effects. As we understand the immune modulating effects of these agents not developed as immune modulators, their use in treatment of cancer could be modified.

13.9.1 Anti-Regulatory T Cell Effects

The IL-2/diphtheria toxin fusion protein denileukin diftitox, the anti-CD25 monoclonal antibody daclizumab and the cytotoxic agent cyclophosphamide relatively selectively deplete regulatory T cells in human cancer patients, and a host of other agents reportedly also reduce regulatory T cell numbers in cancer patients or in preclinical models[12]. These agents are undergoing clinical trials as regulatory T cell depletion agents alone and combined with vaccines and other agents. Please see Chap. 9 for additional details.

Vascular endothelial growth factor[125], prostaglandins[126], estrogen[127], and tyrosine kinases[128] contribute to regulatory T cell differentiation and/or function in some tumors. Agents blocking these signals have demonstrated potential for regulatory T cell depletion in addition to their known mechanisms. For instance, blocking vascular endothelial growth factor reduced the number of intratumoral regulatory T cells in mouse models of colon cancer and melanoma[125], and cyclophosphamide, a standard cytotoxic chemotherapeutic[129], has long been known to inhibit suppressor T cells[130], now recognized as due to regulatory T cell depletion[131], and the multi-tyrosine kinase inhibitor sorafenib reduces regulatory T cells in patients with renal cell carcinomas[128] as a few examples.

13.9.2 Anti-Myeloid Derived Suppressor Cell Effects

5-fluorouracil[132] and gemcitabine[133] are cytotoxic chemotherapeutic agents now also known to deplete myeloid derived suppressor cells in mouse models for cancer. Both drugs are associated with good clinical outcomes. Liposomes selectively kill myeloid derived suppressor cells because they are phagocytosed and then are cell toxic[134]. In 23 patients with renal cell carcinoma, 4 weeks of oral sunitinib (a multi-tyrosine kinase inhibitor) reduced blood myeloid derived suppressor cells by ~50%[135]. These data suggest that these agents (and capecitabine, a 5-fluorouracil pro-drug), could be useful to deplete dysfunctional myeloid derived suppressor cells and thus become part of novel anti-cancer immunotherapy.

13.9.3 Other Effects

Some chemotherapeutic agents can themselves elicit tumor immunogenicity through the calreticulin pathway[136, 137]. Statins, histone deacetylase inhibitors and PPAR agonists, have anti-cytokine properties that are under investigation as cancer treatment or prevention agents[138].

13.10 Sex and Hormonal Effects

Immunity in men and women differ in important regards such as the observation that, compared to males, females generally exhibit better anti-infectious agent immunity[139], have more pronounced allograft rejection[140] and have a generally higher rate of many autoimmune diseases[141]. We recently reported that in a mouse model of B16 melanoma, females resisted tumor challenge better and developed better anti-tumor immunity than males if the immune co-signaling molecule B7-H1 was genetically deleted, or blocked in wild types with an antibody[142]. Recent work from us demonstrates that the defect is due to differential B7-H1-dependent estrogen effects on regulatory T cells[143]. Given the considerable attention to immune co-signaling blockade as novel anti-cancer immunotherapy (see Chap. 8 for details), it will be important to understand these hormonally driven differences and to understand what other sexual dimorphisms could be operative in other strategies. For example, males and females respond significantly differently to inflammatory pathways such as through TLRs[144, 145], which are also under intense investigations as cancer treatment or prevention agents (see Sect. 4 above).

13.11 Tumor Stroma

It is now clear that tumor stroma plays a critical role in tumor growth and tumor immune evasion. Stromal elements include immune cells recruited to the tumor microenvironment. The importance of these cells, including macrophages, dendritic cells, B cells, natural killer cells, and other cells has all been extensively reviewed recently in excellent global reviews[146, 147]. The relevance of myeloid cells to tumor inflammation is discussed above in Sect. 3 above, and myeloid cells are further addressed in Chap. 10.

B cells can contribute to tumor immunopathology in numerous ways. For example, they block macrophages from making the chemokines CCL3 and CCL5 to recruit protective $CD8^+$ T cells and from making the anti-angiogenic chemokine CXCL10. B cells can contribute to cancer metastases[148–150]. Non-regulatory T cells can be cancer-promoting[151] and platelets can promote tumorigenesis including metastatic spread[152]. Relationships between cancer stromal leukocyte populations and treatment outcomes with cytotoxic and immune agents have been reported[153]. Thus, there are many avenues for development of novel therapeutic attack based on altering immune cell trafficking into tumor stroma, or altering their interactions in stroma, that remain to be explored.

Non-hematopoietic cells have also received considerable attention as agents of cancer pathology and immunopathology. For example, cancer-associated fibroblasts protect tumor cells and dysfunctional immune cells from necrosis, induce vascularization and promote tumor growth. They contribute to immunopathology directly by producing pro-inflammatory cytokines and indirectly by attracting and/or altering tumor-associated stromal cells such as macrophages through NF-κB-dependent mechanisms[154, 155]. The role of stromal fibroblasts in tumor immunopathology is becoming better established[46].

Tumor stroma can be targeted directly by stromal-specific immunotherapy that does not recognize tumor and nonetheless mediate significant tumor regressions[156–158]. Tumor-stromal cell interactions that protect from chemotherapy were noted in Sect. 5.1 above. Tumor stroma can increase multidrug resistance gene expression that can reduce the efficacy of chemotherapy[159]. Additional investigations of tumor stroma effects on multidrug resistance gene expression and how that affects immunotherapeutic (and other) treatment approaches are warranted. Cell senescence in the tumor stroma, and its relationship to inflammation and immunopathology has been the subject of several excellent reviews recently[160–162].

13.12 Epigenetic Approaches

Targeting epigenetic changes in tumors has proven a successful strategy, with the FDA approval of the epigenetic modifiers 5-azacitadine (Vidaza; Celgene Corporation), decitabine (5-aza-2′-deoxycytidine, Dacogen; Eisai

Pharmaceuticals) and suberoylanilide hydroxamic acid (SAHA, vorinostat, Zolinza; Merck Pharmaceuticals). Recently, epigenetic modifiers, including 5-azacytidine and others have been demonstrated to alter immune functions, including driving Th1 or Th17 polarization of CD4$^+$ T cells[163, 164]. Azacitidine and 5-aza-2′-deoxycytidine appear to affect natural killer cells differentially[165]. Investigators are now attempting to understand if epigenetic alterations in immune cells will improve anti-tumor immunity.

13.13 Cancer Stem Cells

Cancer stem cells resemble non-malignant stem cells in self-renewal and variable differentiation fates, relative replicative quiescence and high resistance to common cytotoxic agents[166]. Originally identified in acute myeloid leukemia, cancer stem cells have now been tentatively identified in several epithelial carcinomas including the common cancers of colon, breast, and lung[167] and also in relatively less common, but nonetheless important cancers including melanoma, ovarian cancer, gastric cancer, and others[168].

Anti-vascular endothelial growth factor antibody plus cyclophosphamide synergistically reduced cancer stem cell like cells in a xenotransplant glioma model[169]. Various labs are testing a variety of strategies to treat cancer stem cells[170]. As cancer stem cells express antigens distinct from more differentiated cells, they could be amenable to specific immune approaches. Evidence that anti-cancer stem cell immunity can be generated[171] supports optimism for this approach that is still in its infancy.

13.14 Miscellaneous Small Molecules and Approaches

13.14.1 Mitogen-Activated Protein Kinase Inhibitors

p38 mitogen-activated kinases regulate many cellular processes including inflammation[172]. A small molecule p38 mitogen-activated kinase inhibitor improved dendritic cell function and reduced pro-inflammatory cytokine secretion in human myeloid cells in vitro[173]. Several p38 mitogen-activated protein kinase inhibitors are in clinical trials, including for reducing cancer cachexia. Clinical uses of these agents were recently reviewed[174]. A vast number of multi-tyrosine kinase inhibitors are in development and in clinical trials, many of which have potential immune effects that remain little studied thus far. These agents represent an excellent farm team from which to select promising drugs to develop for direct effects on tumors as well as for their immune modulating effects.

13.14.2 Custom Anti-Idiotype Vaccines

BiovaxID is a personalized vaccine to treat non-Hodgkin B-cell lymphomas in combination with GM–CSF. It targets malignant B cells by generating tumor-specific anti-immunoglobulin idiotype antibodies. Its development is through Biovest International, Inc. (a subsidiary of Accentia BioPharmaceuticals, Inc.), under license from Stanford University. Phase I and II clinical trials have established immunogenicity, safety and clinical efficacy, leading to ongoing phase III clinical trials in non-Hodgkin B cell lymphomas[175]. Updated clinical trial results remain encouraging and were recently discussed[176].

13.14.3 Thalidomide Congeners and Derivatives

Thalidomide has been used to treat multiple myeloma because of anti-angiogenic properties and ability to inhibit the production of pro-inflammatory cytokines including IL-6. Thalidomide analogs have been developed that have significantly greater potency in inhibiting pro-inflammatory cytokine production, preventing angiogenesis and with potential direct tumor toxicities, collectively known as IMiDs (immunomodulating drugs)[177]. The IMiD lenalidomide is FDA-approved to treat multiple myeloma. Its congeners, including pomalidomide (under review by the FDA for approval), are being evaluated to treat additional hematologic malignancies[178] and epithelial carcinomas such as prostate cancer[179].

13.14.4 Targeting Adenosine Metabolism

Extracellular adenosine can be increased in the tumor microenvironment where it can be immunologically suppressive or dysfunctional[180, 181]. CD73 is an ecto-5′-nucleotidase that generates adenosine from local adenosine monophosphate[182, 183]. CD39 is an ecto-ATPase that generates adenosine from local adenosine triphosphate. Adenosine generated either by CD39 or CD73, but generally by both working in concert has pleiotropic effects in the tumor microenvironment by augmenting angiogenesis, and the properties of the tumor or local immune cells including invasion, growth, cellular adhesion, and chemotaxis[184]. We and others have shown that anti-CD73 antibodies or small molecule CD73 antagonists can boost tumor immunity or the efficacy of adoptive T cell therapy for cancer in mouse preclinical models (reviewed in[185]).

13.14.5 Levamisole

Levamisole in combination with 5-fluorouracil was FDA-approved in 1990 for adjuvant treatment of Duke's stage C colon cancer. The approval was based on results from two randomized, controlled trials in colorectal cancer patients with resected tumor. Levamisole as a single agent was not effective in the bigger of the two studies, but levamisole plus 5-fluorouracil reduced tumor recurrence by 41% ($p < 0.0001$) and deaths by 33% ($p = 0.006$) versus no treatment with a minimum 2-year follow-up. However, the levamisole effect on 5-fluorouracil was not clearly established. Levamisole is not a regular treatment option for colorectal cancer in current practice guidelines and is mentioned largely for historical purposes.

In melanoma treatment, a meta-analysis of four levamisole trials did not find a significant survival benefit as a result of levamisole versus no treatment and no survival benefit in randomized controlled trials of combinations of levamisole with vaccines (nine trials) or chemotherapy (ten trials)[186]. Nonetheless, levamisole and other anti-parasitics have important immunomodulatory effects[187] that suggest that further investigations into their potential as anti-cancer agents could be warranted.

13.15 Summary and Future Directions

This chapter on miscellaneous approaches makes plain that the field of tumor immunotherapy is rapidly growing and testing ever-increasing numbers of treatment approaches as our understanding of cancer immunopathogenesis increases. It is also easy to see that many approaches defy simple pigeon holing and could well be described under several distinct sections. For example, approaches targeting tumor-stromal fibroblasts could be described as anti-stroma, anti-inflammation through reduced fibroblast-driven inflammatory mediator production, or anti-angiogenic through reduced angiogenesis factor production among other potential labels. Many additional strategies are also under development. For example, CD47 is an anti-phagocytic molecule expressed on many cancers. Blocking CD47 improves tumor phagocytosis in vitro and slows tumor growth in vivo in mouse xenograft models[188, 189]. We recently described how immunotherapy not only could be used as an adjunct to treat multidrug-resistant tumors, but also pointed out that features of *mdr* gene expression driving resistance have already been exploited in specific immunotherapeutic approches[159]. Much additional work lies ahead to understand which of these various approaches merit follow up and translation, and how best to combine them optimally with each other, and with additional strategies. We anticipate many novel approaches to be tested in the next several years, with potential to make significant improvements in clinical outcomes of anti-cancer immunotherapy.

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Chapter 14
Monitoring Antigen-Specific Responses in Clinical Trials of Cancer Immunotherapy

Aude G. Chapuis and Cassian Yee

Abstract Immune-based therapies are designed to generate or augment anti-tumor immune responses to achieve clinical benefit. Monitoring quantitative and qualitative parameters of immune function affords the opportunity to identify endpoints that correlate with, or predict clinical benefit and define the requirements for effective therapy. This chapter discusses structural and functional methods to assess both monoclonal and polyclonal antigen-specific T cell responses in vivo in humans. The most adequate methods to detect responses in blood and tumor tissue are examined with a focus on the information provided by evaluating the phenotype of tumor-specific cells. Because considerable variability in the type and performance of immune-monitoring assays exists, harmonization is required to render meaningful comparisons among the increasing number and complexity of immune-based clinical trials.

14.1 Structural Assessment of Monoclonal T Cells Responses

Structural assessment of cellular immune responses represents an important requisite in defining responses after intervention (active vaccination, adoptive cell transfer) and involves calculating the frequency or absolute number of T cells in blood or tissue that recognize specific antigens on the basis of a structural component (i.e., T cell receptor (TCR) sequence) (see Table 14.1).

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Table 14.1 Comparison of structure-based assays

Assay	Peptide-MHC complexes	TCR tracking	Spectratyping	High-throughput DNA sequencing
Sensitivity	1:2000–1:5000 $CD8^+/CD4^+$	1:100,000 PBMC	NA	1:100,000 lymphocytes
Advantages	Multiple parameter analysis possible on the same cell; cells remain viable after assay; not dependent on T cell function; assessment of polyclonal populations possible; highly reproducible allowing precise quantitation.	Detects T cells based on TCR rearrangement rather than specificity; detects class I and class II responses.	High-throughput analysis; assesses skewing of the TCR repertoire.	Detects T cells based on TCR rearrangement rather than specificity; tracking of polyclonal populations possible; high throughput analysis.
Disadvantages	Requires specific pMHC complex for every T cell specificity examined with prior knowledge of peptide and restricting allele; highly dependent on availability of pMHC complexes.	Terminal assay; multiple parameter analysis not possible; not suitable for following polyclonal responses.	Terminal assay; semi-quantitative; does not precisely identify the CDR3; multiple CDR3s can have the same length thus confounding results.	Terminal assay; multiple parameter analysis not possible; cannot distinguish between $CD4^+$ or $CD8^+$ T cell based on TCR sequence; expensive.
Applications	Longitudinal tracking of spontaneous, adoptively transferred or vaccine-induced responses.	Adoptive T cell therapy of clonal cells; tracking known T cell clonotypes in vivo.	T cell infusions/immune reconstitution after lymphodepleting chemotherapy.	Longitudinal tracking of polyclonal responses in vivo.

NA not applicable, *PBMC* peripheral blood mononuclear cell, *pMHC* peptide-MHC (major histocompatibility complex) complex, *TCR* T cell receptor

14.1.1 Structural Assessment of Specific CD8$^+$ T Cells Using Peptide-MHC Complexes

During development in the thymus, CD8$^+$ T cells commit to the expression of one specific, productive TCR. The sequence that ultimately determines the TCR expressed by a CD8$^+$ T cell is determined by somatic rearrangement of TCRα and TCRβ loci during T cell development. With an estimated 10^{15} possible unique TCR sequences, the potential TCR repertoire is larger than an individual's total T cell compartment estimated at 5×10^{11} cells[1]. However, more than one CD8$^+$ T cell (and TCR) can recognize a given peptide ligand presented by a specific major histocompatibility complex (MHC) molecule. With the exception of immune privileged sites such as the testis[2, 3], MHC class I molecules are expressed on the surface of all nucleated cells. Thus CD8$^+$ T cells can survey the body for intracellular infections or tumor cells that perturb the MHC-associated peptide pool.

In the late 1990s fluorescence-labeled multivalent recombinant peptide-MHC complexes were developed that have now become an essential tool in monitoring and characterizing immune responses[4]. Peptide-MHC (pMHC) class I complexes are produced by refolding a defined MHC allele with β2-microglobulin and a peptide of interest, to generate large quantities of complexes, or refolded protein[5]. To counter the inherent low affinity and high off-rates of a T cell receptor for its specific pMHC, multiple purified pMHC complexes are linked to a backbone scaffold (e.g., streptavidin, dextran, coiled coil domains) and conjugated to a fluorochrome for fluorescence-based detection. Structures with increasing amounts of complexed MHCs (tetramers, pentamers[6], dextramers[7], or Q-dot conjugated multimers[8]) have been used to increase the mean fluorescence intensity and separation of the detected CD8$^+$ T cell population from non-binding populations[9]. Although efforts are being made to establish manufacturing standards, large variability exists between locally produced constructs[10]. Because this technique can be used both to detect and sort for antigen-specific CD8$^+$ T cell populations, in some cases, at frequencies as low as 1 in 10,000 total CD8$^+$ T cells, pMHC constructs have become an invaluable tool to track CD8$^+$ T cell responses over time and allow a multiparametric characterization (see *Phenotype of Antigen-Specific T cells in vivo section*).

Cross-linking of the TCR by pMHC at physiological temperatures can lead to prolonged signaling, alteration of CTL function, and induction of apoptosis which is problematic if CTL are sorted for downstream applications[11, 12]. To circumvent this alteration in peptide complexes, methods have been developed that enable the TCR to bind reversibly to pMHC allowing for selection followed by disengagement, thereby preserving T cell function[10, 13].

As pMHC binding defines a specific T cell population that represents a single fluorescent signal and a unique HLA-peptide combination, this method provides detailed but restricted information on a specific CD8$^+$ T cell response while neglecting other potential responses elicited by other epitopes for the same antigen. Unless the immunodominant response is defined by one or more *known* pMHC

epitopes, the use of multimer-peptide MHC complexes to characterize comprehensive responses towards antigen lack power in providing an overview of the breadth of the immune response. Detection of multiple CD8$^+$ T cell responses using pMHC have been developed in recent years in an attempt to expand the breadth of detection. PMHC (peptide MHC) *combinatorial encoding* analysis of T cell responses uses pMHC complexes bound to multiple fluorochromes[14, 15]. Two-dimensional (2 color) coding and a total of 8 different flurochromes allow for the potential testing of a total of 28 different pMHC in a single patient sample. With additional recombinant MHC molecules available for folding and the continued characterization of new pMHC combinations, standard "cocktails" of pMHC for patients with different HLA types could presumably allow the tracking of multiple antigen-specific T cell populations.

Overall, the use of multimers remains dependent on the ability to generate functioning pMHC complexes for specific haplotypes and previously characterized peptide-MHC combinations. For a more comprehensive approach of the immune response directed towards a specific protein/antigen without the restriction imposed by the ability to generate pMHC complexes, other techniques are available (see Sect. 14.3).

14.1.2 Structural Assessment of Specific CD4$^+$ T Cells Using Peptide-MHC Complexes

Technical difficulties associated with the generation of class II pMHC multimers to detect antigen-specific CD4$^+$ T cells have rendered their widespread use more challenging when compared to class I multimers. As deletion of the transmembrane domains of class II MHC results in the dissociation of the α and β chains, expression of MHC class II in bacterial systems can be inefficient. Furthermore, the pMHC class II chains bind with lower affinity to each other than class I and in most cases the addition of peptide is not sufficient to stabilize the MHC-peptide complex. Thus, more elaborate recombinant expression systems such as insect cells, which lack antigen processing and loading machinery, have been used to generate peptide-free MHC class II molecules, which are in turn loaded with the desired immunogenic peptides in vitro (e.g., HLA DRB1*0101 or DRB1*0401)[16, 17]. Alternative methods pairing the C-termini of the subunits with a "coiled coil" domain or leucine zipper to promote assembly of the native α-β heterodimer have also been developed and produce complexes which are of limited stability[16–18].

Biological differences between MHC class I and II multimers further contribute to the observed low frequencies and low mean fluorescence intensities of antigen-specific CD4$^+$ T cells within peripheral blood mononuclear cells. Class II multimer binding might detect a limited fraction of all CD4$^+$ T cells specific for a given cognate antigen, and the CD4 molecule, unlike CD8, does not strengthen the MHC-peptide interaction on the surface of the cell[19, 20]. Lastly, MHC class II-peptide complexes obtained by peptide loading of empty MHC class II complexes are not as homogeneous as class I-peptide complexes obtained by refolding[21]. These differences

negatively impact MHC class II multimer staining and may explain, in part, the discrepancy observed between MHC class II-peptide multimer staining and functional responses as measured by intracellular cytokine staining or ELISpot[22].

Despite these difficulties, antigen-specific CD4$^+$ T cells have been detected in peripheral blood mononuclear cells[23, 24] allowing valuable direct phenotypic analysis of CD4$^+$ T cell responses. Increasing detection thresholds by magnetically sorting CD4$^+$ T cells, stimulating specific CD4$^+$ T cells with peptide prior to staining[25, 26] and/or coupling functional assays in combination with MHC class II staining experiments offer additional information beyond T cell frequency and can be equally applied to monitoring clinical trials as class I MHC peptide reagents.

14.1.3 Structural Assessment of T Cell Frequencies Using TCR-Tracking

The nucleotide sequence encoding the TCRβ chain is determined by somatic rearrangement of V, D, and J segments and the complete VDJ junction region or CDR3 region is unique to each TCR. Quantitative amplification of the CDR3 region can be used to track the frequency of a defined clonal T cell population[27–29]. Using a monoclonal T cell population (for example, an adoptively transferred T cell clone) as template, primers flanking the constant region and the specific TCR Vβ region, as well as a probe encompassing the unique TCR CDR3 are designed and used in Taqman assays. Although a primer/Taqman probe set must be configured for each tracked clonotype, TCR tracking not only allows for a very sensitive detection of clonal cells (up to 1/100,000 total cells) in RNA/DNA isolated from peripheral blood mononuclear cells but also allows detection of T cells in scarce or restricted tissue samples. To normalize the number of specific TCR copies, two different approaches have yielded comparable results. In the first RNA-based approach, cDNA derived from whole peripheral blood mononuclear cell RNA is used a template for the quantitative TCR-specific PCR. A concurrent quantitative Taqman assay for the CD8β chain (for CD8$^+$ T cells) is used. Based on the assumption that each CD8$^+$ T cell yields proportionally the same relative number of copies of CD8β and TCR, TCR copies are quantified as a percent of total CD8β chain detected. Alternatively, genomic DNA isolated from mononuclear cells can also be used as a template for a quantitative real-time PCR assay. The frequency of the specific T cells in peripheral blood mononuclear cells is normalized, for example, to the house-keeping gene β-actin. Both methods were validated in patients in whom T cells could also be tracked by pMHC complexes and results are nearly identical[30]. This method is exquisitely sensitive, has a lower detection threshold compared to pMHC multimers and can conveniently be used with T cells for which a pMHC complex is unavailable. However, in contrast to T cells detected by pMHC multimers, no further phenotypic characterization can be performed as cells are lysed in the process of detection.

14.2 Functional Assessment of T Cell Responses in Vivo

Functional assessment of cellular immune responses provides information on the ability of antigen-specific cells to deliver a functional feature (e.g., cytotoxicity, cytokine secretion) that is associated with tumor recognition and can also be used to assess T cell frequency, but on the basis of functional and not structural features (see Table 14.2). T cell activation is a consequence of direct cell contact based on the target cell's surface expression of MHC-peptide complexes and co-stimulatory ligands. Whereas the term "affinity" is used define the strength of binding of the TCR with its peptide-MHC ligand, the term "avidity" reflects the binding between the effector and target populations involving multiple receptors/ligands. Because the circulating peripheral T cell pool consists of T cells with a sufficiently low avidity for self-antigens to have avoided negative thymic selection and deletion[31], high-avidity T cells reactive against tumor-associated self-antigens are less frequent than high-avidity T cells reactive to viral associated antigens. In these cases, functional assessment will often underestimate the total number of antigen-specific T cells, but could more accurately represent T cell activity. The concept is discussed in detail in Chapter 3.

14.2.1 Direct Cytotoxicity Assays

To assess the specific cell-mediated cytotoxicity or killing potential of a T cell population towards a target population of cells, the ^{51}chromium-release assay has remained the gold standard since it's inception in 1968[32, 33]. Target cells are labeled with radioactive ^{51}Cr and incubated with an effector cell population, and ^{51}Cr released from the killed target cells within the culture medium is measured within 4–5 h by a γ-ray emission counter. Although several alternatives have been developed to measure direct cytotoxicity based on the release of non-radioactive compounds[34, 35], these alternatives have not been widely adopted by the scientific community in part due to the superior ability of ^{51}Cr to label a wide variety of target cells reproducibly (for e.g., B lymphoblastoid cells lines, CD40-L activated B cells, monocytes, adherent cells, and fibroblasts), low rates of spontaneous release, and its easy applicability. This assay also offers the possibility to determine the avidity of the effector population to limiting amounts of antigen for direct comparison. The major limitation to the ^{51}Cr release assay remains the use of radioactive material requiring adequate disposal and dedicated facilities. This assay also demands significant amounts of pure effector and target cell populations (clonal T cells or purified T cell lines) and provides no information on the behavior, the type of cytotoxicity or the phenotype of effector cells within the assay. Furthermore, autologous tumor cells that would constitute the most accurate measure of cytotoxicity are notoriously troublesome to load with ^{51}Cr and have a high rate of spontaneous release[35]. An emerging alternative to directly quantify killing activity of tumor cells is the caspase cleavage assay, which detects caspase enzyme activation indicative of early stage apoptosis either by direct measurement of caspase

Table 14.2 Comparison of function-based assays

Assay	Direct cytotoxicity	ELISpot	Intracellular cytokine secretion	Proliferative responses	Cytokine arrays
Sensitivity	1:300–1:500 targets	1:50,000–1:100,000 PBMC	~1:500–1:5000 $CD8^+$/ $CD4^+$ T cells	NA	~0.01 pg/ml
Advantages	Most direct assessment of T cell killing potential; easy applicability; titration and comparison of the avidity of different antigen-specific populations possible.	High-throughput analysis of class I and class II responses to multiple peptides; least labor intensive per sample basis; does not require specific pMHC multimer.	Multiple cytokines can be assessed simultaneously on same cell; evaluation of class I and class II responses possible; does not require specific pMHC multimer but simultaneous pMHC binding or phenotypic evaluation of secreting cells possible.	High-throughput; easy applicability; non-toxic; (Resazurin). Specific; detection of proliferation highly suggestive of $CD8^+$ Tcm if no exogenous cytokines are added; multiparameter analysis possible (CFSE dye).	High-throughput; easy applicability; assessment of the largest amount of cytokines (>30) simultaneously.
Disadvantages	Uses radioactivity (^{51}Cr); terminal assay (^{51}Cr); purified effector populations preferred; does not provide information on the type of cytotoxicity exerted by or phenotype of effectors.	Multiparameter analysis not possible.	Terminal assay; labor intensive.	Uses radioactivity; non-specific (^{3}H thymidine). labor intensive; requires 5–7 day cultures; pMHC multimer preferred (CFSE).	Secreting cell cannot be identified or further analyzed; broad assay variability; large inter- and intra-patient fluctuations of cytokine concentrations.
Applications	Comparison of the avidity of different Ag-specific effector populations.	Peptide screens, epitope spreading for vaccine or adoptive immunotherapy.	Assessment of antigen-specific cells generated after immunotherapy.	Complements characterization of antigen-specific cells.	Biomarker identification; assessment of "cytokine storms."

NA not applicable, *pMHC* peptide-MHC (major histocompatibility complex), *CFSE* carboxyfluorosuccinamide ester dye, *PBMC* peripheral blood mononuclear cells

catalytic activity, cleavage of their substrates or labeling using conformation-sensitive antibodies followed by flow cytometric detection[36].

14.2.2 ELISpot Assays

The enzyme-linked immunosorbent assay as applied to cell functional detection (*ELISpot*) involves stimulation of antigen-specific T cells on immunosorbent membrane micro-well plates coated with anti-cytokine capture antibodies. After removal of the cells, localized cytokine production by individual cells can be detected by a second anti-cytokine antibody that is conjugated to a colorimetric reagent, appearing as individual spots on the membrane. T cell frequencies can then be calculated as the number of spots per input number of cells (for example, peripheral blood mononuclear cells) in each micro-well[37]. ELISpots are highly reproducible, sensitive (~1/100,000 events), ideal for detecting low-frequency antigen-specific T cells, and require approximately tenfold less material than intra-cellular cytokine assays (see the following sect. 5). ELISpots are suited for measuring T cell reactivity to peptide pools or peptide libraries requiring multiple parallel testing. A semi-quantitative measure of the avidity of antigen-specific T cells within peripheral blood mononuclear cells can be determined by serial dilutions of cognate antigen. Unlike intracellular cytokine secretion, cells are unaltered by the assay and can be further isolated[38, 39]. Historically, the detection of interferon (IFN)-γ was used as a surrogate to detect all pro-inflammatory T cells capable of cytotoxicity in vivo[40]. However, the induction of IFN-γ- producing T cells has been shown in vaccine trials not necessarily to correlate with tumor regression or viral immune protection[41, 42], and detecting additional effector cytokines such as IL-2, granzyme and perforin (discussed below) could represent a more comprehensive evaluation[43]. Although newer fluorescent ELISpot reagents allow for the assessment of up to 3 simultaneous cytokines, these are not yet available for widespread application and other techniques, such as intracellular cytokine secretion offer a more comprehensive assessment[44, 45].

14.2.3 Intracellular Cytokine Secretion Assays

Intracellular cytokine secretion assays allows the simultaneous detection of several cytokines secreted by a single cell in response to cognate antigen to provide comprehensive information about the in vivo killing potential (most commonly for $CD8^+$ T cells) and cytokine expression profile (for example $CD4^+$ T cells) of antigen-specific T cells. Briefly, effector $CD8^+$ T cells function in multiple ways to cause tumor rejection either by directly killing tumor cells or by changing the micro-environment of the tumor to inhibit further growth. Amongst other mechanisms, cell to cell contact and recognition of peptides presented by MHC class I leads to the exocytosis of lytic granules containing perforin, a toxin that

forms pores in the target cell plasma membrane, serine proteases such as granzymes (particularly granzyme B) and chemokines such as MIP-1α, MIP-1β, and RANTES which promote the recruitment of additional cells to the site of the inflammatory response. Granzymes enter the target cells through the newly formed pores and cleave aspartate residues, which in turn activate the caspase cascade and lead to apoptosis of the target cell. Effector cells can also secrete TNF-α family members (TNF-α, FasL, TRAIL) which induce multimerization of their cognate receptors on target cells resulting in the induction of apoptosis[46]. The most defined role for IFN-γ is enhancement of MHC class I and Fas levels on target/tumor cells that increases their sensitivity to $CD8^+$ T cell killing[47]. Although IL-2 does not have a direct cytolytic effect, antigen-specific $CD8^+$ T cells that secrete IL-2 can use it as an autocrine growth factor promoting their survival and further proliferation after secondary antigenic encounter[48].

Whereas perforin and granzyme are produced constitutively in effector cells delivering immediate cytotoxic effect, cytokines are produced upon relevant stimulation within 4–5 h[49]. Intracellular cytokine staining relies upon the stimulation of T cells in the presence of an inhibitor of protein transport thus retaining the produced cytokines inside the cell. While it remains clear that $CD8^+$ T cells and their cytotoxic capacity are a critical component of the cellular immune response against tumors, precise correlates of tumor lysis remain to be defined[43]. Polyfunctional T cells capable of multiple functions including the production of IL-2, TNF-α, IFN-γ, the chemokine MIP-1β, along with the expression of the surface degranulation marker CD107a, have shown to be generated in broadly protective vaccines such against vaccinia virus[50]. These have also been shown to correlate more reliably with the control of human immunodeficiency virus replication than human immunodeficiency virus-specific T cells with a more limited range of capabilities and might also be necessary for tumor regression[9, 51].

$CD4^+$ T cells likely play a critical role in orchestrating the adaptive immune response to tumors[9]. $CD4^+$ T cells capable of secreting multiple cytokines/chemokines including TNF-α, IFN-γ, and MIP-1β have been generated following tumor vaccination and adoptive transfer[23, 27, 52]. However, a substantial heterogeneity and plasticity exists within $CD4^+$ T cell responses in humans. $CD4^+$ T cells have the potential to differentiate in at least four functionally divergent T cell subsets defined by their cytokine profile: Th1 (producing IL-12 and IFN-γ), Th2 (producing IL-4, IL-5, IL-6, IL-10, and IL-25), Th17 (producing IL-17 and IL22) and regulatory T cells (Tregs, producing TGF-β and IL-10) which may have opposing tumor rejection or tolerant effects. These Th subsets variably have the capacity to convert from one subset to another[53]. See chapter 2 for details of T cell subsets in the tumor micro-environment. Overall, this heterogeneity and differentiation plasticity has limited the ability to define protective immune correlates of $CD4^+$ T cells both after preventive and therapeutic vaccination. Comprehensive analysis of the multifactorial functional parameters engendered after vaccination and/or adoptive transfer in both $CD8^+$ and $CD4^+$ T cell will be necessary to identify factors that correlate with the success or failure of an anti-tumor immunologic intervention.

14.2.4 Assessment of Proliferative Responses

A hallmark of an effective protective T cell response is the ability of antigen-specific cells to proliferate rapidly and robustly in response to re-encounter with antigen, which entails the capacity to produce new effector daughter cells[54, 55]. (^{3}H) Thymidine incorporation assays have been used to evaluate the proliferative function of antigen-specific T cells to cognate antigen[56]. DNA incorporation of (^{3}H) Thymidine during the S-phase of cell division is determined after 16–18 h of exposure by adsorbing the cells to a membrane. In addition to the disadvantages associated with the use of radioactivity, (^{3}H)thymidine incorporation assays are limited due to the inability to differentiate between dividing CD4$^+$ and CD8$^+$ T cells and regulatory CD4$^+$ T cells which have a broad dampening effect (see Sect. 15.5). Non-radioactive, nontoxic compounds such as Resazurin (Alamar Blue®), which is reduced in living cells to the fluorescent compound resofurin offers an attractive alternative to (^{3}H)thymidine incorporation as this allows further characterization of fluorescent cells by multiparameter flow cytometry[57]. Carboxyfluorescein succinimidyl ester can also label lymphocytes without affecting their function and track their ex vivo mitotic activity by the progressive twofold reduction in fluorescence intensity with each cell division. Staining with carboxyfluorescein succinimidyl ester allows simultaneous characterization of dividing cells by flow cytometry based on their T cell subset (CD4$^+$ or CD8$^+$), binding to multimer or expression of surface markers. Results for CD4$^+$ T cells are comparable to (^{3}H) thymidine incorporation assays[58]. Ex vivo carboxyfluorescein succinimidyl ester dilution is also particularly instructive to assess CD8$^+$ proliferative responses after 5–7 days to cognate antigen in the absence of exogenous cytokines (such as IL-2) both in anti-viral and anti-tumor responses, and the results have correlated with persistent transferred cells that had acquired a phenotype of long-lived memory cells in vivo[30, 59]. Alternatively, antigen-specific cells that can be identified by binding to a multimer can be directly assessed in vivo for evidence of division by measuring Ki-67 expression. Ki-67 is an intra-nuclear protein tightly associated with cell replication expressed in all active phases of the cell cycle (G1, S, G2, and mitosis), but absent from resting cells (G0)[60]. Ki-67 expression in multimer$^+$ cells provides a discrete snapshot of the cells' proliferative state without firm knowledge of specific antigen encounter, compared to a 5–7 day retrospective of proliferative history in the presence of controlled quantities of specific antigen provided by the ex vivo carboxyfluorescein succinimidyl ester dilution assay.

14.2.5 Monitoring Extracellular Cytokines

Technologies capable of quantifying multiple cytokines in a limited amount of sample such as serum or plasma, have rendered possible longitudinal quantification of cytokines in response to immune interventions. Although tissues such as whole

blood, plasma, and serum might not reflect events of the tumor micro-environment or identify the cell responsible for the secretion of cytokines[61], these are accessible, and variations in the cytokine levels can be easily detected[62, 63].

Two technologies are available to quantify released cytokines reliably in liquid samples: ELISA and multiplex cytokine bead arrays. Both are excellent screening tools due to their sensitivity and the amount of cytokines they can detect (>30). Cytokine bead assays, although more expensive at this time, allow a rapid flow cytometry-based analysis in very small volumes of specimen by coating polysterene microspheres with antibodies designed to capture the cytokines of interest[62]. Of note, gene expression platforms also support the simultaneous quantification of mRNA of multiple cytokines in cell subsets. However, due to posttranscription modifications and variability in the assays, these may not reflect actual cytokine expression[64]. Although some studies have been able to associate serum/plasma cytokine patterns with favorable/unfavorable responses in Hodgkin lymphoma or dendritic cell-based vaccines in colorectal cancer[65, 66], most studies have failed to establish a correlation of extracellular cytokine patterns obtained by multiple cytokine analysis, with the immunogenicity of candidate vaccines or with therapeutic benefit[61–63, 65–69]. This likely reflects the complexity, nuance, and plasticity of cytokine networks and cytokine feed-back loops within the tumor micro-environment[70, 71]. Nonetheless, studies examining responses to protective infectious disease vaccine have shown that the amount and diversity of cytokine produced in response to cognate antigen may be more relevant to assess than the type of cytokine secreted[72]. Recent studies showing that "cytokine storms" with the release of multiple cytokines correlate with lymphoma eradication after the adoptive transfer the gene-modified cells[73, 74] may point to new hope in our ability to identify global cytokine signatures of prognostic importance.

Furthermore, evaluating cytokine levels is subject to a broad variability which may further confound the results obtained. For example, basal cytokine levels vary for most individuals and within individuals over time, large fluctuations occur and positive values may not be sustained[65], different cut-offs for positive versus negative values have been used for the same cytokines between different studies, and different control populations have been used for comparisons. Efforts aimed at harmonizing results and standards (e.g., The Cancer Immunotherapy Consortium[75] and Minimal Information about T cell Assays project [www.miataproject.org]) are underway to extract the full potential of this powerful technology. Broad-based standardized serial monitoring of the cytokine milieu could become an integral part of future immunotherapy to provide valuable insights into relevant biomarkers[76].

14.3 Assessment of Polyclonal/Polyvalent T Cell Responses

As adoptive transfer studies are evolving towards generating polyclonal T cell products as opposed to monoclonal products requiring shorter ex vivo manipulation time, evaluating the presence and frequencies of polyclonal (same T cell specificity,

different T cell clone) antigen-specific T cells in vivo requires alternate methods of detection and enumeration. Furthermore, these methods can also be applied to polyvalent (different T cell specificity, different T cell clone) T cell responses generated, for example, after vaccination.

14.3.1 Tracking Polyclonal T Cells Using Multimers

Peptide-MHC multimers can be used to quantify the frequency of polyclonal T cells of a defined specificity (for example HLA A*0201-restricted MART1-specific $CD8^+$ T cells) as these will bind to the multimer regardless of their clonality. However, TCRs specific for an array of pMHCs can be elicited following vaccination and, without prior knowledge of the response, multimers are of lesser value. Alternatively, mapping of antigenic epitopes by function-based assays can offer a more exhaustive evaluation of the response elicited (see Sect. 14.3.4).

14.3.2 Tracking Polyclonal T Cells Using Vβ Arrays (Spectratyping)

Somatic rearrangement of V, D, and J segments of the TCRβ chain results in CDR3 segments of variable nucleotide length. Spectratype (or Immunoscope) analysis involves polymerase chain reaction (PCR) amplification of the rearranged CDR3 segments present in complex populations of cells. The PCR products are resolved on polyacrylamide sequencing gels to reveal the array of CDR3 sizes, which may vary in length by 30–60 nucleotides in a Gaussian distribution, within each of the 24 Vβ families[77]. The TCR repertoire of polyclonal T cell products destined for infusion can be assessed and compared to peripheral blood mononuclear cells collected after infusions. A skewing of the repertoire towards a specific Vβ length present within the infusion product is suggestive of the presence of infused T cells. Spectratyping is useful in cases where very large changes to the immune repertoire are likely to be observed, for example, following TIL infusions after near myeloablative conditioning regimens[78]. This technique allows for rapid throughput analysis of multiple samples. However, because the identity of the TCR is based on the CDR3 length and not direct sequencing, spectratyping cannot be used to identify a specific TCR as more than one TCR can have the same length. It also provides limited information when few, but diverse T cells are generated such as after vaccination[79].

14.3.3 Tracking Polyclonal T Cells Using High-Throughput TCR DNA Sequencing

With the advent of high-throughput DNA sequencing technology, methods for sequencing millions of TCRβ chains are available based on multiplex PCR methods and can potentially be applied to track polyclonal/polyvalent T cell responses quantitatively after adoptive T cell therapy or vaccination[80]. A pool of primers to all V and J pairs specifically designed to amplify the complete VDJ junction region such that only the minimal region (60 nucleotides) containing the clone-specific nucleotide information for each TCRβ CDR3 could be amplified and sequenced for several million T cells comprising an individual's immune repertoire[81, 82]. Using genomic DNA as a template, this method has the potential to capture the frequency of individual TCRs in biologic samples with accurate reproducibility and a sensitivity of 1/100,000 TCR-containing lymphocytes. This method also yields nearly identical quantitative results compared to pMHC multimers (also a structure-based assay) as demonstrated by direct assays of infused T cell clones in vivo[83], and has the potential to quantify and follow the expansion/contraction of a polyclonal T cell repertoire over time. As some VDJ rearrangements contain non-templated insertions and deletions at the junctions between the V and D segments, and the D and J segments, current methodologies allow for the identification of 75–90 % of unique CDR3s. If one of the primers falls in a deleted section, the sequence is not amplified and the unique sequence is not identified. Refining and improving this powerful technology has the potential to allow multiparameter tracking in large-scale clinical trials.

14.3.4 Assessment of Polyclonal/Polyvalent T Cell Responses by Screening for Antigenic Epitopes

To evaluate antigen-specific T cell responses elicited after vaccination or adoptive transfer without the constraints imposed by HLA restriction, identification of epitopes recognized by $CD4^+$ or $CD8^+$ T cells in study patients can be performed by testing the reactivity of T cells against pools of overlapping peptides spanning target proteins[84]. Peptides 15–20 amino acids in length offset by 1–5 amino acids bases and spanning target proteins offer the prospect of detecting both CD8-binding (9–11 amino acids) and CD4-binding (12–16 amino acids) epitopes. Longer peptides with a short offset length offer a higher likelihood of multiple epitope hits. Peptide pools are used to stimulate T cells which are then enumerated on the basis of cytokine secretion by ELISpot assay or intracellular cytokine staining (see relevant sections above). To map individual recognized peptides efficiently in each pool, whole peripheral blood mononuclear cells or selected $CD8^+$ T cells (in the case of MHC class I determination) can be performed by screening against smaller sub-pools aligned in a grid, in such a way that every peptide is contained in exactly

2 sub-pools and the intersection between the 2 sub-pools on the grid reveals the responding peptide[85]. MHC restriction can further be determined by HLA typing of subjects, testing responsiveness to HLA-restricted cells lines, epitope prediction algorithms (SYFPEITHI), or comparison to previously identified sequences[86]. Although overlapping peptide screens remain the most efficient tool for large-scale clinical trials, a considerable amount of blood is necessary to screen multiple peptide libraries. This large blood volume requirement in addition to the assay cost are generally considered limiting factors. Furthermore, in situations where the frequency of responding T cells is low, testing the reactivity to individual peptides could require using previously expanded T cells thus adding a supplementary modifying step to the detection process.

14.3.5 Epitope Spreading

Epitope spreading is an extension of the T cell response from one dominant antigenic peptide to include secondary epitopes distinct from and non-cross reactive with the dominant peptide. Epitope spreading is a recognized immune phenomenon in autoimmune diseases mediated by CD4$^+$-induced T cell damage[87]. Tissue debris is taken up by antigen presenting cells that have upregulated expression of MHC class I, class II, and co-stimulatory molecules in response to inflammatory cytokines. The activated antigen presenting cells can in turn prime *de novo* T cells against other target tissue epitopes[88, 89]. Although this phenomenon has been triggered by CD8$^+$ T cells binding to MHC class I in mice, MHC class II TCR ligation, CD40-CD40L interactions, and CD28-mediated co-stimulation are required for epitope spreading to occur with CD4$^+$ T cells[90, 91]. In humans, epitope spreading has been demonstrated after vaccination with peptide-pulsed immature dendritic cells as well as after the infusion of a monoclonal CD4$^+$ T cell clone, and has been correlated with tumor regression[27, 68]. To assess the breadth of epitope spreading, autologous antigen presenting cells transfected with cDNA encoding the sequence of target proteins (for example MART-1, Tyrosinase, gp100, NY-ESO-1, and Mage1-3 in the case of melanoma) and co-cultured with peripheral blood mononuclear cells have been used to assess CD8$^+$ and CD4$^+$ T cell responses[27, 92]. Alternatively, overlapping peptides spanning target proteins can be used to assess responses in a semi-quantitive manner.

14.4 Assessing Antigen-Specific Immune Responses in Target Sites

Clinical efficacy is dependent on the ability of antigen-specific cells to reach tumor tissue[93]. As the characteristics of T cells present in the peripheral blood might not reflect the characteristics of T cells present at the local tumor level[94, 95], efforts to

assess the responses elicited by vaccination or adoptive T cell therapy in the tumor micro-environment must be pursued. The scarcity of, and sometimes invasive procedures necessary to obtain human tumor tissue, particularly in solid tumor malignancies, remain major barriers to routine sampling of potential T cell target sites. When such tissues are available, standard approaches described above could be applied. However, these approaches require some degree of ex vivo manipulation and represent the immune response at a single time point (see Sect. 14.1). Dynamic, non-invasive approaches that provide whole body imaging allow for repeated in vivo assessment without the requirement for tumor sampling (discussed in non-invasive approaches).

14.4.1 Ex Vivo Evaluation of Immune Responses at Target Sites

Some of the standard approaches described in the previous sections that apply to assays to detect antigen-specific T cells in the peripheral blood can also be applied to disaggregated tumor or tissue samples, once lymphocytes are rendered into single cell suspension. Flow cytometry for example can identify tumor- or tissue-associated antigen-specific T cells by staining with peptide-MHC multimers. Multiparametric flow cytometry can be used to determine the phenotype of cells isolated from fresh tumor preparations and represents the most accurate method to determine the quantitative and qualitative nature of the immune response at the local tumor level. Caveats include the need for large amounts of fresh, unprocessed tumor tissue (in some cases, large quantities of tumor is needed to isolate a few T cells); some tumors require extensive manipulation to disaggregate the embedded T cells such that the phenotype of the isolated cells is altered (for example shedding of CD62L[96]); and the inability to assess the histologic location of the T cells in relation to tumor tissue which can be instructive in assessing immune responses. In some examples, memory T cells penetrating intratumoral regions can be predictive of good prognosis[97].

Functional assays including ELISpot represent an attractive semi-quantitative alternative to flow cytometry to determine both the presence and the function of T cells within tumor tissue. Although provisions similar to those relevant to flow cytometry also apply, the amount of tissue required for performing the assay is significantly less. When considering even smaller samples, for which disaggregation is not feasible or desirable, the presence of T cells in peripheral tissue and at tumor sites can be determined in cases where the CDR3/TCR sequence is known beforehand. In the case of adoptive therapy, the clonotype(s) of the infused T cells have already been defined. DNA can be extracted from fresh or formalin-fixed paraffin embedded tissue, and a quantitative or semi-quantitative amplification of the clone-specific CDR3 DNA can be performed. Laser capture microdissection can provide additional information as to the histologic location of DNA-extracted T cells. While this method can be highly sensitive for small amounts of tissue, function and further characterization of infiltrating T cells is limited.

The use of in situ immunofluorescence stains on tissue specimens allows an assessment of the histologic location of the infiltrating T cells, regulatory cells in relation to other immune cells as well as tumor and nontumor stromal components. This highly informative approach was initially limited by the relatively faint stain produced by fluorochrome-conjugated multimers and the relative low frequencies of pMHC-specific T cells in peripheral tissues or tumor. Quantum dot (Qdot)-labeled multimers exhibit similar specificity but have higher signal intensities for fluorescence imaging than multimers labeled with conventional photophores[98, 99]. pMHCs can be formed in vitro and conjugated to Qdots[4, 100]. Tissue sections stained for CD8, the corresponding tumor-specific Qdot multimer, and counterstained with the nuclear dye DAPI produce semi-quantifiable evaluation of specific $CD8^+$ T cells in tissue biopsies by confocal or fluorescence microscopy[100, 101]. This method also has the ability to detect antigen-specific T cells in three-dimensional tissue due to the intense brightness of the signal. When combined with other cell-specific stains (e.g., for tumor, stroma, vascular cells, this yields an information-rich rendering of the tumor micronenvironment). Due to the technology validation and extensive operator expertise, this approach is more suited to smaller well-defined studies than to large-scale clinical trials.

14.4.2 Non-Invasive Cell Tracking

As the use of adoptive immunotherapy becomes more sophisticated and widespread, tools to study the fate of transferred cells non-invasively and longitudinally in vivo in humans will become an essential component of immunologic monitoring[93]. Optical imaging techniques using fluorescence of bioluminescence are widely used in small animal models, and due to the virtual absence of background labeling, bioluminescence allows for semi-quantitative data acquisition. However, this technology is not currently applicable to human studies due to the limited penetration of the light wavelengths[102, 103]. Before pMHCs were widely available, several groups labeled melanoma-specific TIL or CTL with ^{111}Indium (^{111}I) and tracking was performed with computerized gamma ray imaging. Localization of transferred cells to the tumor site was demonstrated to peak 48 h after an initial accumulation to the lung, liver and/or spleen[104–107]. The use of ^{111}I as radioactive tracking agent is restricted by exposure limits in humans, a short half-life, nonspecific uptake by adjacent tissue, and slow biological clearance. Magnetic resonance imaging is an attractive alternative as it offers a concurrent high-resolution imaging. Superparamagnetic iron oxide nanoparticles consist of suspended colloids of iron oxide nanoparticles that can be detected by magnetic resonance imaging based on their property to reduce the T_2 signal such that transferred cells appear dark on T_2 weighted images. This contrasts with the widely used Gadolinium-based agents which exhibit poor intracellular uptake[108]. Superparamagnetic iron oxide nanoparticles were initially approved by the FDA for liver imaging because of the preferential uptake by naturally phagocytic Küpfer cells, and lack of toxicity due to the complete biodegradation within 2–4 weeks[109, 110]. For non-phagocytic cells,

complexing superparamagnetic iron oxide nanoparticles with cationic transfection agents such as protamine ensure successful intracellular magnetic labeling[111]. Pilot clinical studies have used similar agents to label dendritic cells[112], neural stem cells infected near an area of brain injury[113], $CD34^+$ cells injected in cerebrospinal fluid[114], and cadaveric pancreatic islet cells transferred intraportally to diabetic patients[115]. All cells could be identified by magnetic resonance imaging and in one case, for up to 6 weeks in vivo. Limitations of this method include the inability to discriminate live from dead cells as superparamagnetic iron oxide nanoparticles remain in dead cells until they are cleared by macrophages, and dilution of these particles to undetectable levels in the case of rapid division of transferred cells. Perfluorocarbons represent a promising alternative to superparamagnetic iron oxide nanoparticles and constitute a group of fluorine (^{19}F)-containing compounds derived from hydrocarbons by complete substitution of ^{1}H with ^{19}F. ^{19}F possesses comparable magnetic resonance sensitivity to currently used protons (^{1}H) but exhibits no tissue background signal, allowing specific and selective assessment of transferred ^{19}F-containing compounds in vivo in mice[93]. Perfluorocarbons are nontoxic, biologically stable and are cleared through respiration after in vivo administration[116]. Overall, the use of such compounds in clinical trials has the potential to expand our understanding of cell trafficking to tissues in humans substantially.

The ability to manipulate, and now, genetically engineer T cells ex vivo using recombinant vectors provide means to label T cells indirectly with a reporter gene construct. In one embodiment, this could be a vector expressing the herpes simplex virus 1 thymidine kinase (HSV1-tk) gene which is not expressed in human tissues. When administered following transfer of these engineered T cells, an ^{18}F-based reporter probe (e.g., 4-fluoro-3-[hydroxymethyl]butyl)guanine is taken up by the infused thymidine kinase-expressing T cells and phosphorylated by their HSV1-tk thereby retaining the probe in the cell where it can be detected by positron emission tomography[117]. In contrast to direct labels (described above), there is little to no dilution of signal with cell division since the integrated transgene is transmitted to all progeny. Limitations to this system are that nucleoside-based probes such as (4-fluoro-3-[hydroxymethyl]butyl)guanine do not normally cross the blood–brain barrier and the reporter gene HSV-tk, is immunogenic. In this latter instance, HSV-tk-expressing T cells will be eliminated relatively quickly by the endogenous immune response. However, T cells engineered to express the dopamine receptor, somatostatin receptor, sodium symporter or norepinephrine transporter, would be less likely to be rejected since these are all naturally occurring receptors with available pharmaceutical grade probes[118–120].

14.5 Characterization of Antigen-Specific T Cells in Vivo

Combining multimer stains with polychromatic flow cytometry allows simultaneous enumeration and detection of either surface and/or intracellular components expressed by antigen-specific cells within peripheral blood mononuclear cells or

Table 14.3 T cell-associated markers

Aspect of T cells	Markers
Memory phenotype	CD45RA/RO, CD27, CD28, CD127(IL-7Rα), CD62L, CCR7
Cytokine production	IFN-γ, TNF-α, IL-2, IL-4,IL-5, IL-6,IL-9, IL10, IL-12, IL-17, IL-22
Cytotoxicity	Perforin, granzymes A/B/K, FasL, TRAIL
Degranulation	CD107a
Inflammatory response recruitment	MIP-1α/β, RANTES
Activation status	HLA-DR, Ki-67, 4-1BB, CD38, CD69, CD57, members of the TNF receptor super family
Exhaustion	PD-1, TIM-3, CD160, Lag3, 2B4, CTLA-4, BTLA-4
Tissue trafficking	α4β7, CCR9, CLA, CCR10, CCR5, CXCR4
Regulatory function	CD25, FOXP3, CD127 (IL-7Rα)

tissue. Table 14.3 lists T cell-associated markers that have been correlated alone or in combination with functional anti-tumor immune responses and the category to which they pertain. Although the characteristics of cells that mediate tumor eradication have not yet been clearly determined, each category of markers carries characteristics of tumor-specific T cells that play important roles in tumor-control. This list however, is not exhaustive and needs continual update and incorporation into the evolving knowledge of immunological assessment.

14.5.1 Characteristics Associated with Tumor Eradication or Protective Immunity

The ultimate goal of immune therapies is to establish a long-lived resident population of cells that have the potential to eliminate preexisting tumors and ultimately prevent tumor recurrence. Central memory T cells (Tcm) are largely responsible for maintaining long-term memory, and in comparison to more differentiated effector memory T cells (Tem), exhibit enhanced survival, the ability to proliferate rapidly and robustly in response to re-encounter with antigen, and the capacity both to self-renew and to produce daughter cells that can differentiate to become effector cells[54,55]. In mouse models, the enhanced protective immunity provided by Tcm appeared to result from the more robust proliferation of Tcm than Tem after in vivo challenge, which likely reflects in part the distinct ability of Tcm to secrete IL-2 in response to antigen and use it as an autocrine growth factor[121].

Analysis of the cellular immune responses generated after vaccination with successful protective vaccines or naturally controlled infections (e.g., yellow fever, hepatitis B, EBV, CMV) have demonstrated that, in addition to protective antibodies, Tcm are formed[122, 123, 124, 125]. The precise phenotype of Tcm cells remains controversial. However, it is generally agreed that antigen-experienced T cell populations that persist after an acute infection is resolved, that express

CD28, CD27, CD127, CCR7, and CD62L, that proliferate extensively upon antigen re-encounter, and that secrete IL-2 in addition to effector cytokines, contain Tcm[126]. Studies in patients with metastatic melanoma who experienced clinical responses after adoptive transfer of antigen-specific cells, have shown that the persistent cells acquire characteristics of Tcm[127,128,151]. Thus, a systematic analysis of the phenotype of tumor-specific T cells in patients who experience clinical responses to immune interventions has a high likelihood of establishing correlates of immune protection.

14.5.2 Characteristics Associated with T Cell Activation

Markers reflecting the activation status of transferred T cells or cells generated after vaccination for example, could potentially indicate whether cells are successfully encountering antigen and/or expanding as a result of the encounter. However, classically used indicators of T cell activation such as HLA DR, CD38, and CD69 are nonspecific and rarely expressed on antigen-specific T cells in vivo[129]. Members of the TNF receptor superfamily including 4-1BB, as well as CD57 are induced on activated T cells but it is unclear if their overall expression is associated with cell proliferation and survival or signifies the cells are entering a state of senescence[130, 131]. Ki-67 is also rarely detected on antigen-specific populations found in the peripheral blood[41]. Therefore, the expression of activation markers reflecting the real activation status of cells needs further validation.

14.5.3 Characteristics Associated with T Cell Exhaustion/ Inhibitory Regulation

Inhibitory receptors suppress the cytolytic activity of T cell targeting cancer. CTLA-4 and PD-1 are expressed on naturally occurring tumor antigen-specific T cells. Blocking antibodies against these molecules have already been shown to benefit cancer patients[132, 133], discussed extensively in chapter 8. Exhausted $CD8^+$ T cells were shown to express up to seven inhibitory receptors in a chronic lymphocytic choriomeningitis virus murine infectious model including PD-1, Lag 3, CD160, and 2B4. Increased expression of the inhibitory receptors on the cell surface was associated with a cumulative inhibitory effect on antigen-specific secretion of IFN-γ that could further be reversed with inhibitory receptor blockade[134]. Elegant studies of human peripheral blood melanoma-specific $CD8^+$ T cells showed that a majority of cells simultaneously expressed four or more of the inhibitory receptors BTLA, TIM-3, LAG-3, KRLG-1, 2B4, CD160, PD-1, and CTLA-4. However, although gene expression profiling showed differences in gene expression, flow cytometric evaluation of CMV- and/or EBV-specific

populations that retain full functional capabilities did not significantly differ from the exhausted melanoma-specific cells. Furthermore, expression patterns were very different depending on anatomical localization as melanoma-specific T cells isolated for the tumor micro-environment expressed significantly higher levels of exhaustion markers than the ones isolated from the periphery[135, 136]. Systematic assessment of exhaustion markers on adoptively transferred tumor-specific cells in vivo or cells generated after vaccination and correlation with clinical outcome will contribute to determine the requirements for the generation of successful tumor-specific cells.

14.5.4 Homing Characteristics of T Cells

Assessing the trafficking potential of T cells could provide information on the inherent ability of the cells to reach the tumor-containing tissue. For example, the expression of α4β7 on antigen-specific T cells suggests the ability to traffic to gut-associated lymphoid tissue. Expression of the chemokine receptor CXCR4 confers the potential to traffic to the bone marrow; CCR5, the ability to migrate to sites of inflammation; and cutaneous lymphocyte-associated antigen and/or CCR10 the potential to reach cutaneous tissue[137–140]. However, the absence of expression of these markers in the periphery cannot be interpreted as a trafficking deficiency if these cells have migrated to the tumor/tissue. Alternatively, the presence of these markers on peripheral cells in the absence of their trafficking could be interpreted as failure of trafficking ability. Although technically difficult in human clinical studies, directly examining peripheral tissue could address these issues. An understanding of which receptors measured in the peripheral blood correlate with trafficking to tumor will be important to determining if forced expression of such chemokine and counter-receptors would benefit future immunotherapy studies[141].

14.5.5 Characteristics Associated with Regulatory T Cells

$CD4^+$ regulatory T cells (Tregs) are mediators of peripheral tolerance and although their purpose is to operate to prevent autoimmune disease and inhibit inflammation, evidence suggests they are involved in the suppression of effective immune responses to autologous tumor cells[142]. Under normal homeostasis, Tregs constitute approximately 10 % of total blood $CD4^+$ T cells and have suppressor functions on other immune cell populations. Most $CD4^+$ Tregs can be identified by the surface expression of CD25 and the intra-nuclear expression of forkhead box P3 transcription factor (FoxP3). Although FoxP3 at present remains the most specific functional and phenotypic marker for Tregs, characterization by flow-cytometry of T cells that express the $CD3^+CD4^+CD25^+$ phenotype and lack CD127 (IL-7Rα) identifies the majority of FoxP3 expressing $CD4^+$T cells within peripheral blood mononuclear

cells[143, 144]. The use of surface stains to identify Tregs without having to resort to a terminal intranuclear stain allows the cells to be further isolated and characterized. Because not all FoxP3$^+$ CD4$^+$ T cells mediate suppressive functions, assays directly assessing the suppressive function of Tregs are more pertinent[145].

Ex vivo assays to measure the suppressive function of Tregs have involved co-culturing a constant number of stimulated responding CD4$^+$CD25$^-$ cells with increasing numbers of CD4$^+$CD25$^+$ Tregs for 3–5 days. Suppression of responder T cell activity/proliferation has previously been measured either by (^{3}H)thymidine incorporation, dye dilution, or cytokine production[146, 147]. These labor-intensive assays are poorly suited for monitoring Treg suppressive activity in immunotherapy clinical trials, and novel methods based on the suppression of the activation markers CD69 and CD154 on responding effectors after antiCD3/CD28 stimulation could prove to be less technically challenging[148]. Additionally, a few studies have identified Tregs that are specific for tumor antigens[149], and some studies have been able to quantify the responses with MHC class II multimers and track the responses in vivo[150]. Please see chapter 9 for additional details on Tregs.

14.6 Conclusion

Cellular immune responses generated after immune interventions can be assessed by a multitude of structure- and function-based assays that are continuously being adapted and refined. No definitive parameters have yet been established to predict firmly the correlates of tumor regression or immune protection. However, the rapid expansion of knowledge adding to our understanding of the function of the different players of the immune system alongside the continuous discovery of new biomarkers indicates these correlates might be close at hand. Harmonizing and streamlining the performance of immune monitoring to facilitate comparison of human immunotherapy trials and the systematic accumulation of comprehensive observational data, especially in patients who demonstrate clinical responses, will further promote defining the requirements of immune-mediated tumor regression.

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Chapter 15
Issues in Pre-clinical Models, Clinical Trial Design and Analytical Considerations in Developing and Evaluating Novel Cancer Immunotherapies

Marijo Bilusic, Ravi A. Madan, and James L. Gulley

Abstract The role of the immune system in detecting and killing cancer cells has been understood for decades[1–3]. The human immune system has an innate ability to arrest carcinogenesis; however, in some cases, this system fails due to a series of mechanisms that tumor cells use to escape immune surveillance, such as mimicking normal cells, producing immunosuppressive growth factors and cytokines, or forming complex immunological interactions[4]. Differences in clinical responses to immunotherapy differ sufficiently from responses to cytotoxic agents that many aspects of clinical trial design must be re-thought, the subject of this chapter.

15.1 Introduction

The role of the immune system in detecting and killing cancer cells has been understood for decades[1–3]. The human immune system has an innate ability to arrest carcinogenesis; however, in some cases, this system fails due to a series of mechanisms that tumor cells use to escape immune surveillance, such as mimicking normal cells, producing immunosuppressive growth factors and cytokines, or forming complex immunological interactions[4].

The goal of therapeutic cancer vaccines is to induce an antitumor immune response that targets specific tumor-associated antigens (TAAs) through T cell stimulation. The ideal target TAA is unique to, or overexpressed on, the surface of cancer cells. Cytotoxic T cells are able to recognize 9- to 14-mer antigenic peptides expressed within the major histocompatibility complex (MHC) on the surface of all cells. When appropriately activated, T cells can then detect specific TAAs and initiate targeted, immune-mediated cell killing[5, 6].

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Therapeutic cancer vaccines and other novel cancer immunotherapies have as their goal to induce targeted immune responses against cancer cells[7]. They may not produce immediate cancer cell death that decreases tumor volume, but may stabilize disease and delay tumor growth, which may translate into prolonged survival. To be effective, a cancer immunotherapy agent must achieve two major goals: it must stimulate specific immune responses against an appropriate target, and the immune responses must be sufficient to overcome immunosuppressive mechanisms employed by tumors[8].

Standard cytotoxic chemotherapies have immediate effects and may initially reduce tumor size, but after several months or years, the disease will inevitably progress. In contrast, immunotherapy may delay tumor growth by altering host/tumor interface through an active antitumor immune response. Thus, with a cancer vaccine, there may initially be no significant reduction in tumor size, and immunological processes may even induce tumor growth. This phenomenon could explain why several phase III vaccine trials have demonstrated no significant change in disease progression (disease-free survival), while the long-term endpoint of overall survival (OS) has significantly improved[9–11]. Unlike cytoreductive chemotherapy, cancer vaccines require sufficient time to generate an immune response, and evidence of clinical benefit may therefore be delayed[12].

This new paradigm of long-term benefit without immediate and significant reduction in tumor size makes biological sense in terms of immune response, but represents a significant departure from the standard practice of treating patients with cytotoxic drugs and assessing radiographic response every 2–3 cycles. Evaluating the benefit of immunotherapy will require altering the current practice of relying largely on radiographic response and will be more efficiently accomplished if the appropriate patient population can be evaluated[12].

15.2 Patient Selection

Identifying the ideal population for a clinical trial is particularly important in vaccine trials, where only a select group of patients may benefit from treatment. Throughout this chapter "cancer vaccine" is the model for discussion, but the concepts here refer to any cancer immunotherapy. In the current clinical trial model, new vaccines are usually tested in patients with late- or end-stage disease who have been heavily pretreated and have exhausted other treatment options. However, numerous vaccine studies have shown that immunotherapy is less effective in patients with heavy disease burden. Such studies include the GVAX[13, 14] and prostate-specific antigen (PSA) vaccine trials[15] in prostate cancer, the idiotype vaccine trials in follicular lymphoma[16], monoclonal antibody treatment in colorectal cancer[17], and adjuvant immunotherapy trials in melanoma[18].

It is well known that greater tumor burden leads to a proportional increase in regulatory T cells (Tregs)[19, 20], as well as increased levels of indoleamine-2,3-dioxygenase, transforming growth factor (TGF)-β, and IL-10, all of which can

inhibit T cell activation[21, 22]. In addition, patients with very aggressive cancer may not have enough time to develop a significant immune response and thus benefit from treatment. The number of previous chemotherapy regimens also negatively affects response to a cancer vaccine. For example, in trials evaluating a vaccine targeting carcinoembryonic antigen, patients who had been treated with several chemotherapeutic regimens were less likely to have a measurable immune response ($P = 0.032$) than patients who had less prior exposure to chemotherapy[23].

Taking several factors into consideration, the ideal patient for treatment with a therapeutic cancer vaccine has slow-growing and/or low-volume disease, with minimal prior exposure to chemotherapy[24]. In clinical trial design, appropriate patient selection is thus key to accurately assessing the clinical efficacy of therapeutic cancer vaccines.

15.3 Response Evaluation Criteria in Solid Tumors Versus Immune-Related Response Criteria

In addition to patient selection, a crucial factor in clinical trial design is the selection of an appropriate and realistic primary endpoint. The Response Evaluation Criteria in Solid Tumors (RECIST) were developed in 2000 through a collaboration of the European Organisation for Research and Treatment of Cancer, the National Cancer Institute of Canada, and the National Cancer Institute (NCI) of the United States[25, 26]. The vast majority of clinical trials rely on RECIST, evaluating clinical benefit strictly in terms of reduction in size of soft tissue tumors and defining progressive disease as a 20% increase in the cumulative size of target lesions or development of any new lesions[25, 26]. The value of RECIST depends on the type and chemosensitivity of the tumor and on treatment goals. For acute leukemias and highly curable tumors such as testicular cancer, RECIST criteria are irrelevant.

The value of RECIST in immunotherapy trials is questionable in light of the biology of immune response after vaccination. A significant and potentially beneficial immune response may cause transient increases in the size of lymph nodes, which could be identified as progressive disease based on RECIST criteria[27–29]. Indeed, immunotherapy trials in melanoma have suggested that the disease may initially flare in some areas before more clinically beneficial results are seen[30].

New response criteria based on World Health Organization (WHO) and RECIST were evaluated in a few recent studies with ipilimumab[31, 32]. Four distinct response patterns were described: immediate response, durable stable disease, response after tumor burden increase, and response in the presence of new lesions. All patterns appear to be associated with favorable survival compared to patients with progressive disease by WHO criteria.

To evaluate all observed response patterns, Wolchok et al. recently proposed a new set of immune-related response criteria (irRC) for tumor immunotherapy[32, 33]. Response categories defined as immune-related complete response (irCR),

Table 15.1 Description of immune-related response criteria (irRC) (adapted from Wolchok et al.[33])

Measurable response by tumor volume	Nonmeasurable response (non-index lesions; new, nonmeasurable lesions)	Overall response by irRC
100 % decrease	Absent	irCR
≥50 % decrease	Any	irPR
<50 % decrease to ≤25 % increase	Any	irSD
>25 % increase	Any	irPD

irCR = complete disappearance of all indexed and new, measurable lesions
irPR = ≥50 % decrease in tumor volume from baseline
irSD = does not meet criteria for irCR or irPR, in absence of irPD
irPD = >25 % increase in tumor volume from nadir

immune-related partial response (irPR), immune-related stable disease (irSD), and immune-related progressive disease (irPD) are considered clinically meaningful because they appear to be associated with favorable survival. As with irPR and irCR, the response category irPD should be confirmed at two consecutive time points. At baseline tumor assessment, the sum of the products of the two largest perpendicular diameters (SPD) of all index lesions (five lesions per organ; up to ten visceral lesions and five cutaneous index lesions) is calculated. At each subsequent tumor assessment, the SPD of the index lesions and of new, measurable lesions (≥5 × 5 mm; up to five new lesions per organ; five new cutaneous lesions and ten visceral lesions) are added together to calculate total tumor burden. By irRC, new lesions alone do not constitute irPD if they do not increase tumor burden by ≥25%. Appearance of new lesions accompanied by an overall decrease in tumor burden of ≥50% is defined as irPR, while a <50% decrease to ≤25% increase in tumor burden is defined as irSD. Importantly, an early increase in the size of lesions, which may be attributable to inflammation, does not mean that irCR, irPR, or irSD may not be achieved at the next consecutive time point.

If a patient is classified as having irPD, confirmation by a second scan in the absence of rapid clinical deterioration is required. Thus, disease progression is confirmed by an increase in tumor burden of ≥25% over baseline at two consecutive time points ≥4 weeks apart[33–35]. For complete details of the new irRC, see Table 15.1, adapted from Wolchok et al.[33].

15.4 Progression-Free Survival Versus Overall Survival

Progression free survival (PFS) versus overall survival (OS) as an appropriate endpoint in clinical trials has been debated in the literature for several years[36]. PFS is measured as the time elapsed between initiation of treatment and tumor progression or death from any cause, with censoring of patients who are lost to follow-up. PFS is often seen as a more attractive endpoint for clinical trials than OS, as well as a surrogate marker for OS, because it can be determined earlier, is less

influenced by competing causes of death, and is not influenced by second-line treatments. Unlike OS, which is a definitive measurement, disease progression may be subject to measurement errors. Accurate determination of the starting point of disease progression can be problematic, and the quality of PFS measurement can vary among centers and investigators. In addition, the date of progression is in fact a proxy for the true time of progression, which occurs at an unknown point between two successive radiological assessments[37]. Finally, while RECIST is a useful tool for determining antitumor activity, improving survival or quality of life should be the ultimate goal of all cancer therapeutics[26]. While improved PFS without the benefit of improved OS has led to approval by the US Food and Drug Administration of several drugs, including bevacizumab for metastatic breast cancer[38], evidence of the unreliability of PFS as an endpoint in clinical trials is seen in the fleeting effects of bevacizumab in the adjuvant treatment of colon cancer[39]. Over 2,500 men with stages II and III colon cancer were treated with adjuvant chemotherapy with or without bevacizumab administered for either 6 or 12 months. Initially, there was a suggestion that patients on bevacizumab for 12 months had a higher rate of PFS after 1 year; however, the superior PFS result was no longer significant at a later follow-up.

Data from numerous trials of vaccine monotherapy, as well as data from clinical trials combining chemotherapy and cancer vaccines, suggest that in trials involving vaccines, PFS and time to progression (TTP) may not be appropriate endpoints[10, 32, 40, 41]. We have recently seen that cancer vaccines can improve OS without significant changes in PFS. A randomized placebo-controlled phase III trial of sipuleucel-T and a phase II study of the vector-based vaccine PSA-TRICOM are good examples of the importance of appropriate endpoints. The sipuleucel-T trial initially failed to meet its primary endpoint of PFS; however, it did provide evidence of longer OS (25.8 months vs. 21.7 months; $P = 0.032$)[9, 42–44]. This advantage was confirmed in a larger OS endpoint study[40]. Likewise, the PSA-TRICOM study failed to meet its primary endpoint of improved TTP determined by new or enlarging soft tissue tumors or bone metastasis (TTP), but a survival analysis indicated a clear clinical benefit for the vaccine. The median OS was 8.5 months longer in the vaccine arm compared to the control arm ($P = 0.016$), suggesting that in spite of a lack of improved TTP, there was a long-term survival advantage for patients treated with PSA-TRICOM[10]. A confirmatory OS endpoint study opened in 2011 with final results pending.

Finally, similar data were recently demonstrated with another immunotherapy. Ipilimumab is a humanized monoclonal antibody targeting CTLA-4, a cell-surface marker upregulated on T cells following activation. Ipilimumab blocks the negative signaling through CTLA-4 that leads to a turning off of the immune response. In a randomized controlled phase III trial in metastatic melanoma, use of ipilimumab led to improved OS without an improvement in median TTP[45].

Growing public concern about the cost of health care may make it harder to justify the use of agents that improve PFS but do not improve OS. Thus, appropriate endpoints in clinical studies are vital to understanding the benefits of emerging immunotherapeutic agents used alone or in combination with cytoreductive therapies.

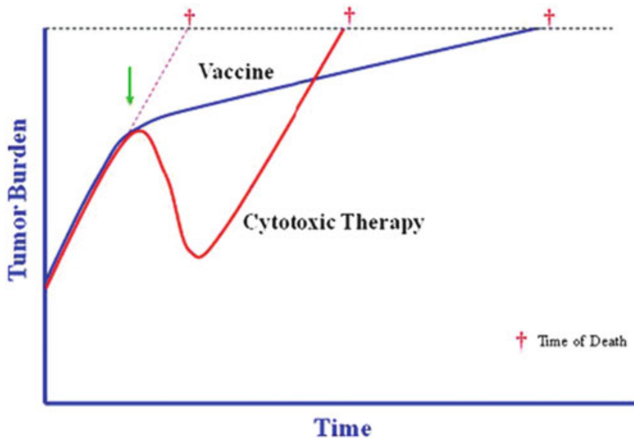

Fig. 15.1 Tumor growth is a dynamic biologic process that is the combined result of cells dividing and other cells dying. Chemotherapy (red line) affects the tumor growth rate only while it is being administered, which may result in a dramatic but transient response. When chemotherapy is discontinued, the growth rate returns to its pretreatment slope, driven by the underlying tumor biology. Immunotherapy (blue line), on the other hand, can alter host biology by inducing an active antitumor immune response, including a memory response. This may not cause an immediate or dramatic change in tumor burden, but continued, cumulative pressure that slows tumor growth rate, especially if started early in the disease course, may lead to substantially longer overall survival. Arrow indicates initiation of treatment; cross indicates time of death from cancer. (Adapted from Madan et al.[41]).

15.5 Effect of Immunotherapy on Kinetics of Tumor Growth

A review of several prostate cancer clinical trials conducted at the NCI in the last decade revealed interesting findings on PSA kinetics. For patients treated with chemotherapy, there was a very close relationship between time on treatment and survival. After treatment was discontinued, pretreatment PSA kinetics resumed, and time to death was predictable based on similar pre- and posttreatment PSA trajectories. For patients treated with PSA-TRICOM vaccine, PSA kinetics did not immediately change while on treatment, but time of death was well beyond what was predicted by the models (Fig. 15.1).

Although immune responses can be initiated within 3 months of receiving a vaccine, it appears that these responses are not sufficient to significantly reduce tumor size, but may eventually decrease tumor growth velocity. Moreover, it is possible that the immune response most relevant to antitumor therapy may not be the one targeted by the vaccine, but a new immune response to other tumor antigens in a phenomenon known as antigen cascade or antigen spreading[46, 47]. For instance, the initial immune response to a vaccine can lead to T cell-mediated killing of tumor cells, causing antigen-presenting cells (APCs) to take up dying tumor cells and present other more relevant antigens to the immune system. This broader antitumor immune response may be more clinically relevant and may lead to a

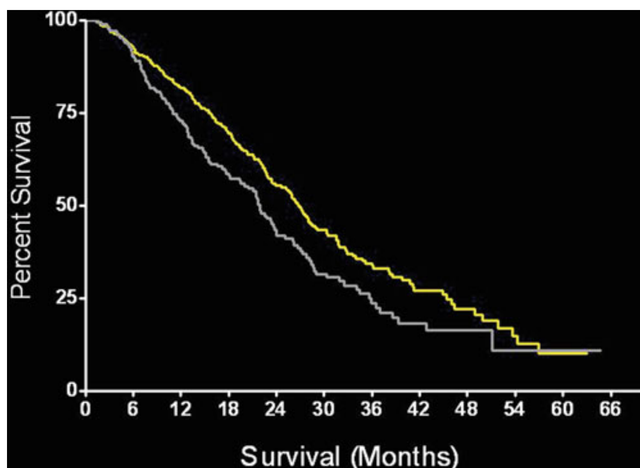

Fig. 15.2 IMPACT trial: Kaplan–Meier estimates of overall survival. Yellow line represents sipuleucel-T group ($n = 341$) and gray line placebo group ($n = 171$) (hazard ratio for death in the sipuleucel-T group, 0.78; 95% confidence interval [CI], 0.61 to 0.98; $P = 0.03$). Separation of Kaplan–Meier curves occurs approximately 8 months after randomization. (Figure reprinted by permission from Kantoff et al., NEJM, 2010[40]).

slower tumor growth rate. Furthermore, this immune response can be maintained or even augmented following subsequent therapies[48].

Unlike chemotherapy, which acts directly on the tumor, cancer immunotherapies demonstrate new kinetics that involve building a cellular immune response, delaying tumor progression, and potentially resulting in improved survival. Several mechanisms have been proposed for this phenomenon[12, 41, 48]. Subsequent therapies may alter the expression of TAAs on tumor cells, making them more susceptible to immune-mediated cell killing, or may enhance the immune response by depleting immune regulatory mechanisms. Chemotherapy-induced cytolysis may expose an activated immune response to additional antigens that can then be targeted in a broader immune response or may trigger a molecular "danger signal" that leads to an enhanced immune response[49, 50]. As seen in the IMPACT trial of sipuleucel-T, the separation of Kaplan-Meier curves occurs approximately 8 months after randomization (Fig. 15.2)[40].

15.6 Immunotherapy and Statistical Concept of Relevance

There has been a significant effort to define pathways and harmonize methods for translational cancer immunotherapy. The Translational Research Working Group published a recommended pathway incorporating various milestones in a flowchart algorithm for translating potential immunologic therapies from the bench to use in clinical trials[51]. A proposed guide to therapeutic cancer vaccine development was recently published by the Cancer Vaccine Clinical Trial Working Group[52].

Proof-of-principle trials are exploratory and share aspects of conventional phase I and II trials. They should be conducted in well-defined and selected patient populations and should investigate disease-specific biologic parameters. Objectives should be to initiate a safety database, determine dose and schedule, and demonstrate biologic activity, defined as any effect of the vaccine on the target disease or host immune system, using biologic markers such as clinical, molecular, or immune response as study endpoints. Immune response is confirmed if demonstrated in two separate, established, and reproducible assays at two consecutive follow-up time points after the baseline assessment. If proof-of-principle trials show such an immune response or other biologic or clinical activity, efficacy trials may be initiated.

A noncomparative phase II trial may use a single group with a historical control. Statistical power to compare outcomes is limited due to small sample sizes, which may not allow definitive conclusions about the superiority of either arm. A phase II trial may also be comparative, powered to show a statistically significant difference between two arms in a well-defined patient population using a well-defined measure of primary outcome. If positive and well conducted, comparative randomized phase II trials can provide evidence of efficacy.

A comparative randomized phase II trial with adaptive component is the phase II component of a phase II/III trial aiming to demonstrate the efficacy of a novel product. It has the stringency, prospective design, and planned conduct of a conventional phase III trial. The phase II component has a specific finish at a prospectively defined trigger point of efficacy. If a prospectively defined efficacy goal is achieved, it will trigger activation of a full phase III trial. If the prospectively defined efficacy goal is not achieved, the study will be terminated.

Defining the trigger point and the parameters to measure is crucial. A trigger point is prospectively defined and may be relatively complex. For example, a trial may use a less definitive endpoint in the first phase (e.g., molecular response), which triggers expansion of the study and a more definitive endpoint (e.g., OS) in the second phase, demonstrating efficacy in the expanded study. Trigger points may not be fully statistically powered to demonstrate superiority and may be independent of the primary efficacy endpoint. Independence of endpoints may avoid paying a statistical penalty.

Adaptation of such a trial may entail not only adjusting sample size but also modifying eligibility criteria to focus on a specific population. Depending on the developmental path of the product under study and findings from earlier trials, more than one efficacy trial may be needed. The concept of efficacy trials allows for early assessment of vaccine efficacy and more rapid and informed development of cancer vaccines.

15.7 Biological Markers as Intermediate Endpoints

Current knowledge suggests that immune protection against cancer is likely a balanced interaction of cellular and humoral immune responses. If a vaccine targets a specific TAA and a subsequent immune response results in a vigorous attack on a

separate TAA via antigen cascade, it may be difficult to know which TAA the immune system is attacking and therefore which to assess. As previously demonstrated, an antigen cascade following a vaccine-mediated immune response may result in the targeting of multiple antigens not specified by that particular vaccine[46]. In addition, the most relevant TAA may vary among patients treated with the same therapeutic cancer vaccine. Thus, if only the response to a specific TAA is assessed, the actual benefit of the vaccine may be underestimated.

Identifying intermediate (surrogate) endpoint biomarkers is absolutely essential to accelerating development of novel treatments for all cancers, but is particularly crucial for cancer vaccines. Intermediate endpoint biomarkers have been pivotal in the approval of drugs for diabetes and chronic myeloid leukemia (CML). The rapid development of ABL inhibitors and their expedited regulatory approval was also due, at least in part, to the availability of highly specific and sensitive biomarkers of response and clinical outcome. Unfortunately, unlike CML, most malignancies do not yet have such easily measurable biomarkers[53].

It is naturally difficult for patients and clinicians to observe tumor progression while waiting for a delayed, long-term therapeutic benefit. And since therapeutic cancer vaccines may never cause an immediate, easily measurable antitumor response, there is an urgent need to develop standardized biomarkers to predict who will likely go on to develop clinical benefit in the absence of an immediate clinical response. Many immunoassays and biomarkers (Table 15.2) have been described in the literature, but none has yet been prospectively validated to correspond with clinical outcomes.

In summary, improved biomarkers that can serve as generalized markers of immune response or as intermediate and surrogate endpoints are urgently needed. These biomarkers would help to identify patient benefit earlier in treatment, guide decisions to discontinue ineffective strategies, and identify active anticancer drugs more efficiently. It would be particularly advantageous if such biomarkers could be measured easily, rapidly, and frequently. See chapter 14 for many additional details.

15.8 Preclinical Models in the Development of Immunotherapeutic Drugs

The challenge in developing a cancer vaccine is effective antigen presentation that elicits antitumor immune responses without triggering autoimmunity. Murine cancer models have been extremely useful for analyzing the biology of pathways involved in cancer initiation, promotion, and progression. However, they do not always adequately represent the many features that define cancer in humans, including long periods of latency, genomic instability, and the heterogeneity of tumor cells and their microenvironment. Most importantly, the complex biology of cancer recurrence and metastasis, integral to outcomes in human patients, is not

Table 15.2 Commonly used immune assays and biomarkers[54]

Assay	Description	Disadvantages
Chromium release assay	Determines ability of $CD8^+$ cells to lyse target cells	Labor-intensive, can use only fresh PBMCs
Tetramers	Measure absolute number of cells that can recognize a particular epitope[55]	Cannot provide information about functionality The exact epitope and MHC class I molecule must be known
ELISPOT	Measures IFN-γ response to antigens (functional information). Has low limits of detection[56]	Significant variability among institutions and readers, restricted to certain HLA types TAA-specific, not useful for immunotherapy with whole tumor vaccines, cytokines, or antibodies[57–59]
CFC (cytokine flow cytometry)	Cytokines produced by T cells and trapped in the cell can be analyzed by flow cytometry Limit of detection is one antigen-specific T cell:10000 PBMCs	Nonspecific background staining
Tregs	Develop in the thymus, do not produce IL-2[60] Major role is to suppress other immune cells[62]	FoxP3 is most accepted marker[61] Presence of small populations of $FoxP3^-$ Tregs
Myeloid-derived suppressor cells (MDSCs)	$CD14^-CD11b^+$ cells MDSCs correlate with more aggressive disease and a poor prognosis[64]	Not clear whether MDSCs mediate antigen-specific or antigen-nonspecific suppression of T cell responses[63]
Cytokine measurement	Evaluates cytokine production by measuring mRNA (RT-PCR)	Requires destruction of the immune cell, which prevents determination of T cell specificity[65]
T cell proliferation assay	Enhanced T cell proliferation appears to be associated with improved outcomes[40]	Poor sensitivity; thus remains unclear if absence of such responses precludes benefit
CD54 (APC activation)	CD54 expression after treatment could be used to assess APC engagement and vaccine efficacy[66, 67]	Needs additional evaluation

adequately reproduced in conventional mouse models used in cancer drug development. The process of developing and approving novel cancer drugs is lengthy and expensive, making animal models that better represent human disease an urgent necessity[68].

Several characteristics of transplantable tumors limit their applicability in immunotherapy trials. Most transplantable tumors were derived many years ago, and mouse strains that were once syngeneic with these tumors may no longer be so. In addition, some transplantable tumors have picked up endogenous viruses and express viral antigens not expressed by their mouse hosts. Therefore, many transplantable tumors may be partially histoincompatible with their “syngeneic” mouse host and/or contain

viral epitopes that make them significantly more immunogenic than naturally arising human tumors. Second, transplanted tumors are typically inoculated subcutaneously or intravenously and therefore do not grow in the anatomically appropriate site. As a result, the animal model does not mimic the organ-specific physiology characteristic of the tumor, and the animal immune system is not exposed to the tumor in a manner comparable to that of naturally occurring malignancies in patients. Third, transplantable tumors generally progress very rapidly following inoculation, whereas spontaneous human tumors usually develop more slowly through a gradual series of cellular changes from premalignant to malignant pathologies. Therefore, the human immune system slowly acclimates to tumors, whereas the immune system of experimental animals is abruptly exposed to transplanted tumors. Fourth, for patients with solid tumors, disseminated metastatic disease is frequently the predominant cause of death, and many cancer vaccines and immunotherapies are aimed at reducing and/or preventing metastasis. Most transplantable mouse tumors, however, are not spontaneously metastatic, so vaccine efficacy studies using these models are not particularly relevant for human metastatic disease.

Discovery of the nude mouse in 1962 was a major breakthrough for cancer research because it allowed human tumors to be studied in another animal. Nude mice are immunodeficient due to lack of a thymus, which is essential for the production of T cells. Thus, they do not reject tumor transplantation from other species. Transplanting a human tumor into a nude mouse allows for study of the tumor in a whole animal system. Unfortunately, this model system cannot be used to study therapeutic cancer vaccines, as they require an intact immune system. Thus, many well-established models of human tumors in mice used for traditional tumor-directed therapies are irrelevant in therapies directed at generating an antitumor immune response.

The most clinically relevant studies are in spontaneous tumor models where tumors arise in an appropriate tissue background, in a host conditioned by the physiological events of neoplastic progression and tumorigenesis, and in the context of a viable immune system. Major drawbacks to the spontaneous tumor models are the amount of time needed to obtain results and the very real possibility of not attaining a complete remission. Paradoxically, the value of this approach is that complete remissions are as elusive in humans as they are in laboratory animals. Solid tumors escape the immune system through many mechanisms, such as downregulation of costimulatory molecules (CD80 and CD86) or MHC class I, secretion of immunosuppressive proteins such as TGF-β and IL-10, failure of immune cells to access the tumors, development of immune cell anergy, failure to respond to antigenic stimulation, or failure to overcome immune tolerance. Learning how to counteract some of these escape mechanisms in spontaneous murine tumors would be valuable in the treatment of human tumors.

Unfortunately, many of these tumor models are not predictive of results in human clinical trials, as numerous therapies that look promising in experimental animals have turned out to be ineffective in humans.

Table 15.3 How standard therapies may enhance therapeutic cancer vaccines

Modality	Proposed mechanism of action
Radiation therapy	Postirradiation-induced upregulated genes: Fas, MHC class I, and ICAM-1[70–72]
Chemotherapy	Upregulation of MHC class I and TAAs on the surface of tumor cells,[73–75] depletion of regulatory T cells (Tregs) (cyclophosphamide)[76], increase in macrophage antitumor activity and apoptosis (doxorubicin) [77], increase in proinflammatory cytokine production (docetaxel)[78]
Hormonal therapy	Induction of T cell infiltration in human prostate[79], enhancement of T cell repertoire, abrogation of immune tolerance[80–82], increase in proinflammatory cytokines, and enhancement of CTL stimulation by reducing the number of Tregs[83]
Targeted therapeutics	Alteration of balance between effector T cells and Tregs/decrease in Treg function (sunitinib, pan-BCL-2 inhibitor GX15-070)[84, 85]

15.9 Combination Therapies

Although therapeutic cancer vaccines hold great promise, their ultimate utility may be in combining them with other standard therapeutic interventions. Increasing data suggest that immune-mediated tumor killing induced by cancer vaccines can be enhanced and promoted by conventional anticancer therapies. Standard treatments may upregulate MHC molecules and TAA expression or may induce apoptosis by increasing the expression of death receptors (such as Fas, TNF receptor, and TNF-related ligand receptors)[48, 69]. Currently, many treatment modalities are being investigated in combination with vaccines, including radiation, chemotherapy, hormonal therapy, and targeted molecular inhibitors (Table 15.3).

The combination of radiation and vaccine has been investigated in several clinical trials[86]. In a clinical study in men with localized prostate cancer, a vector-based vaccine plus standard radiation generated an immune response. In this small study, 11 patients received radiation alone and 19 patients received the combination regimen of vaccine and radiation. Of the 17 patients who completed all 8 scheduled vaccinations, 13 showed increases of ≥threefold in PSA-specific CTLs, which was superior to responses induced by radiation alone[46]. In another phase I clinical trial, patients with advanced hepatoma were administered 8 Gy of radiation, followed 2 days later by an intratumoral injection of autologous immature dendritic cells. Of the 14 patients enrolled, 4 had minor responses and 2 had partial responses, including a patient whose α-fetoprotein (AFP) levels decreased from 128 to 1.6 ng/ml. Evaluation of immune response in 10 patients showed 6 with increased natural killer (NK)-cell activity, 8 with increased AFP-specific immune responses as measured by cytokine-release assay, and 7 with increased AFP-specific immune responses by ELISPOT[87].

Mercader et al. studied 33 patients with biopsy-proven clinical stages T1 to T2b prostate adenocarcinoma without prior hormone therapy or a history of immunosuppressive medications or disease[79]. Seven patients were randomized to a control group that received no treatment. The remaining 26 patients were randomized to

receive preoperative androgen-ablative therapy for 7 ($n = 7$), 14 ($n = 7$), 21 ($n = 5$), or 28 ($n = 7$) days. Mean $CD3^+$ T cell levels within prostate tissues in patients treated with androgen ablation for 7 or 14 days were twofold greater than the control value of T cells within untreated prostate tissues ($P = 0.027$). At day 21 of therapy, mean T cell numbers within the prostate exceeded the mean control value by nearly fivefold ($P = 0.0013$). At day 28 of therapy, T cell levels appeared to decline slightly from the 21-day value, but remained well above the control value ($P = 0.035$).

Cytotoxic chemotherapy currently plays an important role in the conventional treatment of solid tumors. While the primary benefit of chemotherapy derives from its cytotoxic properties, it may also alter tumors phenotypically, enhancing TAA and MHC class I expression, both of which can make cancer cells more amenable to vaccine-induced CTL activity[73–75, 88, 89]. Exposure and subsequent immune response to such an antigen cascade could broaden antitumor immune responses and enhance clinical benefit[46, 90]. There has long been concern that chemotherapy may limit an immune response. However, a phase II clinical trial in metastatic prostate cancer demonstrated that this was not the case. Patients were randomized to receive vaccine alone or vaccine with weekly docetaxel. The results of this study showed an equal increase in PSA-specific CTLs in both arms following 3 months of therapy. Furthermore, immune responses to other prostate cancer-associated TAAs were also detected postvaccination[91].

Administering vaccine prior to chemotherapy may take advantage of the dynamic immune response to TAAs initiated by the vaccine. Subsequent chemotherapy may yield the benefits of cytotoxicity in combination with an ongoing (and perhaps potentiated) immune response. Studies in murine models have indicated that vaccine in combination with docetaxel has a greater antitumor effect than either agent alone[90].

There is a common perception that because many patients treated with a vaccine receive chemotherapy after progression, the chemotherapy alone is actually improving survival, not the antitumor immune response generated by the vaccine. Yet strong preclinical and emerging clinical data suggest that this perception is inaccurate. Several follow-up studies in vaccine trials have also indicated that patients treated with a vaccine do better than expected on subsequent chemotherapy[92, 93]. It is unclear whether this secondary response is due to the cytotoxic therapy's depletion of Tregs that hinder an immune response or that an immune response is enhancing the effect of subsequent hormonal therapy or chemotherapy. Clinical trials are being planned to prospectively validate the benefits of vaccine followed by chemotherapy. One such trial is ECOG E1809, a multicenter randomized phase II trial of docetaxel with or without PSA-TRICOM vaccine in patients with metastatic castration-resistant prostate cancer. Eligible patients have a life expectancy of $\geq$18 months by the Halabi nomogram[94]. Patients are randomized to 3 months of PSA-TRICOM vaccine followed by docetaxel and prednisone versus docetaxel and prednisone up front, with median OS as the primary endpoint. Secondary endpoints are time to radiographic progression, objective response, PSA response, immune response, and association between PSA-specific immune responses, TTP, and OS. This trial was initiated in late 2010 and will enroll 135 patients[95].

15.10 Pitfalls of Translating Preclinical Research into the Clinic

Development of anticancer drugs requires translating information gained from basic research into improved clinical practice by way of clinical trials. Successful drug development starts by identifying targets that cancer cells are absolutely reliant on, so that when these functions are blocked, there is a lethal or cytostatic effect. Once a target has been established, the issue of in vivo validation arises. Traditionally, this has involved treating mice xenotransplanted with human cancer cell lines. Although these models are useful in defining an agent's potential pharmacological properties, in general, they have limited value in defining the potential efficacy of an agent in treating human cancer[53]. There are a number of challenges to be met before new agents can be used in clinical trials. Preclinical studies must demonstrate safety and efficacy, but as mentioned previously, animal models do not always predict how humans will respond to the same intervention. One example of this challenge is TGN1412 (TeGenero AG; Wurzburg, Germany). TGN1412 is a humanized superagonist that works by overriding the body's natural two-step immune signaling system, required for activating naive T cells, by binding to CD28 (T cell coreceptor) cells and triggering a direct T cell immune response. It was developed as a treatment for rheumatoid arthritis, leukemia, and multiple sclerosis, and was supposed to be a new class of monoclonal antibody (mAb) designed to stimulate rather than dampen the immune system.

A phase I clinical trial of TGN1412 was initiated in healthy volunteers in March 2006[96]. Six individuals were infused simultaneously at a dose of 0.1 mg/kg. All of them rapidly became critically ill, developing life-threatening multiorgan failure attributable to massive cytokine release. Investigations have excluded contamination and suggested that the adverse events were true biological effects of the agent. This catastrophic phase I clinical trial has raised serious concerns about the adequacy of the preclinical tests routinely used during the development of novel biologic drugs. The toxicity of TGN1412 is reminiscent of that seen with anti-CD3 mAbs, such as OKT3, which also bypass the need for antigenic stimulation[97]. The agent had not caused serious toxicity in cynomolgus monkeys, even at doses 500-fold greater than those given to human volunteers. Moreover, in vitro effects of TGN1412 on human and primate T cells appeared to be equivalent[98]. There is no explanation for why the dramatic toxicity seen in humans was not seen in preclinical primate studies. Although human and cynomolgus CD28 cells share identical TGN1412-binding epitopes, there are probably small but potentially significant amino acid differences in other domains of the molecules[96].

It is standard practice to introduce new anticancer agents to one or two patients at a time. However, there is growing concern that new agents in first-in-human trials should be administered to patients sequentially, with an appropriate period of observation between dosing of individuals. New rules for testing investigational drugs in humans allow for phase 0 or exploratory trials. These are brief trials lasting several days in which human subjects are given very low doses of experimental drugs before standard in vitro and animal tests are completed. Phase 0 studies do not

examine safety or efficacy, but gather data on the targeting, action, and metabolism of a drug in the body. The goal is twofold: to identify and select potential candidates early on and to generate data that will aid in the design of smarter phase I studies of promising compounds. Phase 0 trials would benefit pharmaceutical manufacturers, who are frequently forced to rely on animal data alone to choose one drug from a panel of candidates to advance to phase I trials. It could also alter the manner in which such drugs are tested on human volunteers in early-stage trials.

There is clearly an urgent need to accelerate translation of preclinical research into improved therapeutic strategies. Critical to future progress will be an increased understanding of tumor biology, identification of key molecular targets, and the discovery and clinical development of rationally designed anticancer drugs. Proper patient selection and establishment of proof of concept could minimize the risk of late and costly drug attrition due to disease heterogeneity, accelerate patient benefit, improve drug approval registration strategies, and result in more frequent and less costly anticancer drug approvals. Disasters such as the TGN1412 trial should be occasions for learning, but should not inhibit efforts to explore new therapies for cancer patients.

15.11 Conclusion

Therapeutic cancer vaccines have been in development for several decades, initially with disappointing results. Recent trials in prostate cancer have renewed hope that initiating an active immune response with a therapeutic cancer vaccine can have long-term clinical benefit for cancer patients. The generally indolent nature of prostate cancer, and a dearth of effective systemic treatments for metastatic disease, may explain why vaccines have been more successful in prostate cancer than in other types of cancer. A broader application of vaccines in prostate cancer, plus experience with many more patients, may propel the development of appropriate biomarkers to assess immune response and lead to the effective use of vaccines in other cancers.

Cancer vaccines generate a cellular immune response that results in altered tumor growth curves and prolonged survival. They have thus changed our understanding of the kinetics of immune response. However, much work remains to be done. First, cellular immune response assays must be standardized and validated as reproducible biomarkers that can be correlated with clinical outcomes. Second, the new irRC, which are able to capture more complex response patterns, should replace RECIST. irRC assess tumor burden as a continuous variable, accounting for index lesions identified at baseline and new lesions that occur after initiation of treatment, based on bidimensional measurements of each lesion. Third, new statistical models describing hazard ratios as a function of time and recognizing differences before and after the separation of Kaplan-Meier curves should be developed to evaluate phase III trials.

It is hoped that the initial successes in prostate cancer will spur investigators to employ vaccines in the treatment of other cancers as well. The use of vaccines in combination with standard therapies may lead to greater benefit than with either treatment alone. Additional clinical trials are required to answer many of these questions, but immune-mediated antitumor treatment may one day be as common as treatment with mAbs and targeted molecular agents, which were also relegated at one time to the realm of mere scientific curiosity. Therapeutic cancer vaccines, which have few side effects and the potential to generate long-term immune responses that add clinical benefit to subsequent therapies, have already transformed cancer care and hold the promise of revolutionizing future cancer treatments.

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Abbreviations

^{51}Cr	Radioactive ^{51}chromium
5-FU	5-Fluorouracil
AAV	Adeno-associated virus
ADCC	Antibody dependent cellular cytotoxicity
APC	Antigen presenting cell
APRIL	A proliferation-inducing ligand
CAR	Coxsackie and adenovirus receptor
CR	Complete response
CTL	Cytotoxic T lymphocyte
CTLA-4	Cytotoxic T-lymphocyte-associated antigen-4
DAMP	Danger-associated molecular patterns
DC	Dendritic cell
DFS	Disease-free
dsFv	Disulfide-bond stabilized Fv (single chain antibody fragment)
EBV	Epstein-Barr virus
FDA	U.S. Food and Drug Administration
FGF	Fibroblast growth factor
FOLFIRI	Chemothertapy for colorectal cancer with irinotecan plus 5-fluorouracil plus leucovorin
FOLFOX	Chemotherapy for colorectal cancer with oxaliplatin plus 5-fluorouracil plus leucovorin
Foxp3	A nuclear transcription factor governing regulatory T cell differentiation and function
GITR	Glucocorticoid-induced tumour-necrosis factor receptor-related protein
GM-CSF	Granulocyte-macrophage colony stimulating factor
GVHD	Graft versus host disease
HAMA	Human anti-mouse antibody
HLA	Human leukocyte antigen
hTERT	Human telomerase reverse transcriptase

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IDO	Indolamine 2,3-dioxygenase
IFA	Incomplete Freund’s adjuvant
IFN	Interferon
IL	Interleukin
L-Arg	L-arginine
LV	Leucovorin
mAb	Monoclonal antibody
MCP-1	Monocyte/Macrophage chemoattractant protein-1
MDC	Myeloid dendritic cell
MDSC	Myeloid-derived suppressor cell
MHC	Major histocompatibility complex
MoDC	Monocyte-derived dendritic cell
MOI	Multiplicity of infection
mRNA	Messenger ribonucleic acid (RNA)
mTOR	Mammalian target of rapamycin
NCI	National Cancer Institute
NK	Natural killer
NO	Nitric oxide
OS	Overall survival
PAMP	Pattern-associated molecular patterns
PCR	Polymerase chain reaction
PD-1	Programmed death receptor-1
PDC	Plasmacytoid dendritic cell
PFS	Progression free survival
pMHC	Peptide-MHC complex
PR	Partial response
PRR	Pattern recognition receptors (such as DAMPs and PAMPs)
PSA	Prostate specific antigen
RANK ligand	Receptor activator of nuclear factor-kappa B
RNA	Ribonucleic acid
scFv	Single chain antibody fragment
TAA	Tumor-associated antigens
Tcm	Central memory T cells
TCR	T cell receptor
TDLN	Tumor draining lymph node
Tem	Effector memory T cells
TGF	Transforming growth factor
Th	T helper cell (a type of $CD4^+$ T lymphocyte)
TIL	Tumor infiltrating lymphocyte
TLR	Toll like receptor
TNF	Tumor necrosis factor
Treg	Regulatory T cell
VEGF	Vascular endothelial growth factor

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