Ernst Schering Research Foundation
Workshop 50

Animal Models of T Cell Mediated Skin Diseases

T. Zollner
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(Editors)

Springer

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With 33 Figures

Springer

Series Editors: G. Stock and M. Lessl

Library of Congress Control Number: 2004106459

ISSN 0947-6075
ISBN 3-540-21067-9 Springer Berlin Heidelberg New York

Springer is a part of Springer Science+Business Media
springeronline.com

Printed in Germany

Editor: Dr. Ute Heilmann, Heidelberg
Desk editor: Wilma McHugh, Heidelberg
Production editor: Andreas Gösling, Heidelberg
Cover design: design & production GmbH, Heidelberg
Typesetting: K+V Fotosatz GmbH, Beerfelden

Printed on acid-free paper – 21/3130/AG-5 4 3 2 1 0

Preface

Our understanding of inflammatory diseases in humans is tightly linked to animal models of these disorders. Significant knowledge regarding the pathophysiology of inflammation was first gained in animals and later documented for humans. In addition, animal models are of key importance for target identification and lead compound discovery and optimization. Indeed, most pharmaceutical companies base decisions on the clinical development of candidate compounds and on results obtained from animal research in disease models. Nevertheless, attrition rates in clinical development are still very high; up to 90% of new compounds fail in clinical phases I–III. Late-stage clinical failure is to a great extent due to lack of clinical efficacy, indicating that there is a strong need for highly predictive in vitro and in vivo models.

We thus wanted to organize a workshop that would provide a forum to present and discuss recent developments and breakthroughs in this exciting and important research field with a particular emphasis on animal models for acute and chronic inflammatory skin diseases. For this 50th ESRF Workshop on "Animal Models of T Cell-Mediated Skin Diseases" held in Berlin from 24 to 26 November 2003, we were pleased to bring together a group of international scientists from seven countries who are leading in their field. The first part of the proceedings of this workshop covers, in addition to methodological aspects, diseases sharing pathophysiological aspects of inflammatory skin disorders. The second part deals with the three major indications atopic dermatitis, psoriasis, and allergic contact dermatitis. We are grateful to the authors for their excellent contribu-

tions to the proceedings. The organizers hope that this publication will be a valuable source for scientists and clinicians alike, since this book provides an overview of the pathophysiology, value, but also limitations of many currently available models of T cell-mediated skin diseases.

Berlin, April 2004
Thomas Zollner, Harald Renz, Khusru Asadullah

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1 Making Gene-Modified Mice

M. Pasparakis

Genetic studies in mice have made a major contribution to our current understanding of mammalian biology. The development over the last three decades of molecular biological techniques allowing the genetic manipulation of the mouse has revolutionised experimental biology and has provided an unprecedented tool for the in vivo analysis of gene function in mammals. Today, this technology has developed to a sophistication that permits the manipulation of the mouse genome with nucleotide precision. This chapter does not aim to provide a comprehensive coverage of the technologies currently available for the genetic engineering of the mouse, a subject covered extensively in numerous excellent laboratory protocol books and review articles. Within the current space limitations, this manuscript rather attempts to outline the potential of this technology and discuss some of its applications in the generation of genetically modified mice.

1.1 Transgenic Mice

1.1.1 Factors Affecting Transgene Expression

The most widely used method for the generation of transgenic mice is by microinjection of DNA into the pronuclei of fertilised mouse oocytes. Transgenes introduced by pronuclear injection are integrated into a random position in the mouse genome and usually contain multiple copies of the injected DNA in a head to tail array. Several factors can affect the expression pattern of transgenes and should be taken into consideration for the design of transgenic constructs (Brinster et al. 1985). Transgenes usually contain a promoter, an open reading frame, and a polyadenylation signal that ensures the efficient translation of the produced messenger RNA. It was recognised very early that the presence of bacterial DNA sequences, such as parts of the plasmid vectors used for subcloning, can interfere with the efficient expression of transgenes and should be removed. In addition, it was shown that the presence of introns is important for efficient transgene expression. Hence, when cDNAs are used as transgenes it is advisable to introduce heterologous intron sequences. Although placement of heterologous introns between the coding se-

quences and the polyadenylation signal has been used successfully in many cases, the presence of an intron downstream of the translation termination codon has the risk of creating a transcript that could be a target for nonsense-mediated decay mechanisms (Hentze and Kulozik 1999). For this reason, it is currently preferred to introduce the intron sequences between the promoter and the coding region of the transgene in order to achieve maximum expression efficiency.

Despite the use of promoter and enhancer elements to drive tissue-specific transgene expression, many transgenic lines do not display the expected expression pattern, and usually several independent founders must be tested in order to obtain a line that shows efficient expression. This is because the position of integration affects transgene expression by influencing the chromatin remodelling processes that are critical for gene transcription. When transgenes are inserted close to heterochromatin, they are often expressed in only a fraction of the cells in a certain tissue, a phenomenon known as position effect variegation. Introduction of locus control regions (LCRs) overcomes position effects and gives position-independent and copy number-dependent transgene expression by providing all the necessary *cis*-acting elements that ensure efficient chromatin remodelling and transcription of the transgene (Kioussis and Festenstein 1997). Use of large genomic fragments for transgene construction ensures that all necessary *cis*-regulatory elements are present, and usually results in highly reproducible expression patterns. Taking advantage of the development of techniques allowing the genetic engineering of bacterial artificial chromosomes (BACs) by homologous recombination in bacteria (Yang et al. 1997), the use of large BAC fragments has become a particularly effective approach for the generation of transgenic mice showing expression patterns that faithfully resemble those of endogenous genes (Gong et al. 2003).

1.1.2 Production of Transgenic Mice Using Lentiviral Vectors

Highly efficient generation of transgenic mice was recently demonstrated upon infection of single-cell mouse embryos with lentiviral vectors (Lois et al. 2002). Lentiviral transgenes were expressed efficiently and were transmitted to the progeny of transgenic founders,

showing that this approach produces stable transgenic lines. The advantages of using lentiviruses for the production of transgenic mice are the high efficiency of transgene integration and the relatively simple experimental protocol used, which does not require specialised microinjection equipment and expertise in the generation of transgenic mice. Furthermore, lentiviral vectors can be used to produce transgenic animals in different species, thus providing a method for transgenesis in animals where generation of transgenics by pronuclear injections is not possible or is very inefficient (Hofmann et al. 2003). The main limitation of lentiviral transgenesis is the relatively small size of transgenes (10 kb) that can be inserted into the lentiviral vectors (Lois et al. 2002). In addition, the insertion of lentiviruses in multiple genomic locations in a single embryo could complicate the generation of well-characterised transgenic lines carrying a single integration site, since extensive breeding would be needed for transgene segregation.

1.2 Tetracycline-Regulated Transgene Expression

The most widely employed approach for inducible transgene expression is the tetracycline-regulated system (Gossen et al. 1994). This system is based on the tetracycline-controlled transactivation of gene expression, using fusion proteins of the bacterial tetracycline repressor (TetR) with the viral VP16 transactivation domain to drive expression of genes under the control of a minimal promoter containing tet operator (tetO) sequences. There are two versions of the tetracycline system. In the original version referred to as Tet-Off, the tetracycline transactivator (tTA) binds and drives expression of the tetO promoter in the absence of the inducer (Doxycycline, Dox), but addition of Dox prevents its binding and stops expression. The Tet-On system was developed later and makes use of a reverse tetracycline transactivator (rtTA) that cannot bind to the tetO promoter in the absence of the inducer, but binds and drives expression in the presence of Dox (Fig. 1). Application of the tet systems in mice requires the presence of two transgenes, one expressing the tetracycline transactivator under the control of a tissue-specific promoter, and another containing the gene to be regulated by the tet system

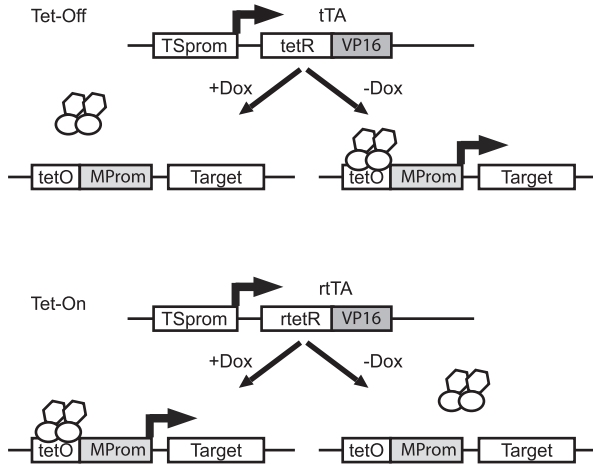

Fig. 1. Tetracycline-regulated gene expression. The tetracycline transactivator (*tTA*, *rtTA*) is expressed under the control of a tissue-specific promoter (*TSprom*) and regulates the expression of a target transgene controlled by a tetracycline responsive minimal promoter (*MProm*) in the tet-Off system, addition of Dox represses expression whereas in the tet-On system, Dox activates expression

under the control of the tetO promoter (Mansuy and Bujard 2000). Although placing both components in one transgenic construct or co-injection of the two transgenes resulting in co-integration has occasionally led to good results (Ray et al. 1997; Schultze et al. 1996), in most cases two different transgenic lines are generated and subsequently inter-crossed to produce double transgenics. The main advantage of generating independent lines is that it allows the screening of several founders in order to select the ones showing the correct expression pattern before they are inter-crossed. In addition, each transactivator-expressing transgenic line can be combined with several different tetO lines and vice versa, thus providing more flexibility in using each line to express different transgenes under the control of different regulatory elements. A particularly useful modification of this system allows two different transgenes to be co-regulated placing the tetO sequences between two minimal promoters (Baron et al. 1995). This approach allows, for example, the simulta-

neous expression of both a transgene and a reporter gene that can be used to monitor the kinetics and specificity of expression.

1.3 Modification of Endogenous Genes in the Mouse

1.3.1 Mouse Embryonic Stem Cells

Transgenic technology allows the introduction and expression of exogenous DNA into the mouse genome. The targeted manipulation of endogenous genes in the mouse was made possible by the isolation and culture of pluripotent cells derived from the inner cell mass of mouse embryos at the blastocyst stage. These embryonic stem (ES) cells can be maintained in culture for several generations without losing their pluripotency, and when introduced into developing mouse embryos they can colonise all tissues including the germ-line, giving rise to chimeric mice derived partly from the host embryo and partly from the ES cells. The demonstration that chimeric mice can transmit the ES cell-derived genome to their progeny, in combination with the development of gene-targeting techniques in ES cells, provided mouse geneticists with an unprecedented tool for the manipulation of the mouse genome (Robertson et al. 1986; Thomas and Capecchi 1987).

1.3.2 Gene Targeting by Homologous Recombination in Mouse ES Cells

Gene targeting, defined as targeted modification of an endogenous genomic locus, takes advantage of homologous recombination to replace a part of an endogenous gene with a modified DNA fragment. A standard gene-targeting vector contains two fragments of homologous sequences flanking a selectable marker, usually a *neo* expression cassette that provides resistance to G418. Since homologous recombination is a relatively rare event, a negative selection marker is often introduced at either end of the homologous sequences to select against clones containing random integration of the targeting vector (Fig. 2a; Mansour et al. 1988). Thymidine kinase (*tk*) expression

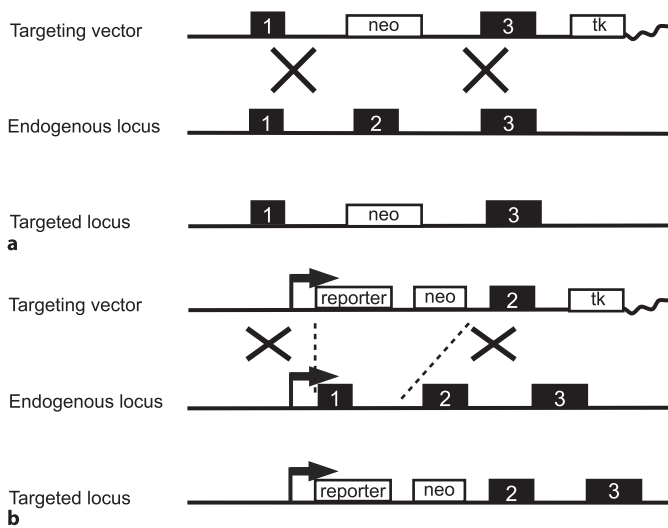

Fig. 2 a, b. Gene targeting by homologous recombination in ES cells using the positive-negative selection strategy. **a** The targeting vector containing a positive (*neo*) and a negative (*tk*) selection marker is introduced in the ES cells and replaces the endogenous locus by homologous recombination. Selection with G418 and ganciclovir is applied to enrich for the homologous recombinant ES cell clones. **b** A reporter gene is inserted into the targeted locus and is expressed under the control of the endogenous promoter

cassettes, which allow negative selection using ganciclovir, or diphtheria toxin expression constructs have been successfully used to enrich for homologous recombination events. The frequency of homologous recombination may vary significantly between different genetic loci; however, the usage of isogenic DNA for the preparation of the targeting vector (te Riele et al. 1992) and the design of homologous arms of at least 3–4 kb length usually guarantee reasonable targeting frequencies (Hasty et al. 1991).

1.3.3 Generation of Knockout Mice

Gene targeting by homologous recombination in ES cells has been used extensively for the generation of "knockout" mice carrying tar-

geted inactivation of specific genes. A gene knockout is usually achieved by the insertion of a neo selection marker that either disrupts or replaces an essential part of the target gene (Fig. 2a). A disadvantage of this strategy is that the selectable marker cassette remains in the targeted locus and can in some cases interfere with the expression of neighbouring genes, thus complicating the analysis and interpretation of the phenotype of the knockout mice (Olson et al. 1996). A useful addition to this basic principle is the introduction of a reporter gene, for example beta-galactosidase or green fluorescent protein (GFP), into the targeted locus so that it is expressed under the control of the endogenous promoter, facilitating the analysis of the expression pattern of the targeted gene during mouse development and also in the adult (Fig. 2b).

1.4 Site-Specific DNA Recombinases in Gene Targeting

1.4.1 The Cre/loxP and Flp/FRT Site-Specific DNA Recombination Systems

The introduction of site-specific DNA recombinases to the battery of tools available for gene-targeting experiments opened new possibilities for the manipulation of the mouse genome. The DNA recombinase most widely used in gene-targeting experiments is the bacteriophage P1-derived Cre recombinase, which recognises and mediates site-specific recombination between 34-bp recognition sequences referred to as loxP sites (Sauer 1998). The orientation of the loxP sites determines the final outcome of the recombination exchange; when the two loxP sites are in the same orientation the sequence between them is deleted, and when the two loxP sites are in the opposite orientation, the sequences they flank are inverted (Fig. 3). The recombination reaction is reversible, and although when the sequence is deleted the probability of it being re-inserted is very low, when the loxP sites are in reverse orientation, Cre-mediated inversion is constant as long as the recombinase is present, reaching equilibrium between the two orientations. A second site-specific DNA recombinase used in gene-targeting experiments is Flp, which recognises 48-bp sequences called FRT sites (Rodriguez et al. 2000; Schaft et al.

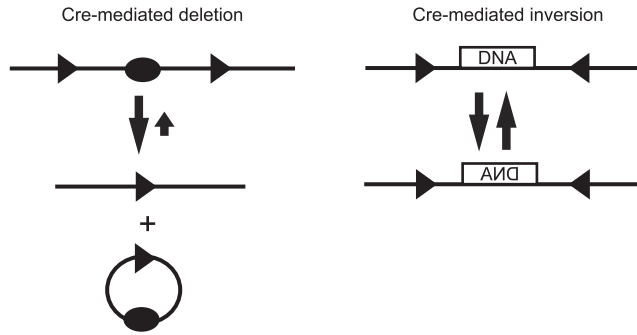

Fig. 3. The Cre/loxP site-specific DNA recombination system. The outcome of Cre-mediated recombination depends on the orientation of the two loxP sites (shown here by *arrowheads*). If the loxP sites are in same orientation, the sequence in between them is deleted. If they are in the opposite orientation, then the loxP-flanked sequence is inverted. Both reactions are reversible, but practically, reversion is only observed in the latter case since re-insertion of the excised circularised sequence is a rare event. The same principles apply to the Flp/FRT recombination system

2001). Flp/FRT-mediated recombination follows the same principles as the Cre/loxP system. Cre is usually the preferred recombinase in gene targeting since it shows higher efficiency than Flp; however, the Flp/FRT system provides an alternative tool that can be used for these experiments. The combination of the Cre/loxP and Flp/FRT recombination systems provides an additional level of flexibility in the use of site-specific DNA recombination for the manipulation of the mouse genome.

1.4.2 The Cre/loxP System in Gene Targeting

1.4.2.1 Deletion of Selection Marker Cassettes from Targeted Loci

Selection marker cassettes used for knockout experiments contain strong promoters that can interfere with the expression of neighbouring genes, complicating the analysis of the phenotype of knockout mice (Olson et al. 1996). The use of loxP-flanked (usually referred to as “floxed”) selection cassettes allows the Cre-mediated excision

of the selection marker after the homologous recombination has been accomplished. The deletion of the selection marker can be performed either in vitro by transient transfection of Cre recombinase, or in the mouse by crossing to a Cre-deleter transgenic mouse strain.

The use of the Cre/loxP system to remove the selection marker cassette from targeted loci has allowed not only the generation of "clean" knockouts but also the introduction of non-selectable functional modifications into endogenous mouse genes. In such experiments a two-step approach is followed where in the first step part of the gene is replaced by homologous recombination, and in a second step the floxed selection marker is removed by expression of Cre recombinase. These "knock-in" mutations can vary from single-nucleotide changes to replacement of parts or of all the coding region of a gene with a heterologous coding sequence (Fig. 4). The ability to replace the coding region of a gene with a heterologous sequence also provides an alternative approach for the generation of transgenic mice. In order to express a transgene with precisely the same expression pattern as the endogenous gene, the transgene can be inserted

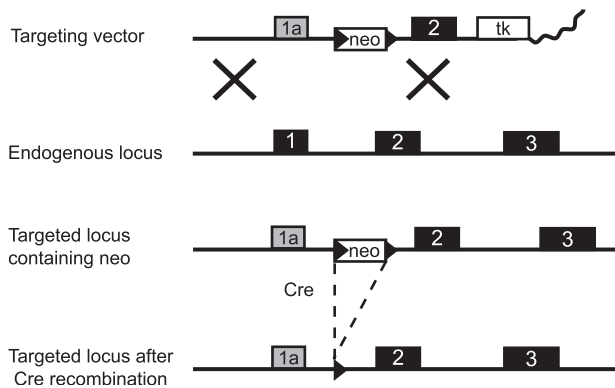

Fig. 4. Knock-in of a mutation and subsequent removal of the selection marker from the targeted locus. Alterations ranging from single-nucleotide changes to replacement of parts of a gene with a heterologous sequence are introduced by homologous recombination. The selection marker is subsequently removed by Cre-mediated recombination, since it often interferes with the expression of the targeted gene

by homologous recombination into the endogenous locus and thus be expressed under the control of the endogenous *cis*-regulatory elements. Since this approach leads to the disruption of the endogenous gene, it can only be used for the generation of transgenic mice when heterozygous inactivation of the endogenous gene does not cause a phenotype.

The removal of the floxed selection cassette from the targeted locus can be achieved either by expression of Cre recombinase in vitro, or by crossing the mouse carrying the targeted allele with a Cre-deleter transgenic strain. In vitro deletion is usually a faster approach but requires a second transfection/selection step that may decrease the efficiency of the targeted ES cell clones to generate germ-line chimeras. Deletion of the floxed cassette in vivo, on the other hand, is time-consuming since it requires additional mouse breeding. In an elegant experimental approach, a self-deletable selection cassette (ACN) was designed that facilitates the removal of the selection marker from the targeted locus without requiring additional transfection/selection of the cells or mouse breeding (Bunting et al. 1999). This cassette contains a neo selection marker under the control of the RNA polymerase II promoter and an expression cassette with Cre recombinase placed under the control of the sperm-specific angiotensin-converting enzyme (tACE) promoter, and is flanked by loxP sites. When ES cells targeted by homologous recombination using the ACN cassette are used for the generation of chimeric mice, Cre is expressed and efficiently deletes the floxed ACN cassette during spermatogenesis, resulting in the germ-line transmission of the targeted locus without the selection marker.

1.4.2.2 Deletion of Large Genomic Fragments

Another application of the Cre/loxP system is to delete large genomic fragments that cannot be targeted in a single homologous recombination event. In this case, two sequential targeting experiments are performed to insert two loxP sites flanking the fragment to be deleted. Subsequently, the floxed genomic fragment is deleted by transient expression of Cre recombinase in ES cells. Since the efficiency of Cre-mediated recombination drops as the distance between the two loxP sites increases, strategies allowing the selection of cells carrying the recombined alleles need to be employed for the effi-

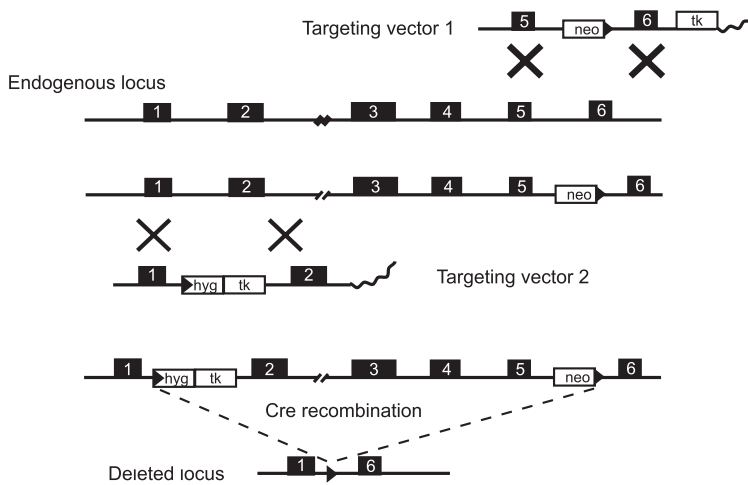

Fig. 5. Deletion of large genomic fragments using the Cre/loxP system. Two sequential gene-targeting experiments are performed introducing loxP sites flanking the genomic sequence to be deleted. The *tk* cassette is used as a negative selection marker in the first targeting, and also in the last step to enrich for the cells carrying the excised locus upon Cre recombination

cient isolation of cells carrying large deletions, for example by introducing a *tk* cassette into the locus (Fig. 5). An alternative approach is based on the separate introduction of two halves of an HPRT mini gene into the targeted locus, which are brought together upon Cre recombination, allowing the positive selection of the recombined clones in an HPRT-deficient ES cell line. This approach has been used successfully to engineer large chromosomal rearrangements in the mouse (Yu and Bradley 2001; Zheng et al. 2000).

1.5 Conditional Gene Targeting

The generation of mutant mice carrying disruption of specific genes revolutionised the study of gene function in mammals, since it allowed the analysis of the effect of gene inactivation in the context of a living organism. A large number of genes have been knocked-

out in the mouse, providing invaluable information about the function of these genes in mouse development and physiology. However, when a gene is important for mouse development, its inactivation may lead to early embryonic lethality. Although such knockouts reveal the role of these genes in development, they do not allow the analysis of gene function in tissues of the adult mouse. In addition, disruption of genes that display pleiotropic functions in multiple cell types often results in complex phenotypes, complicating the analysis of the function of these genes in individual tissues.

Conditional gene targeting using the Cre/loxP system overcomes these problems by allowing spatially and temporally controlled inactivation of selected genes (Rajewsky et al. 1996). This is achieved by using tissue-specific and/or inducible expression of Cre recombinase to inactivate genes in vivo. In this method, homologous recombination in ES cells is first used to introduce loxP sites flanking an

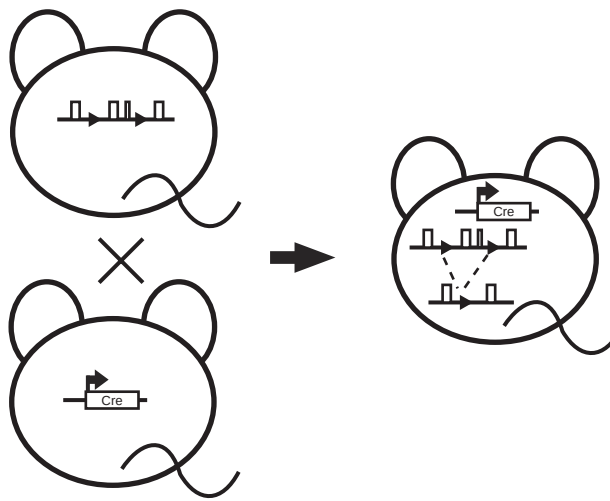

Fig. 6. Conditional gene targeting using the Cre/loxP system. For conditional gene targeting, a mouse carrying a loxP-flanked but fully functional allele is generated by homologous recombination in ES cells. By crossing to a Cre transgenic mouse, the loxP-flanked allele is inactivated only in cells expressing Cre recombinase

essential part of a gene, generating a mouse that carries a functional floxed allele. In a second step, the mouse carrying the floxed gene is crossed to a transgenic mouse expressing Cre recombinase under the control of a tissue-specific and/or inducible promoter. In the resulting double transgenic progeny, the floxed allele will be deleted only in the cells expressing Cre, resulting in tissue-restricted gene inactivation (Fig. 6).

1.5.1 Generation of loxP-Flanked Conditional Alleles

Several factors should be taken into consideration for the design of targeting vectors for the generation of floxed alleles. Since the floxed gene should be fully functional before Cre recombination, it is important to introduce the loxP sites in areas of the gene that are not important for gene transcription, splicing, mRNA stability, and translation. Insertion of loxP sites in the 5′ or 3′ untranslated region of a gene is not recommended, since it may interfere with mRNA translation. In addition, placement of loxP sites in the promoter area carries the risk of interfering with gene expression. Thus, it is generally preferred to place loxP sites inside introns or at the 3′ of a gene downstream of the polyadenylation signal. It is important not to place loxP sites too close to exon/intron junctions, in order to avoid interference with splicing. The efficiency of Cre-mediated recombination is inversely proportional to the size of the floxed fragment to be deleted. Thus, in order to facilitate easier deletion of the loxP-flanked alleles, it is important to keep the length of the floxed genomic fragments as short as possible.

The selection marker must be removed from the targeted allele upon homologous recombination in order to avoid interference with the expression of the floxed gene or of neighbouring genes. For this reason, selection cassettes flanked by loxP or FRT sites are used for such gene-targeting experiments. When a loxP-flanked selection marker is used, the targeted locus contains three loxP sites upon homologous recombination. In this case, in order to remove the floxed marker from the targeted gene without deleting the loxP flanked genomic fragment, a partial excision must be achieved. Partial excision of the floxed marker is usually performed in vitro by transient

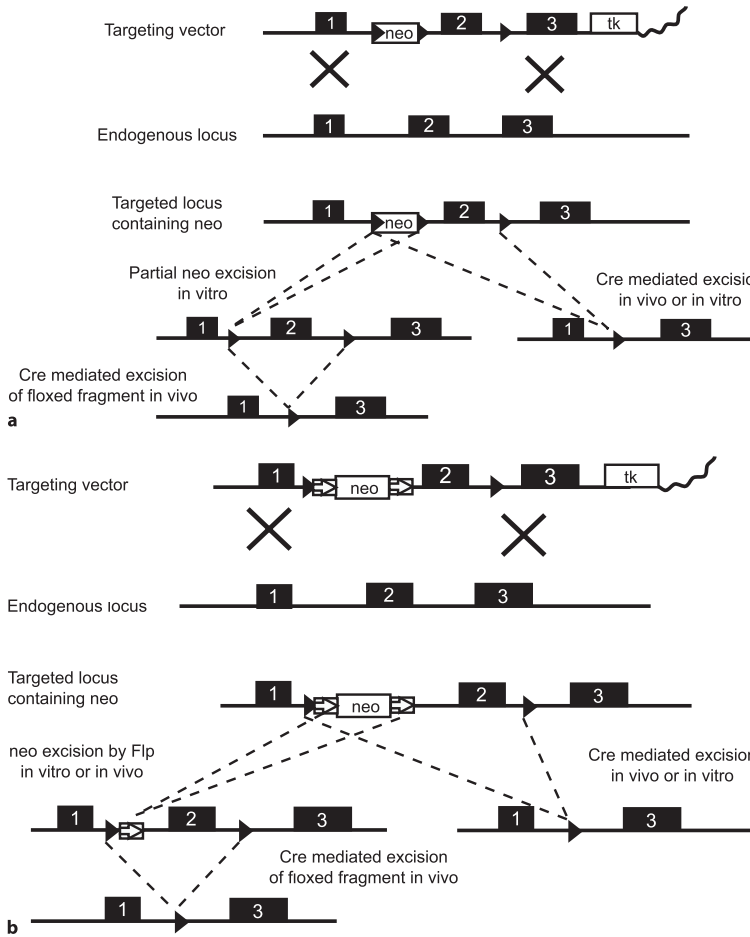

Fig. 7 a, b. Generation of loxP-flanked conditional alleles. **a** loxP-flanked selection marker. Upon homologous recombination the targeted allele contains three loxP sites. Partial excision of the selection marker creating a locus containing two loxP sites is achieved in vitro. The loxP-flanked allele can be deleted in vivo in a tissue-specific manner by crossing to Cre transgenic mice. Complete deletion resulting in the "conventional" knockout of the gene can be achieved either in vitro or in vivo. **b** FRT-flanked selection marker. Upon homologous recombination the FRT-flanked selection marker is excised by Flp-mediated recombination in vitro or in vivo, generating the loxP-flanked conditional allele

expression of Cre recombinase in targeted ES cells (Fig. 7a). For partial recombination resulting in the removal of the selection marker, it is important to use a weak Cre expression vector for transfection (e.g. pIC-Cre), since strong Cre expression vectors (e.g. PGK-Cre) will result in complete deletion of all the locus contained between the three loxP sites (Gu et al. 1994). An alternative approach is to use an FRT-site-flanked selection marker that can be excised by expression of Flp recombinase without affecting the loxP-flanked genomic locus (Fig. 7b). Although Flp is not as efficient as Cre, deletion of FRT-flanked selection cassettes can be performed successfully in vitro by transient expression of Flp recombinase (Schaft et al. 2001) or in vivo by crossing to Flp-deleter transgenic mice (Rodriguez et al. 2000).

1.5.2 Conditional Transgene Activation

The Cre/loxP system can also be used for the activation of transgenes in a spatially and/or temporally controlled manner. This is particularly useful when a transgene is toxic and its expression must be very tightly controlled during development or in many tissues of the adult. Conditional transgenes usually contain a floxed "STOP" cassette, a DNA sequence that prevents transcription and translation, placed between the promoter and the coding region of the transgene. The expression of these transgenes is tightly controlled by the presence of the STOP cassette and can get activated only in cells where Cre recombinase is expressed and deletes the floxed STOP sequences (Fig. 8). This system has been used successfully to express toxic genes such as the diphtheria toxin A subunit (DTA) in a highly restricted pattern in experiments aiming to ablate specific cell types (Grieshammer et al. 1998). Another application of Cre-controlled transgene activation is to restrict expression only in certain cells where both promoters, the one driving the transgene and the one driving Cre, are co-expressed (Fig. 8). This is particularly useful when there is no available promoter that would drive expression restricted to this cell population. The use of inducible Cre transgenes offers another level of regulation by allowing temporal control of target transgene activation.

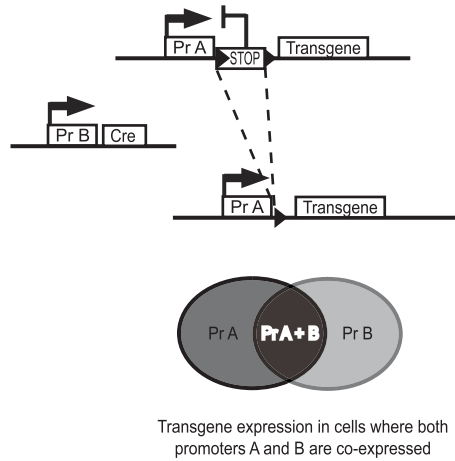

Fig. 8. Conditional transgene expression using the Cre/loxP system. Cre-mediated deletion of a floxed STOP cassette results in transgene expression in tissues where both promoters, the one driving Cre (Promoter B) and the one driving the transgene (Promoter A), are co-expressed

1.5.3 Cre-Mediated Transgene Inversion

When the loxP sites flanking a genomic sequence are placed in the opposite orientation, Cre expression will lead to the inversion of the floxed sequence. Since this reaction is reversible, Cre recombination will be constant as long as Cre is present in the cell, and is expected to reach equilibrium between the two orientations in a given cell population. However, it has been shown that loxP sites with mutated asymmetric 34-bp sequences favour the forward direction of the inversion reaction, since the loxP sites generated after the first recombination display lower recombination efficiency. By using such mutant loxP sites it is possible to achieve efficient unidirectional inversion of target sequences in vitro and in vivo (Oberdoerffer et al. 2003). A useful application of Cre-mediated inversion is to switch between expression of two different transgenes under the control of the same promoter (Fig. 9).

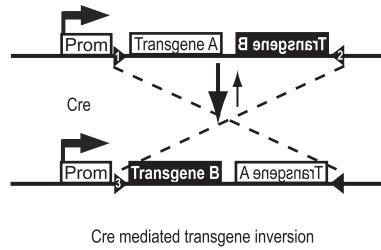

Fig. 9. Cre-mediated transgene inversion. Using mutated asymmetrical loxP sites (which favour unidirectional Cre recombination) placed in the opposite orientation, it is possible to efficiently switch between expression of two different transgenes under the control of the same promoter

1.5.4 Inducible Cre Recombination

Tissue specificity can be achieved by using appropriate promoters to drive Cre expression. In many cases it is desirable to be able to control not only the tissue specificity but also the timing of Cre expression. Temporal control of Cre recombination is made possible by either controlling expression of Cre recombinase at the transcriptional level using the tetracycline system, or by regulating Cre activity using fusion proteins of Cre with the hormone-binding domain of steroid receptors. When using the tet system, a critical parameter is to obtain a tightly controlled tetO-Cre transgenic line that does not show leakage of Cre expression in the absence of the transactivator but can be efficiently induced by the removal (Tet-Off) or addition (Tet-On) of the inducer. Such a tightly controlled tetO-Cre transgenic line can be used to drive expression of Cre under the control of different transactivator lines (Schonig et al. 2002).

Fusion proteins between Cre and mutated ligand-binding domains of the progesterone or the oestrogen receptors have been used to regulate Cre activity in mammalian cells (Kellendonk et al. 1996; Metzger et al. 1995). These Cre-LBD fusion proteins are kept inactive in the cytoplasm in the absence of the inducer, but in the presence of the synthetic ligand (RU486 or tamoxifen, respectively) they bind and accumulate in the nucleus, where they mediate recombination of loxP-flanked sequences. Although this system has been used suc-

cessfully in certain tissues and particularly in the epidermis (Indra et al. 1999; Vasioukhin et al. 1999), the very high doses of inducer required for activation, and the low efficiency and slow rate of recombination induction, which often result in mosaic patterns of recombination, are potential disadvantages that could be overcome by the development of improved versions of the fusion proteins showing higher sensitivity to the ligand and increased inducibility.

1.6 Cre Transgenic Lines

1.6.1 Generation of Cre Transgenic Lines

The generation of transgenic lines showing efficient expression of Cre recombinase is essential for conditional gene-targeting experiments. Many existing Cre transgenic lines have been produced using pronuclear injections, and in most cases many different founders had to be tested in order to select a line that shows a satisfactory expression pattern. A problem often encountered during the characterisation of Cre transgenic lines is the transient expression of the Cre transgene early during mouse development, resulting in mice with deletion of the floxed alleles in nonspecific tissues, often including the germ line. Such nonspecific Cre expression has been observed even in some cases where Cre was expressed under the control of well-characterised promoters that had been previously shown to drive highly restricted tissue-specific expression of different transgenes. This problem is caused by integration site-dependent position effects that may lead to transient expression of the Cre transgene during mouse development in nonspecific tissues. Such transient expression in early embryonic stages would remain unnoticed with most other transgenes; however, Cre expression at this stage permanently deletes the floxed sequences, and thus all adult tissues derived from these cells will also show deletion of the floxed alleles. In order to ensure tightly regulated Cre expression, BAC-based transgenes have been used for the generation of tissue-specific Cre transgenic lines with very good results (Casanova et al. 2001). An alternative approach that guarantees tightly controlled Cre expression is to knock-in the Cre coding sequence under the control of an

endogenous promoter by homologous recombination in ES cells (Rickert et al. 1997). A disadvantage of the latter approach is that Cre insertion disrupts the function of the endogenous gene, and thus it is useful only when heterozygous inactivation of that gene does not result in any phenotype.

1.6.2 Characterisation of Cre Transgenic Lines

The generation of Cre transgenic lines showing strict specificity and high efficiency is critical for successful conditional gene-targeting experiments. For this reason each Cre transgenic line must be rigorously tested to demonstrate that it shows the expected pattern of Cre recombination. This analysis has been made easier by the generation of Cre-reporter lines where Cre-mediated recombination activates expression of a reporter transgene such as beta-galactosidase or GFP. Such Cre-reporter transgenes usually contain a floxed "STOP" cassette between a ubiquitous promoter and the reporter gene. Cre-mediated excision of the STOP cassette activates expression of the reporter in a pattern mirroring the pattern of expression of Cre recombinase. The most widely used Cre reporter lines have been generated by using a knock-in approach to introduce reporter constructs under the control of the ubiquitously expressed endogenous ROSA26 locus (Mao et al. 2001; Soriano 1999). Other existing Cre-reporter lines have been produced by selection of single-copy reporter transgenes driven by ubiquitous promoters. A useful modification of this approach is the generation of Cre-reporter lines that allow the Cre-mediated switching between two different reporter genes, where a lacZ reporter is expressed before recombination and is excised by Cre to turn on expression of alkaline phosphatase as a second reporter that marks Cre-expressing cells (Lobe et al. 1999).

1.7 Considerations Regarding the Application of the Cre/loxP Recombination System for Conditional Gene Inactivation

The usage of the Cre/loxP system offers great advantages for conditional gene modification in the mouse. However, the practical appli-

cation of this technology is not always straightforward and may prove a very complicated task for the inexperienced user. Several considerations should be taken into account for the successful outcome of conditional targeting experiments. Cre-mediated recombination is a stochastic event and does not happen at the same time in all cells where Cre is expressed. The probability of Cre recombination occurring in a given cell depends on the levels of Cre recombinase in this particular cell, and thus the efficiency of Cre recombination is affected by the timing of Cre transcription initiation and the strength of the promoter used. Cre recombination in a certain tissue may saturate at near 100% or lower levels, depending on the Cre transgene. In addition, the excision of the loxP-flanked sequences from the genome of a cell does not simultaneously make this cell deficient for the targeted gene product. Cells usually contain certain levels of mRNA and protein that have been produced by the target gene before excision, and these levels must be diminished before the cell becomes functionally deficient for the targeted gene product. The delay between Cre-mediated excision of the floxed alleles and the disappearance of the protein produced by the target gene depends on the half-lives of the mRNA and protein and may vary considerably between different genes (Fig. 10). This delay may be par-

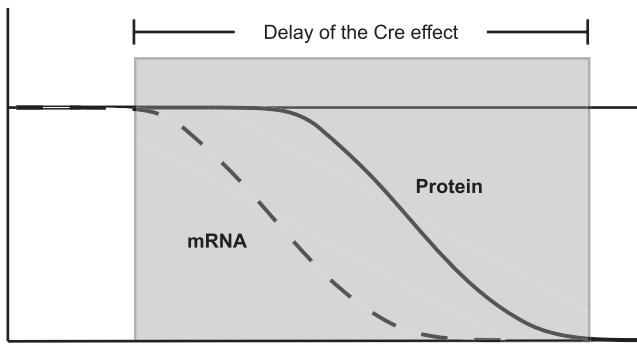

Fig. 10. Delay of the Cre effect in conditional gene targeting. The excision of the loxP-flanked gene does not simultaneously make a cell deficient in the gene product. The delay between Cre-mediated excision of the floxed allele and the disappearance of the gene product depend on the half-lives of its mRNA and protein

ticularly important in cases where rapidly renewed dynamic cell populations (such as lymphocytes) are targeted, and should be taken into consideration for the interpretation of the results obtained from such experiments (Pasparakis et al. 2002; Schmidt-Supprian et al. 2003).

The efficiency of Cre-mediated recombination achieved in a particular experiment may depend also on the effect of the target-gene inactivation in cell viability or proliferation capacity. If inactivation of a gene leads to death or growth disadvantage of the cells, then selection against the cells having undergone Cre recombination will result in reduced recombination efficiency in this population (Pasparakis et al. 2002; Schmidt-Supprian et al. 2003).

1.8 ES Mice

Experiments with genetically modified mice, in particular using conditional gene targeting, are time-consuming since they require extensive mouse breeding. A recently developed technology allows the quick production of mice carrying multiple modifications into their genomes and promises to reduce the time required for mouse experiments by overcoming the need for multiple breedings. This method takes advantage of the observation that when ES cells are injected into tetraploid blastocysts, which are produced by electrofusion of two-cell-stage embryos, the resulting embryos are derived almost exclusively from the diploid ES cells while the extraembryonic tissues arise largely from the tetraploid host cells (Nagy et al. 1993). Recently it was shown that it is possible to produce viable completely ES cell-derived mice using tetraploid blastocyst complementation; however, for reasons that are not fully understood, only ES cell lines isolated from F1 embryos produced by crosses between different mouse strains are efficient for the production of viable “ES mice” (Eggan et al. 2001). By employing sequential targeting experiments for the modification of endogenous loci and/or introduction of the required transgene(s) in cultured ES cells, and subsequently using these ES cells for complementation of tetraploid blastocysts, mice carrying multiple genomic modifications can be obtained without additional breeding. The generation of ES cell lines from mice carry-

ing one or more modifications in their genome further facilitates such experiments by reducing the number of sequential targetings required for the production of ES cells carrying all required genomic modifications (Seibler et al. 2003). The main disadvantage of this method is the low efficiency of the production of ES mice that requires the injection of ES cells into a large number of tetraploid blastocysts in order to obtain sufficient numbers of experimental animals.

1.9 Concluding Remarks

Current advances in the technologies available for the manipulation of the mouse genome have revolutionised experimental biology and have provided an unprecedented tool for the in vivo analysis of gene function in mammals. Studies in gene-modified mice have made a major contribution to our current understanding of the molecular mechanisms governing mammalian embryonic development and physiology. Furthermore, this technology has allowed the generation of mouse models of numerous human genetic diseases, thus providing an in vivo experimental system for the study of the molecular and cellular mechanisms of disease pathogenesis. The application and further improvement of this technology in combination with current developments facilitating large-scale genome sequencing and annotation and the high-throughput analysis of gene expression and protein complex formation and function will undoubtedly play a central role in biomedical research in the years to come, hopefully leading to the better understanding of pathogenic mechanisms and to new, more effective therapeutic approaches to human disease.

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2 High-Throughput Gene Knockouts and Phenotyping in Mice

M. W. Moore

Deltagen has developed a database of in vivo mammalian gene function information based on using homologous recombination to knock out genes in mice. These genes were selected for inclusion in the database based on their potential as tractable drug targets, and belong to gene families such as G-protein coupled receptors, channels, kinases, and proteases. The company has generated approximately 900 lines of knockout mice to date and has generated comprehensive

phenotypic data for 750 of these targets, utilizing an extensive, integrated analysis program to assess the function and potential pharmaceutical relevance of these genes. The phenotypic data for these 750 targets were provided to pharmaceutical and biotechnology customers in DeltaBase, a relational Oracle-based database with a user-friendly, web browser-enabled interface. The body of gene function information available in Deltagen's database provides an advantage to drug discovery efforts by reducing the time required for target validation.

Deltagen believes that its knockout mouse lines and related phenotypic information have several advantages over other knockout mice and phenotypic information that may be available in the public literature. Most importantly, Deltagen offers uniformity and a consistent analysis platform, including genetic background, across all its mouse lines, as each mouse line was handled in the same manner and underwent the same battery of phenotypic testing.

Furthermore, selected targets were moved forward to advanced phenotyping that included models in inflammation such as rheumatoid arthritis (RA), inflammatory bowel disease (IBD), and acute and chronic models for contact dermatitis.

These gene knockout mice are a useful tool in defining the function and disease relevance of mammalian genes for the purposes of discovering and validating novel drug targets. Many of the drugs available or in development are directed at affecting a currently known biological target. Deltagen provides gene knockout mice that help identify novel drug targets that relate to a particular disease.

Deltagen knockout pipeline
1. Target selection
2. Construct generation
3. Probe generation
4. Tissue culture
5. Screening and confirmation
6. Microinjection
7. Chimeras, F1, F2 mice
8. Genotyping
9. Phenotypic analysis

Deltagen scientists systematically analyzed public DNA and EST databases to select a collection of targets from gene families that have

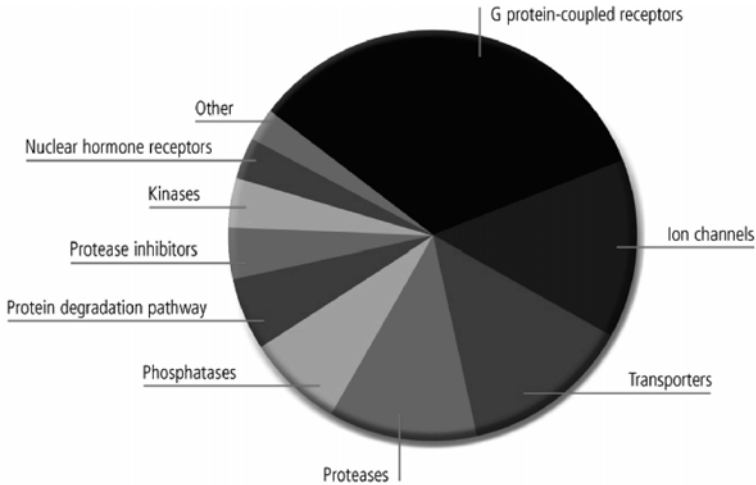

Fig. 1. Source of genes for Deltagen's knockout pipeline

had a history of being tractable targets for pharmaceutical development (Fig. 1).

High-throughput synthesis of targeting vectors for homologous recombination
- Functionally essential gene domains are specifically deleted
  Increases probability of a null mutation
  Domain replaced with a lacZ gene to assay expression
- "Traditional" targeting vectors made by nontraditional methods
  Proprietary methods invented at Deltagen
  Significant improvements incorporated
- High-throughput
  Efficiently automated
  Targeting vectors for 200 gene targets per month
  Vectors contain average genomic sequence of 10 kb

The targeting vectors were constructed to have both a deletion in the target gene and an insertion of a selectable marker cassette that also serves to interrupt the protein coding sequence. The identity of the gene target and the integrity of the vector were confirmed by DNA sequence analysis outward from the insertion site of the Neo (or other selectable marker) cassette.

2.1 ES Cell Work

Embryonic stem (ES) cell lines are gelatin-adapted and handled in a 96-well format. All lines are maintained in liquid N_2 on- and off-site. Deltagen currently uses ES lines derived from 129 mouse strains. ES cells were grown in DMEM media containing fetal calf serum and LIF (leukemia inhibitory growth factor), which maintains the cells in a pluripotent state.

Targeting vectors were linearized with a restriction enzyme that cleaves the vector backbone. The linearized targeting constructs were introduced into ES cells via electroporation. Approximately 400 individual colonies were picked following drug selection in G418 (for Neo cassettes), expanded for DNA preparation, and screened for homologous recombination by PCR analysis. The PCR screening procedure used a target gene-specific oligonucleotide that is not present on the targeting vector and an oligonucleotide corresponding to the Neo (or other selectable marker) cassette. Oligonucleotides outside the targeting vector were used to identify homologous recombinants instead of random integrations of the targeting vector.

2.2 Microinjection

Team members collect, inject, and implant blastocysts. In total, 120–160 blasts per ES line are injected over several days by multiple team members, allowing a maximum production of 3,000 blasts per week. The chimeras are weaned, scored, and then mated. The team then sets up 12 high-percentage males with two C57Bl/6 females each; they are rotated regularly after germline transmission to increase colony size. The teams manage multiple targets and breed and assign F2 mice to phenotypic programs. All lines are currently maintained in serial backcross to C57Bl/6 females (Figs. 2, 3).

All aspects of gene knockout methodology, mouse production, and phenotyping are managed by custom databases. Cohorts of F2 mice are bred to go into the following phenotypic assays (see Fig. 4):

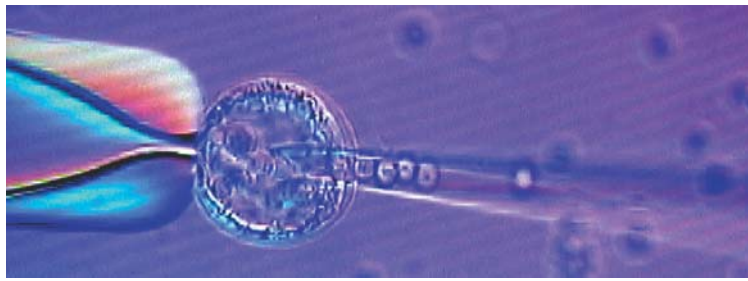

Fig. 2. Microinjection of a blastocyst

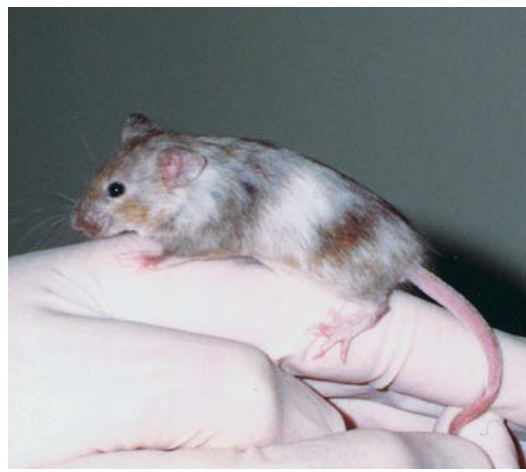

Fig. 3. Example of a chimeric mouse

- Physical examination
- Densitometry
- Necropsy examination
- Histology
- Serum chemistry
- Hematology
- Embryonic/perinatal lethality analysis
- Expression analysis
- LacZ staining

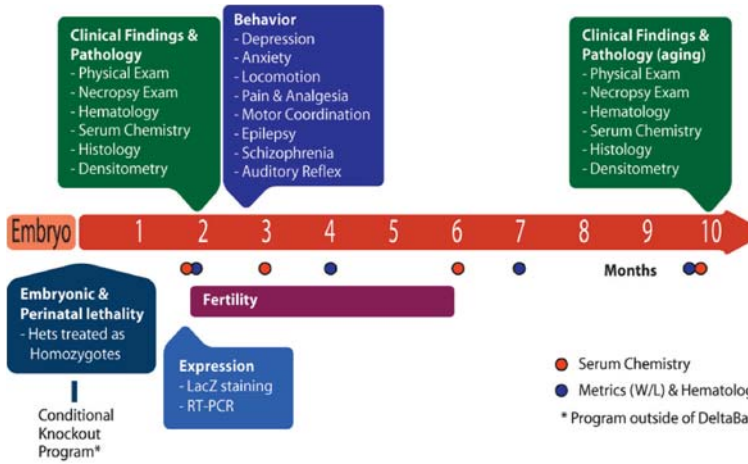

Fig. 4. Identifying gene function

- RT-PCR
- DeltaBase 750, mice and data
- "Post-release" targets, 138 targets with mice but no phenotypic data
- 86 targets at chimera stage
- 352 targets with knockout ES lines
- 564 targets with completed constructs

2.3 Identifying Gene Function

High-Throughput Phenotypic Analysis. The timeline in Fig. 4 describes various programs. Each box represents a separate cohort of mice. All targets go through these programs except for behavior, which is limited to those targets with nervous system expression profiles ($\sim 75\%$).

All mice are observed so as to screen for physical changes that cover 56 different categories and are defined by a controlled medical language system (Figs. 5–7).

Serum chemistry (Hitachi 912 automatic analyzer)

Electrolytes	*Liver function (enzymes)*
Sodium Na	Alkaline phosphatase ALP

Potassium K	Alanine aminotransferase ALT
Chloride Cl	
Bicarbonate Bicarb	
Renal function tests	*Liver function (other)*
Blood urea nitrogen BUN	Protein total T Prot
Creatinine Creat	Albumin Alb
	Globulin Glob
	Bilirubin total Bil T
Inorganic ions	*Lipid profile*
Calcium Ca	Cholesterol Chol
	High-density lipoproteins HDL
Other	Low-density lipoproteins LDL
Glucose Glu	Triglycerides TG

Mice are analyzed at 7–9 weeks of age and again at 10 months of age for all of the above-listed metabolites, enzyme functions, lipids, and proteins

2.4 Pathology

The pathology studies were performed on 7–9-week-old cohort of mice and again at 10 months of age (Fig. 8).

2.5 Necropsy

In total, 57 tissues were evaluated grossly, organ and body weights were measured, and digital images were collected.

2.6 Histopathology

The 57 tissues are evaluated histologically, with a minimum of 218 digital images per target collected.

The following histopathology images are collected:

Adipose tissue, brown	Liver
Adipose tissue, white	Liver/gallbladder
Adrenal glands	Lungs
Aorta	Lymph nodes
Bone marrow	Mammary gland
Bone, cranium	Ovary/oviduct

Bone, femur
Bone, sternum
Bone, stifle joint
Bone, vertebrae
Brain, cerebellum
Brain, cerebrum
Brain stem
Cecum
Cervix/vagina
Clitoral gland
Colon
Duodenum
Epididymis
Esophagus
Eyes
Gallbladder
Harderian glands
Head
Heart
Ileum
Jejunum
Kidneys
Pancreas
Pituitary gland
Preputial gland
Prostate/coagulation gland
Rectum
Salivary glands
Sciatic nerve
Seminal vesicles
Skeletal muscle
Skin
Spinal cord
Spleen
Stomach
Testes
Thymus
Thyroid gland
Tongue
Trachea
Urinary bladder
Uterus

2.7 Hematology

The following hematological parameters were evaluated: CBC, five-part WBC differential, red cell indices, and platelet count. In addition, a peripheral blood smear was performed.

Deltagen conducted lacZ reporter expression analysis on nearly all targets that incorporated the lacZ reporter. In general, frozen tissue sections from 7–10-week-old heterozygous mice were used to determine lacZ expression (Fig. 9).

In some cases, early developmental expression, insertional silencing, or insertional mutations may result in no lacZ expression in adult tissues. Images of representative tissues that demonstrate lacZ expression were included in DeltaBase.

RNA expression was also tested using RT-PCR, which in later DeltaBase targets was expanded to include white and brown adipose tissue. Total RNA was isolated from the organs/tissues listed below from adult C57Bl/6 wild-type mice. RNA is DNaseI treated and re-

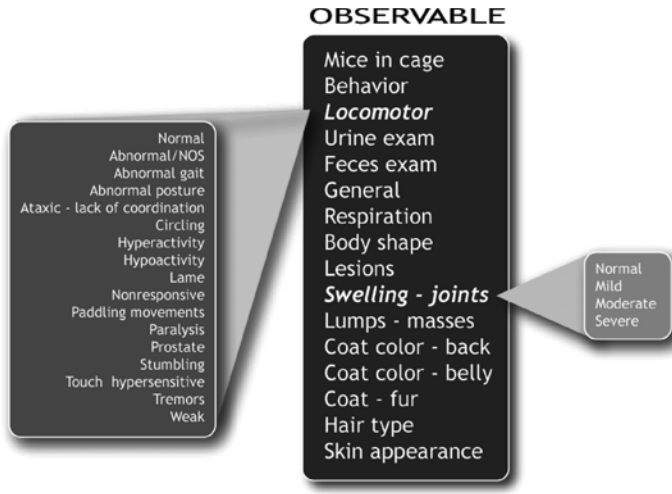

Fig. 5. Mice are screened for physical changes

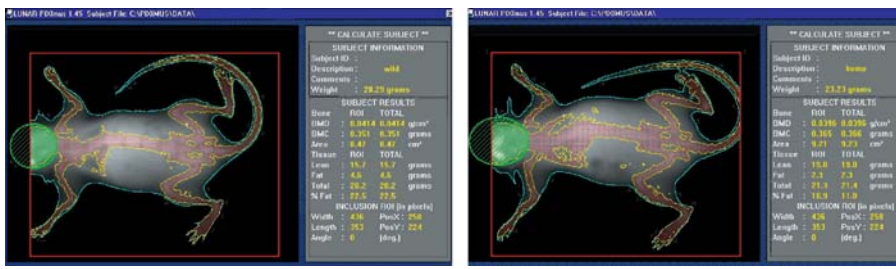

Fig. 6. Example of GPCR knockout. Decrease in percentage body fat

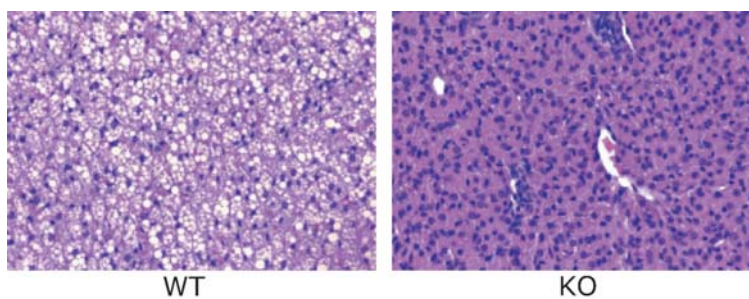

Fig. 7. Intrascapular brown adipose tissue (×40). Knockout mice have decreased cytoplasmic lipid vacuolation. *KO* knockout, *WT* wild-type mice

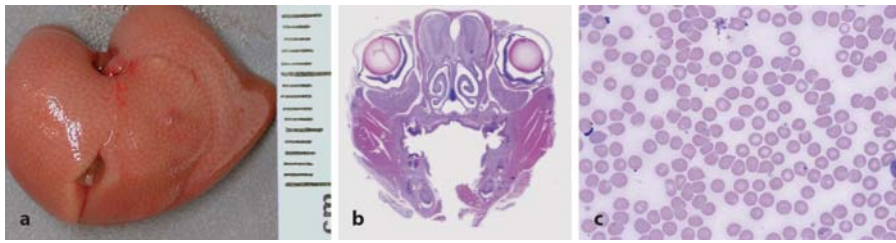

Fig. 8. Examples from pathology, histopathology and hematology obtained from knock-out mice

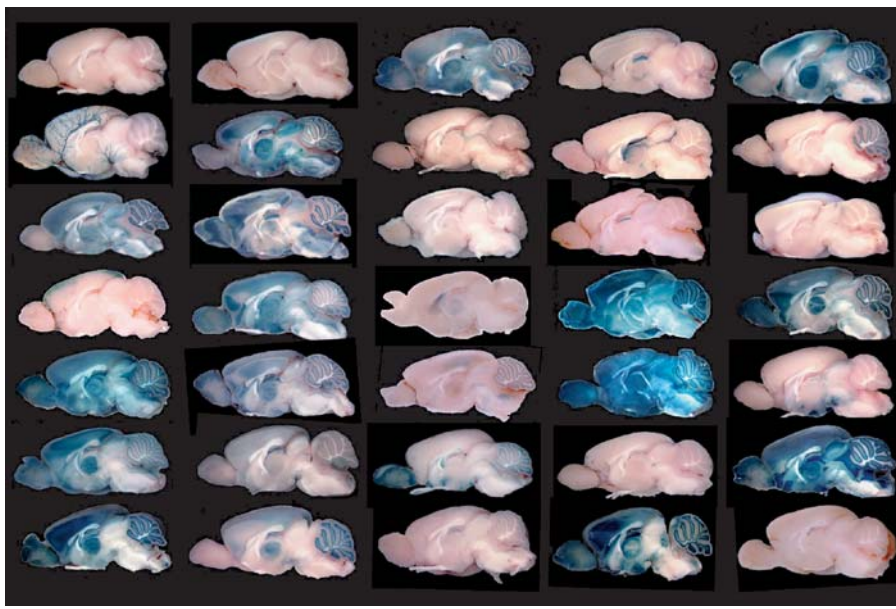

Fig. 9. Tissue sections

verse transcribed using random primers. The resulting cDNA is checked for the absence of genomic contamination using primers specific to nontranscribed genomic mouse DNA. The cDNAs are assayed for both quality and concentration by RT-PCR for the ubiquitously expressed gene hypoxanthine-guanine phosphoribosyltransferase (HPRT) or beta-actin.

Tissues analyzed by RT-PCR included: brain, cortex, subcortical region, cerebellum, brainstem, olfactory bulb, spinal cord eyes, heart, lung, liver, pancreas, kidneys, spleen, thymus, lymph nodes, bone marrow, skin, gallbladder, urinary bladder, pituitary gland, adrenal gland, salivary gland, skeletal muscle, tongue, stomach, small intestine, large intestine, cecum, testis, epididymis, seminal vesicle, coagulating gland, prostate gland, ovaries, uterus, and white fat.

2.8 Behavioral Assays

For targets that demonstrated CNS expression based on lacZ data or on the fact that the gene target is a member of a family implicated in the development or function of the CNS, mice were subjected to a battery of behavioral assays. For example, many GPCRs and ion channels were included in the behavioral studies. Mice that do not exhibit target gene expression in the CNS or neuroendocrine systems were not, by default, subjected to behavioral analysis.

Seven to ten male knockout mice 10–12 weeks of age were analyzed and compared with seven to ten wild-type mice. The behavioral assay data were delivered approximately 3–6 months following the delivery of the first-pass histopathology analyses.

2.8.1 General Points

All behavioral tests were performed on the same group of adult males 10–12 weeks of age ($n = 7$–10 per group for wild-type and knockout). The mice were run through the behavioral programs in the order of tests described in Sects. 2.8.2–2.8.7. There was a 1–2-day rest period between each test. Females were not studied due to variability caused by the estrous cycle. For targets where adult male knockout mice could not be obtained (e.g., early lethality), heterozygous males of the same age were substituted.

Animals were tested within the same time window during the light cycle. Continuous background noise was played throughout the testing. Between subjects, instruments were cleaned with a dilute

bleach solution, wet and dried to prevent the growth of microorganisms and to eliminate mouse odors.

The background strains 129, C57BL/6, and the F1 hybrid (129×C57BL/6) mice had been tested to establish baseline measurements. The standard tests were performed on the offspring of intercrossed F1 mice (F2N1 mice). Pharmacological validation was performed at least quarterly and provided in DeltaBase with the behavioral datasets.

2.8.2 Open Field Test

Mice were screened for behavioral abnormalities by evaluating the response to a novel environment using an open field test. Seven to ten adult wild-type and homozygous males were used in each experiment. Animals were group housed prior to testing. Activity of individual mice was recorded for a 10-min test session and monitored by photobeam breaks in the x-, y-, and z-axes.

Measurements taken include total distance traveled, total number of rearings, and percent of session time spent in the central region of the test apparatus. Increases or decreases in total distance traveled over the test time may indicate hyperactivity or hypoactivity, respectively. Alterations in the regional distribution of movement may indicate anxiety phenotypes, i.e., increased anxiety if there is a decrease in the time spent in the central region.

2.8.3 Righting Reflex

The mouse was placed on its back in a plastic V-shaped trough. A normal mouse will immediately turn itself upright. This is a subjective test without time recordings. A trained observer recorded the response for all WT and KO mice in a target. The results were recorded as either “normal” or “abnormal” for this response.

A 0.25% bleach solution was used to clean the plastic troughs between cages of mice.

2.8.4 Auditory Reflex

The mouse was placed on a plastic trough on the top of its cage. A sharp noise was stimulated approximately one foot from the mouse. A normal mouse will react with either a startle response or at least an immediate ear twitch. A trained observer recorded the response for all WT and KO mice in a target. The results were recorded as either "normal" or "abnormal" for this response.

2.8.5 Tail Suspension Test

The tail suspension test was performed to screen for phenotypes involving depression (either increased or decreased). Seven to ten adult wild-type and homozygous males were used. Animals were group housed prior to testing.

Mice were suspended by their tail on a metal hanger in an acoustically and visually isolated setting. Total immobility time during the 6-min test period is determined using a computer algorithm based on measuring the force exerted by the mouse on the metal hanger. An increase in immobility time for mutant mice compared to wild-type mice may indicate increased "depression".

2.8.6 Rotarod

The Accelerating Rotarod was used to screen for motor coordination, balance, and ataxia phenotypes. Seven to ten adult wild-type and homozygous males were used. Mice were placed on a rotating rod, which accelerates from 0 to 60 revolutions per minute, for a 3-min maximum test period. Each animal was assessed three times with 10-min rest periods between trials. A decrease in the speed of the rotating rod at the time of fall compared to wild-types may indicate decreased motor coordination.

2.8.7 Nociception

The Hot Plate Test was used to screen for phenotypes involving altered thresholds for acute pain sensitivity. Seven to ten adult wild-type and homozygous males were used. Mice were placed on a 55 °C hot plate for up to a maximum of 60 s. The latency to hind paw licking was measured. Alterations in latency to hind paw licking may indicate changes in pain thresholds.

The following is a summary of the phenotypes detected using the aforementioned assays for the 750 gene knockouts that compose DeltaBase:

Cardiovascular diseases	6	
Central nervous system	3	
Dermatology	3	
Endocrinology	5	
Gastroenterology	3	
Genitourinary diseases	13	
Hematology	10	
Immunology	11	
Metabolic diseases	45	
Musculoskeletal diseases	9	
Neurological diseases	118	
Neuroscience	158	
Oncology	11	
Reproduction	16	
Respiratory diseases	4	
Totals	311	Genes with phenotypes or ∼40%
	103	Embryonic lethal or 14%

The gene families that comprise DeltaBase are as follows:
Acetyl transferase (2)
Alpha/beta hydrolase (3)
Apoptosis pathway (4)
Carbohydrate metabolism (6)
Cell adhesion (3)
Channel (103)
Chemokine (1)
Cyclase (5)
Cytokine signaling (4)
Disease-associated protein (1)
EGF domain protein (4)
G-protein signaling (13)

GPCR (195)
Growth factor (3)
Growth factor inhibitor (3)
Interleukin/toll receptor (1)
Ion binding protein (3)
Ion channel inhibitor (1)
Kinase (25)
Lipid metabolism (2)
Neuronal function (4)
Novel protein (1)
Nuclear hormone receptor (15)
POZ/BTB domain (2)
Phosphatase (32)
Phosphodiesterase (11)
Phospholipase (2)
Protease (108)
Protease inhibitor (20)
Protein degradation pathway (24)
Receptor tyrosine kinase (13)
Reductase (4)
SH3 domain-containing protein (1)
Seven transmembrane (6)
Sodium-hydrogen exchanger (4)
Sterol sensing domain (3)
Sulfotransferase (5)
Thrombospondin domains (3)
Transcription factor (2)
Transferase (1)
Transmembrane (20)
Transporter (87)

Figure 10 organizes the percentages of targets that yielded a phenotype in each assay. For example, fewer than 3% of all targets displayed a fertility phenotype, whereas nearly 10% of all targets displayed a histopathology phenotype.

Figure 11 compares the percentage of targets exhibiting phenotypes across gene families and screens.

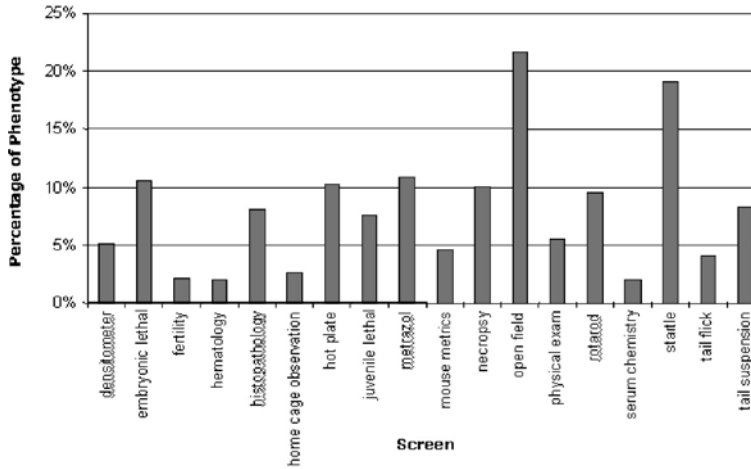

Fig. 10. Percentage of phenotype by screen

2.8.8 Additional Phenotypic Analysis Not Included in DeltaBase

Screening packages:
- Metabolism/obesity
- Immunology
- Cardiovascular
- Analgesia
- Angiogenesis
- Vision
- Neurology

Immunology challenge models:
- Contact hypersensitivity (CA)
- Irritant contact dermatitis
- Thioglycollate mediated peritonitis
- LPS induced cytokine secretion
- AP-II rheumatoid arthritis (RA), 7 days
- Anti-CII mAb challenge
- AP-II inflammatory bowel disease (IBD), 21 days
- Dextran sulfate sodium (DSS) challenge

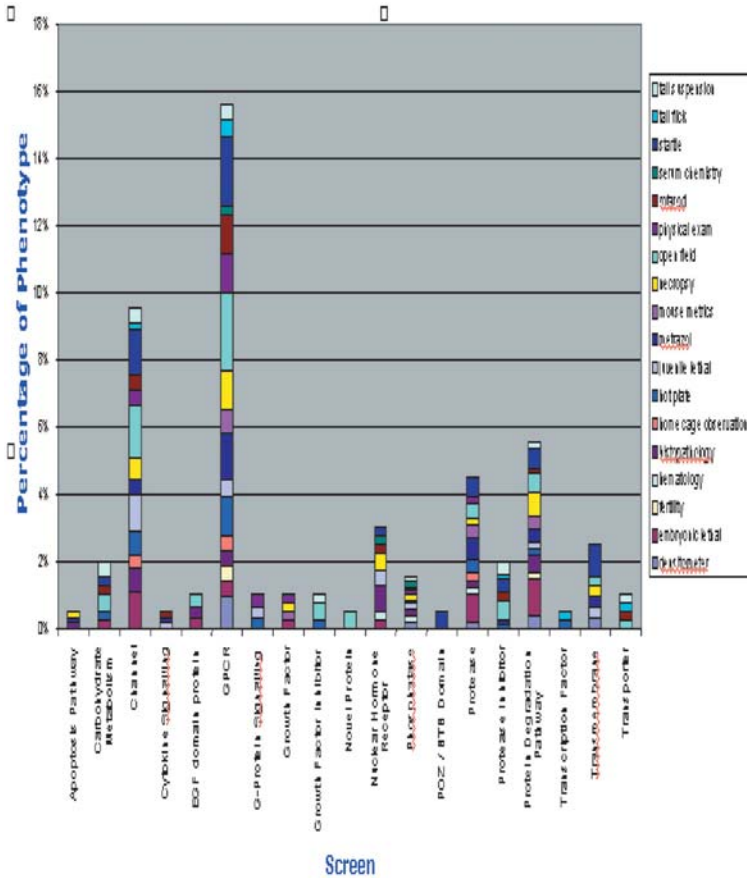

Fig. 11. Percentage of phenotype by family and by screen

2.9 Concluding Remarks

Deltagen has produced 750 gene knockouts with phenotypic analysis. This systematic production and analysis has allowed an exceptionally high level of model production and target validation. In the areas of contact allergy, these screens were very productive. In the allergic contact dermatitis screen, 56 targets were completed with 13

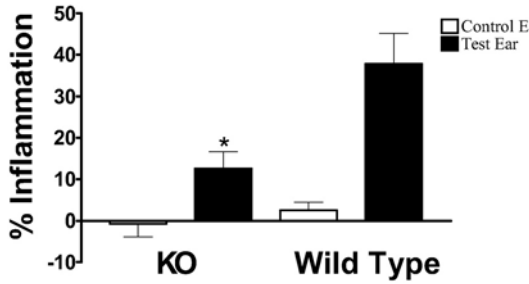

Fig. 12. DT-ACD1 knockout mice show decreased inflammation in allergic contact dermatitis

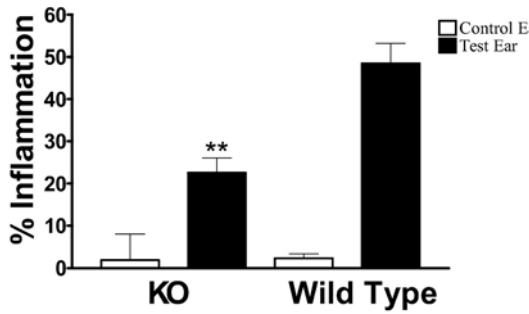

Fig. 13. DT-ICD1 knockout mice show decreased inflammation in irritant contact dermatitis

targets displaying phenotypes (23.2%) and 9 showing decreased inflammation (Fig. 12). For the irritant contact dermatitis screen, 31 targets were completed and 4 targets displayed phenotypes (12.9%) (Fig. 13).

3 Modelling Gene–Environment Interactions in Th1- and Th2-Dominated Diseases of Laboratory Animals

G. A. W. Rook, R. Martinelli, L. Rosa Brunet

3.1 Introduction

Several types of chronic inflammatory disorder are becoming much more common in the rich developed countries. These include inflammatory bowel diseases (IBD) such as ulcerative colitis, and Crohn's disease, certain autoimmune diseases (type 1 diabetes and multiple sclerosis) and allergies. The latter includes atopic dermatitis, clearly of interest to this symposium which is focused on skin disease. It will be argued below that the rapidly increasing prevalence of these disorders can be attributed to changing patterns of microbial exposure in modern life, leading to a failure of immunoregulatory mechanisms in individuals with certain genetic polymorphisms of the innate immune system. This view is referred to as the "Old Friends" hypothesis, and is an updating of the previous "Hygiene Hypothesis". In addition, this chapter will include discussion of certain aspects of psoriasis, because although it is unclear whether its prevalence is increasing in developed countries, psoriasis has some epidemiological association with diseases which *are* increasing in prevalence (such as Crohn's disease; Najarian and Gottlieb 2003) and shows signs of immunoregulatory dysfunction, such as excessive Th1 activity and IL-10 deficiency.

3.2 The Hygiene Hypothesis

The "Hygiene Hypothesis" as it exists in 2003 is very different from the hypothesis that emerged in the late 1990s. By that time, the view that changing patterns of infectious disease might lead to changes in the incidence or presentation of various immunological disorders had appeared intermittently in the medical literature for decades. In 1996 Strachan and colleagues, who were interested in the striking increase in the prevalence of allergic disorders in the developed coun-

tries, gave a new impetus to the idea when they observed that allergies were less common in children from large families with older siblings, especially older brothers (Strachan et al. 1996). A flood of further epidemiological data, particularly studies of farming communities (Braun-Fahrlander et al. 2002; Riedler et al. 2001) and military personnel (Matricardi et al. 2000) provided circumstantial support for the view that diminished exposure to certain microorganisms might lead to an enhanced risk of allergy.

3.2.1 Findings That Undermined the Early Rationalisation of the Hygiene Hypothesis

Most authors treated the Hygiene Hypothesis as though it was synonymous with a "Th1/Th2 see-saw hypothesis". In other words it was suggested that Th1 responses are needed to downregulate Th2 responses, and that the hygienic conditions of modern life provide too little stimulus for Th1 cells, with a consequent non-specific increase in Th2 activity. With hindsight it is clear that this view was deeply flawed. First, Th1 responses are *not* physiological regulators of Th2 activity. Indeed individuals with an almost complete lack of Th1 responses due to congenitally defective IFN-γ receptors do not have increased Th2 activity (Lammas et al. 2000). The earlier literature suggesting that in vivo Th1 cells downregulate Th2 responses must be re-interpreted in the light of the existence of distinct subsets of regulatory T cells (T_{reg}). When the presence of T_{reg} is rigorously excluded and cloned polarised Th1 cells are used in cell transfer experiments, Th1 cells may even exacerbate ongoing Th2-mediated inflammation (Hansen et al. 1999). Indeed, synergy between Th1 and Th2 cells is emerging as a major mechanism of severe immunopathology in some infections (Hernandez-Pando et al. 2003), and it is often forgotten that IFN-γ is in fact a dominant cytokine in human asthma (Krug et al. 1996).

The death blow to the "Th1/Th2 see-saw" interpretation was the realisation that countries experiencing increased incidences of these Th2-mediated disorders were simultaneously experiencing parallel increases in Th1-mediated autoimmune disorders and IBD (Rook 2000). Thus there is a striking correlation between Type 1 diabetes

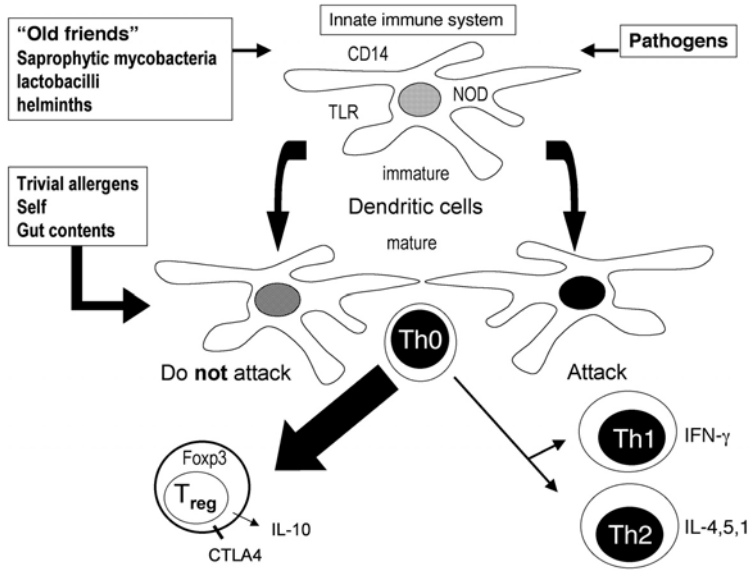

Fig. 1. The "old friends" hypothesis. A cartoon of mechanisms that might explain the links between patterns of microbial exposure and susceptibility to disorders of immunoregulation in man and animals. The "old friends" (harmless organisms that have been interacting with the mammalian immune system throughout our evolutionary history) are recognised by components of the innate immune system (including CD14, Toll-like receptors (TLRs), and NOD1 and NOD2) in such a way that DCs mature to a phenotype that drives formation of regulatory T cells (T_{reg}). Pathogens, on the other hand, drive maturation of DC that activate Th1 or Th2 effector mechanisms. Some, perhaps all T_{reg} express the transcription factor Foxp3, and often CTLA-4 and IL-10. Because the "old friends" cause maturation of DC that drive regulation, they act as adjuvants for the development of regulatory cells that recognise other harmless antigens processed by the same DC, such as self, allergens, and gut contents. Polymorphisms within the innate immune system result in altered efficiency of T_{reg} induction, and so may lead to increased susceptibility to allergies, autoimmunity, or IBD

(Th1-mediated destruction of β cells in the pancreas) and allergies, both within and outside Europe (Stene and Nafstad 2001). Similarly, IBD is increasing in the same countries (Lindberg et al. 2000; Sawczenko et al. 2001). These parallels have been extensively reviewed (Bach 2002). Clearly the explanation for the increased prevalence of

allergic disorders cannot be a lack of stimuli for Th1 activity if Th1-mediated diseases are also increasing. We are witnessing a simultaneous increase in both Th1 and Th2 immune responses against targets that should be tolerated, such as trivial levels of allergens, self, and the contents of the gut, leading to allergies, autoimmunity, and IBD respectively. The current hypothesis (expanded in detail later) is that the balance of regulatory T cells (T_{reg}) to effector T cells (T_{eff}), whether Th1 or Th2, is more important than the balance of Th1 to Th2 cells (Fig. 1).

3.2.2 Does Th1/Th2 Balance Play a Role?

Although Th1-mediated and Th2-mediated inflammatory disorders are increasing in parallel within populations (Bach 2002; Stene and Nafstad 2001), there does seem to be some segregation of Th1 and Th2 at the level of the individual in some studies. For example, people suffering from the Th1-mediated autoimmune diseases, type 1 diabetes or multiple sclerosis, are less likely to be allergic (Douek et al. 1999; Tremlett et al. 2002). However, the Th1/Th2 balance of the individual might reflect genetic background and past history of infections and vaccinations. This in turn might determine the type of disorder of immunoregulation that will occur in that individual when T_{reg} activity is compromised. Psoriasis is interesting in this context. A recent report suggests that this condition can be treated with IL-4, in which case this Th1-mediated disease at least can be modulated by a Th2 cytokine (Ghoreschi et al. 2003). So although there is good evidence that Th1 activity is not a physiological regulator of Th2 cells (Lammas et al. 2000), it remains possible that Th2 activity can sometimes modulate Th1 responses. However, it must not be forgotten that some T_{reg} express IL-4 (Seddon and Mason 1999).

3.2.3 Imbalance Between Effector (T_{eff}) and Regulatory T Cells (T_{reg}): Lessons from Foxp3

The ability of decreased activity of T_{reg} to explain the simultaneous increases in all three major groups of immunoregulatory disorder has

now been repeatedly reviewed (Rook and Brunet 2002; Umetsu et al. 2002; Yazdanbakhsh et al. 2002). Support for this view has come from recent analyses of genetic disorders of human and mouse attributable to mutations in the transcription factor Foxp3 (Brunkow et al. 2001; Wildin et al. 2001). Foxp3 is fundamental to the development of T_{reg} (Fontenot et al. 2003; Hori et al. 2003). In both species the resulting pathology encompasses precisely the spectrum of diseases (allergies, autoimmunity, and IBD) that is increasing in the developed world.

3.3 The "Old Friends" Hypothesis

But why should diminished exposure to microorganisms result in inadequate priming of T_{reg}? In fact, T_{reg} are probably part of the normal immune response to pathogens. After the effector response has reduced pathogen numbers to low levels, T_{reg} may help to terminate the response and allow for the development of concomitant immunity (Belkaid et al. 2002). But clearly, excessive early activation of T_{reg} by pathogens would lead to progressive disease. Therefore the organisms most likely to have a highly developed ability to drive T_{reg} rather than T_{eff}, and perhaps to bypass the phase of T_{eff} induction altogether (Schultz et al. 2002), are harmless "old friends" and commensals that have been present throughout our evolutionary history, and that have become part of mammalian physiology. In addition to the unidentified (but clearly non-pathogenic) factor present in German cowsheds (Riedler et al. 2001), four groups of organism have been highlighted in this context; lactobacilli (Kalliomaki and Isolauri 2003), saprophytic environmental mycobacteria (Rook and Stanford 1998; Zuany-Amorim et al. 2002), helminths (Yazdanbakhsh et al. 2002), and certain viruses that are ubiquitous in developing countries (McIntire et al. 2001).

Lactobacilli were an integral part of the hunter-gatherer diet, and were common in poorly preserved vegetables and in beverages prepared by malo-lactic fermentation (Kalliomaki and Isolauri 2003). Interestingly lactobacilli are present in greater quantities in the guts of children from areas with a low incidence of allergies (Sepp et al. 1997). Similarly the saprophytic mycobacteria are ubiquitous in mud

and untreated water, and used to be consumed in such quantities that detectable levels of tuberculostearic acid accumulated in lymphoid tissue (Hanngren et al. 1987). The presence of mycobacteria in the mammalian evolutionary past is indicated by the presence of CD1-restricted T-cell subsets that appear to recognise only mycobacterial lipids and glycolipids (Dutronc and Porcelli 2002). Exposure remains high in developing countries, where most of the population has skin-test positivity to multiple mycobacterial species (Stanford et al. 1981). In contrast, this is rare in the rich countries. Helminths are endemic in developing countries, and cause little or no pathology in the majority of infected individuals. This is achieved by the induction of T_{reg}. Indeed, the ability of helminths to drive T_{reg} activity is an integral component of the host-parasite relationship. When this mechanism fails, an exaggerated immune response to the parasite ensues, with massive lymphadenopathy and pathology (Satoguina et al. 2002; Steel and Nutman 2003). Virus infections characteristic of childhood in the developed countries appear not to protect from allergies (Matricardi et al. 2000), which fits with the fact that children in inner cities are heavily exposed to these infections and still have a high and rising risk of allergies. However, antibody to infections transmitted by the faeco-oral route correlated with a lower incidence of allergic symptoms (Matricardi et al. 2000). Subsequently it has emerged that a T-cell membrane protein, TIM-1, that controls the development of airway hyperreactivity and production of Th2 cytokines in mice is a homologue of the human hepatitis A virus (HAV) receptor, which may explain the inverse relationship between HAV infection and the development of atopy (McIntire et al. 2001).

Each of these groups of organism has been implicated in down-regulation of allergic responses in humans, either by direct clinical studies or (less convincingly) by epidemiological correlation (Arkwright and David 2001; Camporota et al. 2003; Kalliomaki et al. 2001; Matricardi et al. 2000; van den Biggelaar et al. 2000). Moreover, components of *Schistosoma mansoni* (helminth) and *Mycobacterium vaccae* (saprophytic environmental mycobacterium) have been shown to induce T_{reg} in vitro (van der Kleij et al. 2002) and in vivo, respectively (Zuany-Amorim et al. 2002).

3.3.1 Experimental Evidence for the Induction of Allergen-Specific T_{reg} by "Old Friends"

A GMP preparation of *M. vaccae*, which has been used in clinical trials, has been shown experimentally to induce T_{reg} (Zuany-Amorim et al. 2002). The specificity of the T_{reg} induced was tested in a cell transfer system. It was demonstrated that the T_{reg} induced have specificity for the allergen to which the animals were exposed, but that once the regulatory properties of the T_{reg} have been triggered they can exert bystander inhibition on the response to other allergens (Zuany-Amorim et al. 2002).

3.3.2 The Physiological Role of Bystander Suppression

This bystander phenomenon may be of fundamental importance to immunoregulatory disorders. When SCID mice (which lack T lymphocytes) were reconstituted with T_{eff} with a normal range of specificities, they developed severe colitis. The colitis persisted if they were given cloned transgenic T_{reg} specific for a single epitope of ovalbumin. However if ovalbumin was added to the diet, the bystander suppression exerted by the ovalbumin-specific T_{reg} was able to alleviate the colitis, despite the fact that the colitis will have been triggered by multiple antigens within the food and gut flora (Groux et al. 1997). These observations emphasise the point that the "old friends" discussed earlier may have two distinct roles. Experiments in vitro and in vivo suggest that they can act as "T_{reg} adjuvants", maturing antigen-presenting cells (APCs) to a phenotype that tends to direct T cells towards specific regulatory function (van der Kleij et al. 2002; Zuany-Amorim et al. 2002). However the "old friends" may also provide a crucial background of regulation, because their constant presence leads to a steady background activation of T_{reg} that recognise the "old friends" themselves. Again this argument points towards organisms that will have been taken in orally throughout mammalian evolution (such as saprophytic mycobacteria and lactobacilli) and organisms that were permanently present in the tissues (such as helminths).

3.3.3 T_{reg} May Not Suppress When the Innate Immune System Detects "Danger"

Why does bystander suppression not constantly incapacitate the immune system? Conceptually the answer must be that T_{reg} downregulate immune responses that should not be there, such as responses to self, gut contents, and trivial levels of harmless allergens. But when "danger signals" are present, the T_{reg} must stop regulating and allow full immune responses. In an in vitro system, T_{reg} failed to function when danger signals were provided by addition of CpG motifs or endotoxin (Pasare and Medzhitov 2003). Similarly, T_{reg} can block graft-versus-host disease while permitting anti-tumour responses (Edinger et al. 2003). Finally, it has been noted that T_{reg} express an unusual pattern of Toll-like receptors (TLRs), and these may also play a role in the modulation of their function (Caramalho et al. 2003).

3.4 Innate Immunity and Induction of T_{reg}

How then are the "old friends" recognised as harmless, and how does that recognition lead to the activation of T_{reg}? Similarly, how do danger signals sometimes stop T_{reg} from developing, as outlined in the previous paragraph? The answer probably lies in the innate immune system. The pattern recognition receptors (PRRs) of the innate immune system recognise pathogen-associated molecular patterns (PAMPs). (The use of the word "pathogen" here is misleading since these receptors are also involved in recognition of commensals and even of self components such as heat-shock proteins (HSPs)). The PRRs then drive rapid protective mechanisms before the adaptive response can be generated, and then direct the adaptive response towards appropriate attack mechanisms or towards immunoregulation. It is therefore of particular interest that polymorphisms of genes involved in the innate immune system are proving to be relevant to the incidence of immunoregulatory disorders. The parallels between the components of the innate immune system that are involved in recognition of bacteria and the components that have been shown to be relevant to the allergies have been reviewed elsewhere (Rook et al. 2003). A few examples are given below.

3.4.1 NOD2 (CARD15)

Bacterial cell wall muramyl peptides can signal via recently discovered intracellular PRRs of the innate immune system, NOD1 (CARD4) and NOD2 (CARD15). Interestingly, polymorphisms of NOD2 are associated both with susceptibility to Crohn's disease, a predominantly Th1-mediated condition (Ogura et al. 2001) and also to allergies, a Th2-mediated condition (Kabesch et al. 2003). Similarly, in patients from Newfoundland, polymorphisms of NOD2 were associated with susceptibility to psoriasis that was independent of HLA-Cw*0602, so NOD2 may be the first non-MHC gene to be associated with psoriasis (Rahman et al. 2003). These are therefore examples of genetically impaired recognition of bacterial components leading to a variety of Th1-mediated or Th2-mediated immunoregulatory disorders in different individuals.

3.4.2 Toll-Like Receptors

Ten Toll-like receptor (TLR) types are known, and these may act as homo- or heterodimers with other TLR. They are PRRs that bind conserved microbial derivatives such as LPS, lipoproteins, peptidoglycans, glycosylphosphatidylinositols, double-stranded RNA, CpG motifs in DNA, and flagellin. All microorganisms contain ligands for many TLRs. Nevertheless when whole live organisms are added to TLR-expressing cells, a more restricted pattern can emerge. For example, Gram-negative bacteria tend to trigger TLR4, whereas mycobacteria tend to trigger TLR2 (Heldwein and Fenton 2002).

3.4.3 TLR and Susceptibility to Disorders of Immunoregulation

Recent data suggest the possibility of links between polymorphisms of TLR-2 and susceptibility to asthma. Exposure to the farming environment during early life significantly protects from allergic disorders (Riedler et al. 2001), and this beneficial effect is dependent upon a polymorphism of TLR-2. Blood cells from the children from these farms express significantly higher amounts of mRNA encoding

CD14 and TLR2 (but not TLR4) than do those from non-farmers' children (Lauener et al. 2002). Currently TLR-4, which is triggered by LPS, looks less interesting. LPS, which is stable, and easily assayed in the environment and bedding, may represent a stable marker for microbial exposure in the farming environment (Braun-Fahrlander et al. 2002). One recent study found no evidence that genetic variation in TLR4 contributes to asthma susceptibility (Raby et al. 2002).

Striking changes in the expression of TLRs and CD91 (a receptor for HSPs) have been observed in psoriatic skin, but we are not aware of any evidence that polymorphisms of these molecules alter susceptibility to the condition (Baker et al. 2003; Curry et al. 2003).

3.4.4 Toll-Like Receptors and T_{reg}

Activation of human dendritic cells (DCs) via TLR4 and TLR2 may result in different immunological outcomes (Re and Strominger 2001). Unlike TLR4, TLR2 does not lead to release of IL-12 but rather of inhibitory IL-12 p40 dimers (O'Neill 2002) and IL-23, which is formed when p40 dimerises with p19. IL-23 has a different spectrum of activities from IL-12, and unlike IL-12, causes proliferation of murine $CD45RB^{low}$ T cells, that might include T_{reg} (Oppmann et al. 2000). Human DCs pre-incubated with a schistosomal extract caused T cells to secrete IL-10 (Van der Kleij et al. 2002). However, if TLR2 was blocked with an antibody, the T cells secreted Th2 cytokines, but no IL-10 (Van der Kleij et al. 2002). Heat-shock proteins can also trigger TLR, including TLR2 (Asea et al. 2002; Bulut et al. 2002), and can drive T_{reg} (van Eden et al. 2003).

It is not yet clear why the same TLR can sometimes drive potent inflammatory responses, and yet be essential for initiating regulation. As stated above, the anti-inflammatory effects of lactobacilli require TLR9, but there is nothing special about their DNA. It seems that the lactobacilli present their DNA in a "package", or in the context of other signals to the innate immune system, that permit their recognition as "old friends", and consequently allow the activation of regulation (Rachmilewitz et al. 2002). Such findings imply that

mimicking the effects of the "old friends" with single molecules may be difficult. The "code" for T_{reg} induction is likely to be complex so that pathogens do not too easily acquire it.

3.4.5 CD14 and Inflammatory Disorders

CD14 is expressed by myeloid cells, and secreted by them, and by the liver. It is not a signalling molecule, but it plays a crucial role in binding many microbial components (notably LPS and LAM) and facilitating their interaction with cell membrane-associated signalling molecules such as TLR. A polymorphism in the promoter region of the CD14 gene controls CD14 expression on monocytes and sCD14 levels in the sera of healthy subjects. In some studies of allergic individuals (Baldini et al. 1999) but not others (Sengler et al. 2003), such polymorphisms correlate to the levels of circulating IgE. Low CD14 in amniotic fluid or breast milk has been associated with increased risk of atopic eczema and of allergic sensitisation (Jones et al. 2002). The blood cells of children of farmers, who have a reduced risk of allergies associated with their exposure to the farming environment, expressed higher levels of mRNA encoding CD14 (Lauener et al. 2002).

CD14 has also attracted interest in relation to psoriasis. Keratinocytes of psoriatic skin express membrane-bound CD14 (mCD14), and soluble CD14 (sCD14) is elevated in the sera of psoriatic patients. However, a study of 63 Finnish patients with psoriasis and 126 non-psoriatic controls suggested that the enhanced CD14 expression in psoriasis is not attributable to functional variants of CD14 (Karhukorpi et al. 2002).

3.4.6 NRAMP1 (SLc11a1) in Diseases of Immunodysregulation

The Nramp1 gene was originally described as Ity/Lsh/Bcg, a gene controlling an early phase of resistance of inbred mice to intramacrophage pathogens. Stratification by BCG vaccination unmasked a potential genetic risk factor for atopy in the region of the Slc11a1 locus (Alm et al. 2002). Moreover IBD, multiple sclerosis, and rheu-

matoid arthritis have been associated with various polymorphisms (Kojima et al. 2001; Kotze et al. 2001; Rodriguez et al. 2002). Similarly, in a mouse model of allergy induced by immunisation with ovalbumin in alum, Nramp1^{r} animals showed less release of Th2 cytokines, IgE, and mast cell granules into the airways after aerosol challenge compared to congenic Nramp1^{s} mice (Smit et al. 2003). We are unaware of any studies of Slc11a1 in psoriasis.

3.4.7 CD1-Restricted T Cells

A major component of the early response to microorganisms (in particular mycobacteria) is mediated by CD1-restricted T cells and NKT cells (Dutronc and Porcelli 2002). CD1c-restricted T cells play an important role in the early maturation of DCs, and in driving the DC phenotype towards one that will encourage development of Th1 responses (Vincent et al. 2002). Ligands that interact with CD1 would be expected to modulate this process. CD1-restricted cells recognising glycolipid antigens might be involved in psoriasis (Nickoloff 1999). Interestingly, airway challenge with house dust mite causes a striking influx of CD1c + DC into the airways of asthmatics (Jahnsen et al. 2001).

3.4.8 Immunoregulatory Cytokines

There have been numerous studies of polymorphisms of cytokines and their receptors. We will only consider IL-10 as it is involved in immunoregulation. Monocytes from severe asthmatics secrete less IL-10 (Tomita et al. 2002), and a haplotype associated with low IL-10 production occurs in patients with severe asthma (Lim et al. 1998). Similarly it has been suggested that differences in the IL-10 secretion levels due to polymorphisms might contribute to the differences in the clinical course or time of presentation of psoriasis (Hensen et al. 2003; Kingo et al. 2003). IL-10 has shown potential in the treatment of psoriasis (Asadullah et al. 2003).

3.5 Animal Models for Studying Interactions Between Genes and Commensal or Environmental Microorganisms

The preceding discussion points to the need for animal models that can be used to study the effects of varying the microbial input, and the dependence of such effects on polymorphisms within the innate immune system. Some of the work indicating the effects of the microbial environment will be outlined in the following sections, and where this has been looked at in relation to the innate immune system, this will be indicated.

3.5.1 Models of Inflammatory Bowel Disease and Microbial Exposure

Interleukin 10-deficient (IL-$10^{-/-}$) mice remain healthy under germ-free conditions, but some IL-10-deficient strains develop colitis when given a normal specific pathogen-free (SPF) bowel flora. This is due to a poorly controlled immune response to the bowel flora. Interestingly, colonisation of germ-free IL-$10^{-/-}$ mice with *Lactobacillus plantarum* does not induce colitis, and can protect from subsequent introduction of SPF flora. Moreover *L. plantarum* also alleviates the established colitis in SPF IL-$10^{-/-}$ mice (Schultz et al. 2002). These protective effects may depend upon TLR9, which binds CpG motifs (Rachmilewitz et al. 2002). Similarly, antigen preparations from anaerobic bowel commensals given by gavage to Balb/c mice alleviate colitis induced by dextran sulphate, which is also attributable to an immune response to gut contents (Verdu et al. 2000). Interestingly, the colitis seen in SPF IL-$2^{-/-}$ mice also depends upon the presence of intestinal bacterial flora (Contractor et al. 1998).

HLA-B27 transgenic rats provide another model. Normal luminal bacteria play an essential role in initiating and perpetuating the chronic colitis, gastritis, and joint disease in these animals (Rath 2002), though the skin and genital inflammatory lesions are unaffected by the germ-free state. Obligate anaerobic bacteria, especially *Bacteroides* species, may play a predominant role, since metronidazole prevents colitis (Rath 2002).

3.5.2 Models of Allergic Disorders and Microbial Exposure

A number of observations suggest a role for commensal intestinal flora in allergic disorders. For example, in germ-free mice IgE production cannot be switched off by orally induced tolerance (Sudo et al. 1997). Similarly, neonatal antibiotic treatment can deviate the immune system towards Th2. These effects are prevented if commensal intestinal flora are re-introduced (Sudo et al. 1997, 2002). Such observations imply a role for T_{reg} induction in the gut in these animal models of allergy. It was recently proved that a preparation of an environmental saprophytic mycobacterium can induce allergen-specific T_{reg} ($CD4^+$, $CD45RB^{low}$, $IL\text{-}10^+$) that not only inhibit allergic symptoms, but are also active in a passive transfer model. Their function in the allergic recipient animals was blocked by a combination of anti-IL-10 and anti-TGF-β (Zuany-Amorim et al. 2002a, b). The same material is active via the oral route, though it has not been formally shown that the material acts via induction of T_{reg} when used in this way (Laura Rosa Brunet, Jon Hunt, and Graham Rook; unpublished observations).

Another model is provided by the DS-Nh (DS $Nh/^+$) mouse. These animals develop dermatitis spontaneously when they are housed in a conventional environment. When they are raised under SPF conditions, similar clinical and histopathological symptoms were inducible with repeated percutaneous immunisation of heat-killed *Staphylococcus aureus* on the back (Hikita et al. 2002). The dermatitis can occur in the absence of *S. aureus*, but is less severe (Watanabe et al. 2003). There are likely to be complex interactions between the gut flora (absent/SPF/normal), and the presence or absence of the inducing organism (*S. aureus*), and polymorphisms within the innate immune system.

3.5.3 Models of Autoimmune Disease and Microbial Exposure

Not all models of autoimmune disease are influenced by microbial flora, but many are. For instance, non-obese diabetic (NOD) mice reared in gnotobiotic environments have worsened diabetes (Kukreja and Maclaren 2002). Similarly, female PVG/c strain rats maintained

under specific pathogen-free conditions until weaning were found to be significantly less susceptible to the induction of autoimmune thyroiditis by thymectomy and irradiation than conventionally reared rats of the same strain (Penhale and Young 1988). However the most striking effects are seen in various models of autoimmune arthritis. Although the mechanisms were not understood, it was known in the 1980s that germ-free rats were moderately susceptible to adjuvant arthritis, and that this susceptibility could be enhanced or decreased simply by reconstituting the bowel flora with different bacterial species (Kohashi et al. 1985). Thus, bacterial flora can influence susceptibility to arthritis either way, causing either exacerbation or attenuation of disease. This variability may highlight the need to distinguish between "old friends" that are inducing immunoregulation, and "triggering" organisms that induce disease in appropriate hosts either as target antigens, or as a result of some critical cross-reactivity. Susceptibility to pristane-induced arthritis, which involves immune responses to hsp60, requires exposure to other arthritic animals, presumably to something present within their microbial flora (Thompson and Elson 1993). Similarly, neither the ankylosing enthesopathy in B10.BR mice (Rehakova et al. 2000) nor the inflammatory peripheral joint disease of HLA-B27 transgenic rats will occur if the animals are raised in a germ-free environment (Taurog et al. 1994).

The previous paragraph describes models in which the microbial flora exacerbate the arthritis, but in general the bacterial flora appear to protect. For example, collagen-induced arthritis is increased in germ-free DA rats compared to conventionally raised rats (Breban et al. 1993). Similarly, germ-free Wistar rats are much more susceptible to adjuvant arthritis than are conventionally raised Wistar rats (van de Langerijt et al. 1994). Moreover, transfer of spleen cells from the "dirty" rats to the clean rats is sufficient to reduce the susceptibility of the latter (Moudgil et al. 2001). Thus in various rat strains, background exposure to harmless microorganisms can influence Th1-mediated autoimmunity, and the last experiment quoted suggests a role for T_{reg}. The mechanism has been studied further in streptococcal cell wall arthritis. This is a chronic, erosive polyarthritis that can be induced in susceptible Lewis rats by one i.p. injection of an aqueous, sterile suspension of streptococcal cell wall. Interest-

ingly, F344 rats housed under conventional conditions are resistant to this treatment because commensal bacterial bowel flora induce neonatally a state of tolerance to arthritogenic epitopes in the streptococcal cell wall material. This state of tolerance is maintained throughout life. In contrast, germ-free F344 rats are as susceptible to streptococcal cell wall-induced arthritis, as are Lewis rats. This susceptibility of germ-free F344 rats rapidly disappears if the bowel flora are reconstituted. In contrast, Lewis rats are arthritis-prone even when they have normal bowel flora, because this tolerance is deficient and/or easily broken (van den Broek et al. 1992).

3.6 Conclusions

In conclusion, the "old friends hypothesis" seeks to explain the increasing prevalence of many chronic inflammatory disorders as a failure of immunoregulation secondary to decreased exposure to certain microorganisms that have been present throughout our evolutionary history. It is argued that these organisms are effectively part of our physiology, and are recognised as harmless by the innate immune system, which then activates immunoregulatory circuits, including T_{reg} and regulatory APCs. The "old friends" consequently act as adjuvants for other antigens such as self, allergens, and gut contents, and so limit chronic inflammatory immunoregulatory disorders. These mechanisms can be revealed by altering the microbial exposure, or by studying the presence of polymorphisms of the innate immune system, since these two factors provide a series of classical gene–environment interactions. An important consequence of this understanding is the fact that the enormous variability of animal models of inflammatory disorders in different laboratories can now be exploited to investigate these gene–environment interactions. Experiments based on these differences, perhaps involving collaborations between laboratories, or the use of gnotobiotic mice reconstituted with one or a few microbial species, can be applied to those animal models where variable microbial exposure is already known to exert a profound influence.

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4 Animal Models of Experimental Asthma

M. Wegmann, H. Renz

4.1 Immunopathology of Bronchial Asthma

Allergic bronchial asthma (BA) is a chronic inflammatory disease of the airways with increasing incidence, prevalence, and mortality over the last few decades. The complex pathology and pathophysiology includes chronic airway inflammation, increased mucus production, bronchial hyperresponsiveness, and a variable degree of airflow limitation (Bousquet 1990). Another hallmark of chronic allergic asthma is the observation of structural changes within and beneath the airway's wall as defined by subepithelial fibrosis, increased smooth muscle mass, and epithelial metaplasia (Hegele and Hogg 1996). Although advancement has been achieved in the understanding of several components of BA, its underlying mechanisms, resulting progression, and remodeling are still not precisely described (Table 1).

Both, genetic and environmental factors contribute to the development of BA (Cookson 1999). Chronic inflammation of the bronchial

Table 1. Characteristics of bronchial asthma

Airway inflammation
Central
Peripheral
Airway hyperresponsiveness
Irreversible broncho-obstruction
Airway remodeling
Subepithelial fibrosis
Goblet cell hyper-/metaplasia
Increased mass of airway smooth muscle cells

mucosa, which varies with the severity of the disease, has been suggested as being responsible for some pathological changes in airway structure and function. The characteristic leukocyte subsets within the inflamed tissue consist of eosinophils and T-lymphocytes, but B-lymphocytes, dendritic cells, and mast cells also contribute to the complex immunopathology (Haley et al. 1998; Vignola et al. 1998). Activated CD4-positive T cells have been detected in bronchial biopsies and broncho-alveolar lavage fluids of asthmatic patients, and absolute numbers of these cells correlate with severity of the disease (Walker 1994). Therefore CD4-positive T cells are suggested to play a central role in regulation and perpetuation of chronic asthma (Corrigan and Kay 1992). These T cells are characterized by the production of Th2-type cytokines including IL-4, IL-5, IL-9, IL-13, and GM-CSF (Robinson et al. 1992; Izuhara et al. 2002). The production of allergen-specific IgE by B-cells is under the control of Th2-type cytokines IL-4 and IL-13 (Wills-Karp et al. 1998). IgE binds to high-affinity Fcε-receptors (Fcε-RI) on mast cells and basophils (Kinet 1990; von Bubnoff et al. 2003). Allergen contact induces cross-linking of these receptors and results in mast cell degranulation with a release of histamine, leukotriene, prostaglandin D2, and perhaps other preformed and newly synthesized mediators. These mediators directly or indirectly cause broncho-constriction, increased mucus production, and mucosal edema (Bradding 1996). IL-5 is a major maturation and differentiation factor for eosinophils, and seems to be important for homing and activation of eosinophils during allergen-specific immune response. Eosinophil-derived media-

tors such as major basic protein (MBP), eosinophil cationic protein (ECP), and IL-13 are suspected to be involved in the development of airway hyperresponsiveness (AHR) (Drazen et al. 1996; Shi et al. 1998, 2000).

Many of these results are based on observations made in bronchoalveolar lavage fluids, bronchial autopsies, and sputum samples from asthmatic patients. Studies in experimental animal models have contributed greatly to the understanding of underlying mechanisms. Currently, the most used experimental animal is the mouse, because of the availability of gene-targeted and transgenic animals and because of the variety of specific reagents available for the investigation on the cellular and molecular levels of allergic airway inflammation. A widely used model is the development of acute allergic airway inflammation after short-term exposure to aerosolized antigen in systematically sensitized mice (Renz et al. 1992). Investigations in this animal model have elucidated the crucial role of CD4-positive lymphocytes and Th2-type cytokines, including IL-4, IL-5, and IL-13, in the pathogenesis of asthmatic inflammation (Renz et al. 1992; Gavett et al. 1994; Kaminuma et al. 1997; Hogan et al. 1998 b). Furthermore, murine experimental models provided important insight into IgE-independent mechanisms of allergic inflammation (Hogan et al. 1997; Korsgren et al. 1997), the critical role of eosinophils in the afferent limb of the allergic response (Shi et al. 2000), effector mediators and adhesion molecules regulating leukocyte recruitment, and, more recently, the importance of a neuro-immunological component controlling several events of asthma pathology and pathophysiology (Braun et al. 1999; Päth et al. 2002; Kerzel 2003).

4.2 Models of Experimental Asthma: Lung Function

A hallmark in modeling allergic asthma represents the possibility to monitor lung function in non-anesthetized spontaneously breathing mice. Two different systems have been successfully established: whole-body plethysmography (Hamelmann 1997) and head-out body plethysmography (Glaab et al. 2001). Both whole-body and head-out

body plethysmography are noninvasive procedures that permit measurement of respiratory function and particularly development of airway responsiveness in conscious, intact mice. These methods avoid several disadvantages of invasive procedures such as the need for anesthesia associated with potential drug-induced changes in airway responsiveness or changes in sensitivity to cholinergic challenge, necessity for mechanical ventilation, inability to take repeated on-line measurements, and inability to administer extended and repeated challenges with aerosol or local pharmaceutical agents. It is well documented that whole-body plethysmography is suitable to assess broncho-obstruction, which correlates with alterations in lung function. During measurement, the animal is placed in a plastic box without restraint. Only box pressure (P_b) is measured, and a dimensionless parameter, known as enhanced pause (P_{enh}), is derived from the shape of the P_b decay during expiration and the ratio of the inspiratory and expiratory maxima of P_b. P_{enh} increases during broncho-obstruction and correlates in anesthetized mice with pulmo-

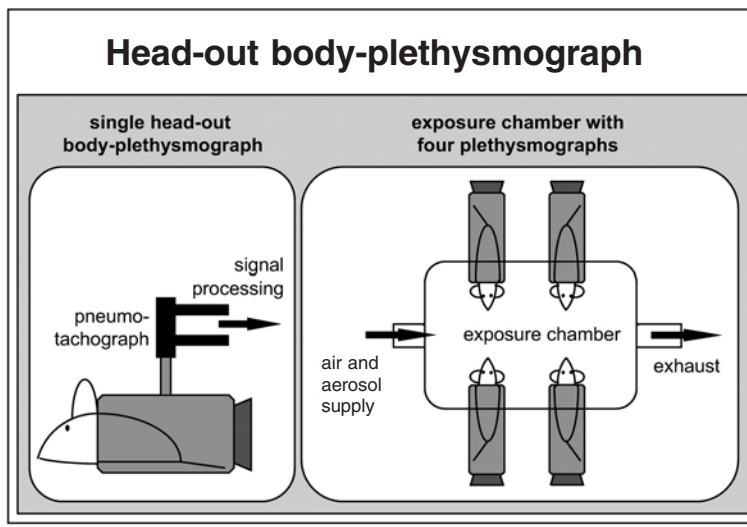

Fig. 1. Schematic drawing of the head-out body-plethysmograph for measurement of respiratory variables in non-anesthetized mice. (The system was modified from Vijayaraghanavan et al. 1993)

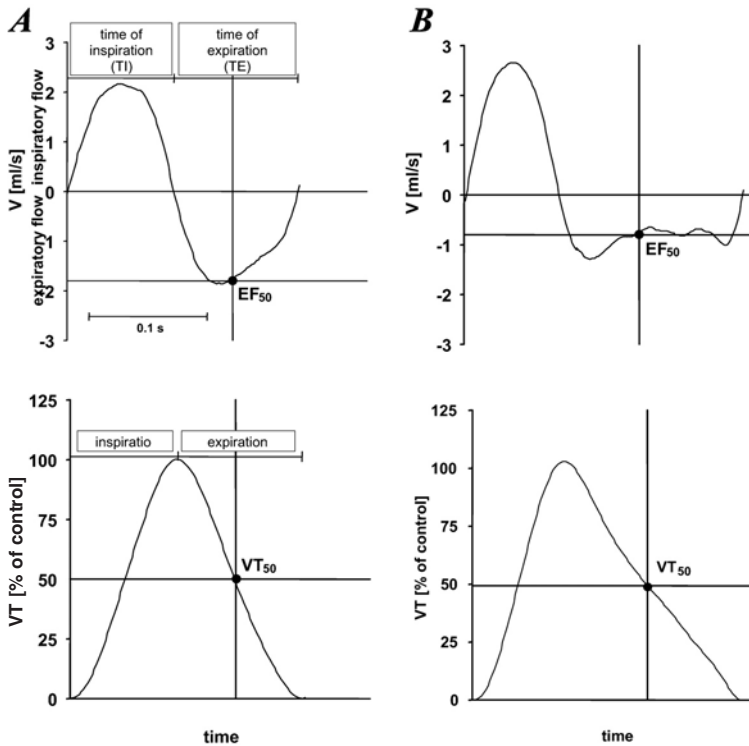

Fig. 2 A, B. Characteristic breathing pattern of normal breathing, non-anesthetized BALB/c mice. **A** Normal breathing pattern of BALB/c mice. **B** Characteristic breathing pattern of BALB/c mice during exposure to methacholine (20 mg/ml); decrease in EF_{50} at VT_{50} indicates airflow limitation

nary resistance, while it is apparently independent of breathing pattern and frequency (Hamelmann et al. 1997).

In comparison, head-out body plethysmography obviates efforts to compensate adiabatic conditions that occur through temperature and humidity changes by inspired and expired air (Glaab et al. 2001). Usually, four plethysmographs are attached to a head exposure chamber that is ventilated by a continuous airflow (Fig. 1); there is no need to replace air inside each plethysmograph. This method allows the continuous on-line recording of tidal flow patterns as a tool

Table 2. Respiratory breathing pattern of the BALB/c mouse derived from tidal flow

Respiratory parameters	Definition	Unit	Baseline value (BALB/c mouse)
Tidal volume (VT)	Amount of air inhaled/ exhaled per breath	ml	0.15±0.01
Time of expiration (TE)	Time from maximum to minimum VT	s	0.14±0.01
Time of inspiration (TI)	Time from minimum to maximum VT	s	0.08±0.01
Respiratory rate (F)	Number of breaths per minute	min^{-1}	268±29
Tidal midexpiratory flow (EF_{50})	Airflow during expiration at 0.5 VT	ml/s	–1.92±0.19

in the assessment of broncho-obstruction. With this method broncho-obstruction causes characteristic changes of the airflow pattern in mice (Fig. 2), which is indicated by a decrease in tidal midexpiratory airflow (EF_{50}). EF_{50} is the airflow at mid-tidal volume during expiration. A decrease in EF_{50} during challenge with β-methacholine via the airways was demonstrated to correlate with an increase in lung resistance in mice (Alarie 1966; Vijayaraghavan et al. 1993) as measured by the invasive method of Amdur and Mead (1958). Several relevant parameters of lung-function measurements can be directly calculated from the airflow pattern. They include respiratory rate (f), tidal volume (VT), time of inspiration (TI), and time of expiration (TE). Table 2 illustrates these data for the commonly used mouse strain BALB/c. Using the decrease of EF_{50} as a parameter of broncho-obstruction, it is possible to evaluate airway responsiveness in response to β-methacholine (Glaab et al. 2001), broncho-constriction as a result of the allergic acute-phase or late-phase reaction to inhaled allergens, and development of stable airway obstruction (Neuhaus-Steinmetz 2000).

4.3 Models of Experimental Asthma: Chronic Inflammation

Murine models have been extensively used to test novel approaches for asthma therapy. In this regard, numerous functional antagonists or inhibitors of functionally relevant mediators have been examined (Kanehiro et al. 2001; Oh et al. 2002). Many novel experimental modalities have been initially explored in the mouse. They include DNA immunization (Hertz et al. 2001) and the use of antisense oligonucleotides (Finotto et al. 2001).

Although animal models of acute allergic airway inflammation are valuable to investigate immunological mechanisms underlying bronchial asthma, it must be recognized that they have significant limitations. The acute model is not able to reflect a number of important structural changes in the airways as they occur in human allergic asthma. These changes include goblet cell metaplasia, increased smooth muscle mass, deposition of connective tissue fibers, and epithelial metaplasia, all of which are associated with the development of persistent and/or progressive airflow limitation (James et al. 1989). Chronic airway inflammation with the cellular components of T cells and eosinophils has been suggested to be responsible for airway remodeling, but the underlying pathogenetic mechanisms remain poorly understood. Therefore, the development of suitable animal models that develop airway remodeling and mimic this complex phenotype of human asthma as closely as possible represents an important challenge.

Several successful attempts have been made in this field. Temelkowski et al. (1998) employed controlled low mass antigen exposure for up to eight weeks in systematically sensitized mice to induce airway remodeling in BALB/c mice. Animals subjected to this protocol developed marked allergic inflammation of the proximal airways, goblet cell metaplasia, subepithelial collagen deposition, and increased bronchial reactivity to β-methacholine. Other sensitization and challenge protocols resulted in similar structural changes of airways with increased numbers of myofibroblasts in airway mucosa (Palmans et al. 2000; Sakai et al. 2001; Kenyon et al. 2003).

In an attempt to model structural changes in the airway, we have established a mouse model of chronic experimental asthma with the use of inhalative allergen challenges of mice following systemic sen-

sitization to ovalbumin (OVA). Allergen challenges took place on two consecutive days every week for up to 12 weeks. After chronic allergen exposure, animals developed persistent and progressive inflammation of the airways that was accompanied by goblet cell hyperplasia as well as metaplasia, deposition of elastic and collagenous fibers in the lamina adventitia, and increased numbers of myofibrocytes. Detailed analysis of airway inflammation in BAL fluids and in airway tissues at different anatomical levels revealed eosinophils as the predominant leukocyte subpopulation in acute allergic inflammation, lymphocytes were present to a lesser extent at this stage. After 12 weeks of allergen challenges a different distribution was noted, and the numbers of eosinophils and lymphocytes were almost the same. Development of chronicity of tissue inflammation was accompanied by a decrease in intraluminal inflammation, as indicated by a lower number of leukocytes in BAL fluids. Furthermore, we detected a progression of airway inflammation from proximal to distal airways. Allergen-induced acute airway inflammation was restricted to proximal airways, whereas chronic allergen exposure was associated with infiltrations throughout the entire airway tree, from proximal to distal airways. Furthermore, inflammation of distal airways was associated with deposition of elastic and collagenous fibers, differentiation of fibroblasts to myofibroblasts and goblet cell hyperplasia. Assessment of lung function revealed an increased airway responsiveness to β-methacholine compared to control or short-term (acute) challenged OVA-sensitized mice. In addition, these chronically exposed mice developed persistent airflow limitation as indicated by a decrease in the midexpiratory airflow over time (Table 3).

Inflammation and structural changes in the small airways represent an important feature of our model, since several recently published human studies have pointed out the role of small airways in the pathogenesis of bronchial asthma. In patients with severe asthma, several important histological changes in distal airways have been observed: infiltration with eosinophils in the airway wall and in distal air spaces, mucus plugging, and smooth muscle hypertrophy (Beasley et al. 1989; Hogg 1993). In humans, small or peripheral airways are defined as airways from the 7th–19th generation of airway branches with a diameter of 0.5–2 mm (Holz et al. 2000). They differ anatomically from proximal airways. The most prominent dif-

Table 3. Phenotyping human asthma, acute vs. chronic experimental asthma

Characteristics of human asthma	Acute	Chronic
Airway inflammation		
Central	++	++
Peripheral	–	++
Airway hyperresponsiveness	+	++
Irreversible broncho-obstruction	–	+
Airway remodeling		
Subepithelial fibrosis	–	++
Goblet cell hyper-/metaplasia	+	++
Increased mass of airway smooth muscle cells	–	+

ferences are the dependency on pulmonary surfactant to protect against excessive volume-related changes in diameter, total absence of cartilage, low numbers of ciliated epithelial cells, and a relatively greater total cross-sectional area. Due to these characteristics, small airways are less resistant to airflow, and their walls are more compliant and have a limited ability to clear secretions. In healthy individuals, small airways account for only 25% of the total lung resistance (Kraft 1999). Nevertheless, peripheral airways are much more sensitive to disease-related changes, because every reduction in airway diameter results in a profound increase in airway resistance. Small airway wall thickening may be caused by smooth muscle hyperplasia, mucus plugging, or inflammation in association with tissue edema, or a combination of the above. All of these events cause a marked decrement of luminal diameter and result in increased airway resistance and airflow limitation in asthmatic patients. The critical role of small airways in asthma pathology is supported by a study in asthmatic children and adults with long-lasting asthma (Yanai et al. 1992). In these patients, small airway resistance accounted for 34%–51% of total airway resistance. Chronic inflammation and airway remodeling in small airways might, therefore, play a major role in the development of stable airflow limitation in patients with chronic BA.

Brochoconstriction is another important component of increased airway resistance and is well characterized for central airways. However, more recent data indicate that small airways also contribute to hyperreactivity, with Wagner and coworkers' (1998) investigation of

small airway reactivity in humans. In addition, Ellis et al. (1994) examined the contractile reactivity of isolated peripheral and central bronchi. Exposure to a given concentration of ragweed allergen resulted in smooth muscle cell contraction in both peripheral and central bronchi, but peripheral bronchi were more sensitive to the stimulus, contracted faster, and released relatively more leukotrienes and histamine compared to central bronchi. Our animal model reflecting the component of persistent allergic inflammation of small airways in association with increased airway reactivity and airflow limitation represents, therefore, a suitable model for the investigation of the underlying mechanisms. Nonetheless, animal models that mimic the phenotype of human diseases as closely as possible still have their limitations. The assessment of lung function, in particular, in murine models of allergic asthma must be carefully handled, in order to detect significant differences in the microanatomy and microphysiology compared to humans. The interpretation of these data must consider marked morphological and mechanical differences between human and murine lung.

Whereas in humans the left lung consists of two and the right lung of three lobes, in mice the left lung is not divided and the right lung consists of four lobes (Table 4). In contrast to human airways, branching in mice is neither symmetric nor dichotomic, resulting in a continuous decrease in luminal diameter over short distances. Smaller bronchi that branch from the main bronchus are characterized by a loss of cartilage rings. Furthermore, smaller bronchi and bronchioli can be completely obliterated via airway smooth muscle

Table 4. Morphological differences between human and murine lung

	Mouse	Human
Anatomy	Right lung: 4 lobes Left lung: 1 lobe	Right lung: 3 lobes Left lung: 2 lobes
Main bronchus (∅)	~ 1 mm	~ 10–15 mm
Bronchioles (∅)	~ 0.01–0.05 mm	<1 mm
Terminal bronchioles (∅)	~ 0.01 mm	~ 0.6 mm
Respiratory bronchioles (∅)	Absent	~ 0.5 mm
Alveoles (∅)	~ 0.0039–0.0069 mm	~ 0.2–0.4 mm

constriction. Another characteristic of the murine lung is the complete absence of respiratory bronchioli. Of course, the most obvious difference between human and murine lung is the size. Whereas in human lung bronchioli exhibit a luminal diameter of more than 1 mm, murine bronchioli exhibit a luminal diameter of 0.01–0.05 mm, implying completely different respiratory mechanics of small airways in the two species (Foster 1983; Eckert and Randall 1988; Boggs 1992).

4.4 Models of Experimental Asthma: Suitable for Development of Novel Immunomodulators?

Only with great caution can observations made in animal models of human diseases be transferred to patients. A relevant example is the use of anti-IL-5 monoclonal antibodies in human asthma therapy. CD4-positive T cells seem to be essential for regulating the allergic immune response, including control of the important effector cells, the eosinophils. Eosinophils are associated with tissue damage and development of bronchial hyperresponsiveness. Most allergic and also nonallergic asthmatics have bronchial eosinophilia. There is a significant association between eosinophil activation and asthma severity as well as with airway hyperresponsiveness (Bousquet et al. 1990). Activated eosinophils are able to induce epithelial shedding by release of highly toxic products such as major basic protein (MBP), eosinophil cationic protein (ECP), eosinophil-derived neurotoxin (EDN), and oxygen free radicals (Gleich et al. 1993). Once activated, eosinophils release many of the preformed or rapidly synthesized mediators which in turn increase vascular permeability (Collins et al. 1993; Busse and Sedgwick 1994; Ying et al. 1995), contract human bronchial smooth muscle (Rabe et al. 1994) and trigger bronchial hyperresponsiveness (Leff 1994). Furthermore, eosinophils can release elastase (Lungarella et al. 1992), metalloproteases (Ohno et al. 1992) and certain growth factors (Ohno et al. 1992; Walz et al. 1993) and therefore play an important role in the process of tissue remodeling and fibrosis. These biological activities resulted in the concept that eosinophils play a central role in the immunopathogenesis of allergy and asthma. In this regard they represent a

cellular component of the innate and adaptive immune systems. In terms of innate immunity, they trigger acute inflammation, cell recruitment, and cytotoxicity. In terms of adaptive immunity they are able to present antigens via MHC-class II antigens, and they are involved in T-cell instruction and polarization via the cytokines IL-4 and IL-12.

This important functional capacity of eosinophils makes this cell population an attractive target for therapeutic intervention in bronchial asthma. This therapeutic concept is based on the hypothesis that removal of eosinophils would therefore improve the clinical situation in asthmatic patients. One important mediator that seems to be responsible for many, but not all, of the biological functions of eosinophils is the cytokine IL-5. The biological activities of IL-5 include development (Sanderson 1988), differentiation (Clutterbuck and Sanderson 1988), recruitment (Lopez et al. 1988), activation (Wang et al. 1989), and survival of eosinophils (Sanderson 1990). In IL-5 knock-out mice (Foster et al. 1996) and in IL-5 transgenic mice (Lee et al. 1997), the relationship between IL-5 and eosinophilic inflammation has been intensively investigated. Biological and functional removal of eosinophils via neutralization of IL-5 has been tested using several approaches in the murine system. Mice were treated with anti-IL-5 monoclonal antibodies, and mice have been generated which either overexpress IL-5 or show a deletion of the respective gene. Detailed analysis of the available literature in this regard indicates that the relationship between eosinophilia and development of airway hyperresponsiveness and the dependency on IL-5 remains unresolved. Bronchial hyperresponsiveness was absent in IL-5 knock-out mice (Foster et al. 1996), but could not be abolished in wild-type mice treated with monoclonal anti-IL-5 antibodies (Marthur et al. 1999). Other experimental studies presented evidence, that anti-IL-5 antibodies prevent from tissue eosinophilia, but do not have an effect on bronchial hyperresponsiveness (Hogan et al. 1998 a; Corry et al. 1998). Further, overexpression of IL-5 is associated with development of bronchial hyperresponsiveness (Lee et al. 1997). Differences in the mouse strains used, the antigen delivered, and the assessment of lung function may explain at least to some extent the conflicting findings. However, the animal studies provided the basis for initiation of clinical trials. The results of these

trials are quite well known. In summary, single or repeated administration of monoclonal anti-IL-5 antibodies resulted in rapid and sustained removal of eosinophils from peripheral blood. It still remains controversial whether this removal also extends into the airway mucosa. More recent data suggest that despite removal from the periphery, anti-IL-5 treatment might not be sufficient to result in an equivalent depletion of these cells from airway mucosa. However, the clinical results were rather disappointing, with only minimal effects on airway inflammation, no effects on airway hyperresponsiveness, unaffected FEV1-values, and the presence of the allergic late-phase response (Flood-Page et al. 2003).

It still must be taken into consideration that all results in the animal models were based on the acute inflammatory situation. No experiments were performed in a model reflecting chronic or persistent airway inflammation with all the additional signs of airway remodeling. These data exemplify the relevance of carefully examining the clinical phenotype of such murine models before any extrapolation into the clinical situation of patients can be made.

4.5 Conclusions

Animal models which mimic the hallmarks of human bronchial asthma are urgently needed. Such models provide important insight into the pathophysiology of the disease. However, the phenotype of such models must be carefully assessed. Most of the models currently available reflect the stage of acute asthmatic responses with airway inflammation, airway hyperresponsiveness, and sometimes mucus production. However, bronchial asthma represents a chronic disease with chronic and persistent airway inflammation, involvement of the smaller airways, structural changes within and beneath mucosal tissues, and persistent airway obstruction. Advances have been made in further developing models which mimic this phenotype much more closely. These models are particularly relevant in terms of developing novel therapeutic approaches for immuno-intervention in this complex disease. In the future it will be necessary to develop preventive strategies as well as strategies which specifically and sufficiently interfere in structural changes of the airways, since none of

the currently available therapies are able to prevent or stop the beginning of airway remodeling.

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5 Models of Rheumatoid Arthritis

R. O. Williams

5.1 Introduction

Rheumatoid arthritis (RA) is a chronic disabling disease affecting around 1% of the population. Much progress has been made in re-

cent years towards the identification of mediators that contribute to the pathogenesis of RA, and a number of studies have pointed to a pivotal role for tumour necrosis factor-alpha (TNFα) in the disease process. Indeed, the success of biological inhibitors of TNFα (Elliott et al. 1993, 1994a, b; Moreland et al. 1997; Weinblatt et al. 1999) in the clinic is a testament to the pathological significance of this cytokine in RA. However, there is still a lack of knowledge of the underlying causes of the disease and it is for this reason, together with the need for more durable remedies, that animal models of arthritis continue to be studied. Animal models of arthritis are used in a wide variety of different studies, including preclinical testing of novel therapies, analysing mechanisms of drug action, identifying both pro- and anti-inflammatory mediators, analysing genetic susceptibility factors, and in the search for markers of disease progression.

5.2 Models of Arthritis Induced by Immunisation

5.2.1 Adjuvant Arthritis

Adjuvant arthritis was the first model of RA to be described and can be induced in rats by a single injection of Freund's adjuvant, containing *Mycobacterium tuberculosis* (Pearson 1956). Clinical arthritis starts at around 10–45 days after injection and generally subsides after 1 month. The chief pathological features of adjuvant arthritis include oedema, infiltration into the joint of mononuclear and polymorphonuclear cells, pannus formation, periostitis, and erosion of cartilage and bone. Although an association between immunity to 65-kDa heat shock proteins and the induction of adjuvant arthritis has been suspected (van Eden et al. 1988), no single mycobacterial immunogen has been shown to be responsible for the arthritogenic response in this model (Holmdahl et al. 1992). Rather, the induction of adjuvant arthritis has been attributed to a mycobacterial cell wall component, muramyl dipeptide, which is immunostimulatory but does not evoke a specific immune response (Kohashi et al. 1982). In addition, a number of adjuvants which lack immunogenic properties have been shown to induce arthritis in susceptible strains of rats, including avridine (Chang et al. 1980), incomplete Freund's adjuvant

and pristane (Holmdahl et al. 1992). An arthritis bearing many similarities to RA is also observed in mice following administration of pristane (Bedwell et al. 1987). Doubts as to the immunological nature of these diseases were dispelled by the findings that (a) anti-T-cell treatments prevent the induction of arthritis (Holmdahl et al. 1992; Larsson et al. 1985), (b) susceptibility is influenced by genes within the MHC (Lorentzen and Klareskog 1996; Vingsbo et al. 1995), and (c) arthritis can be adoptively transferred by T cells (Kleinau and Klareskog 1993; Svelander et al. 1997).

The mechanism of arthritis induction following immunisation with adjuvants is unknown, but one possibility is that following immunisation there is an increase in the activity of antigen-presenting cells (APCs; Warren et al. 1986), leading to the presentation to autoreactive T cells of a hitherto unrecognised or "sequestered" endogenous antigen. The possibility that human RA could also be triggered by exposure to environmental factors with adjuvant-like activity has been highlighted by studies in which it was found that arthritis could be induced in DA rats by percutaneous exposure of adjuvant oils (Kleinau et al. 1994), or even a mineral oil-containing cosmetic product (Sverdrup et al. 1998).

5.2.2 Antigen-Induced Arthritis

Antigen-induced arthritis is seen in mice, rats, and rabbits following intra-articular injection of protein antigen (e.g. methylated bovine serum albumin) into the knee joints of animals that have been previously immunised with the same antigen (Brackertz et al. 1977; Dumonde and Glynn 1962). The histopathological appearance of antigen-induced arthritis bears similarities to RA, including synovial lining layer hyperplasia, perivascular infiltration with lymphocytes and plasma cells, lymphoid follicles, pannus and cartilage erosions. However, unlike RA, antigen-induced arthritis is a monoarticular disease that affects only injected joints. Susceptibility to antigen-induced arthritis is not MHC class II restricted, and this makes the model useful for studies involving transgenic and gene knock-out mice. For example, Busso et al. (1998) studied the evolution of anti-

gen-induced arthritis in urokinase gene knock-out mice in comparison with wild-type mice and were able to demonstrate a role for fibrin in the maintenance of chronic inflammation.

5.2.3 Streptococcal Cell Wall-Induced Arthritis

A single intraperitoneal injection of an aqueous suspension of sonicated streptococcal cell walls (SCWs) has been shown to cause chronic arthritis in rats and mice. Pathological changes of relevance to RA include infiltration of polymorphonuclear cells, $CD4^+$ T cells and macrophages, hyperplasia of the synovial lining layer, pannus formation, and erosion of cartilage and bone. Susceptibility to SCW-induced arthritis varies between strains. For example, Lewis (LEW/N) rats develop severe chronic disease, whereas histocompatible Fischer (F344/N) rats develop mild arthritis that rapidly subsides. This difference in susceptibility between the two strains has been attributed to a defect in the synthesis of corticotropin-releasing factor in the Lewis rat leading to sub-optimal activation of the hypothalamic-pituitary-adrenal axis (Sternberg et al. 1989a, b).

5.2.4 Collagen-Induced Arthritis

Collagen-induced arthritis (CIA) occurs in rats, mice, and primates following immunisation with type II collagen in adjuvant. The pathological changes that occur in CIA include synovitis with infiltration of polymorphonuclear and mononuclear cells, pannus formation, erosion of bone and cartilage, and fibrosis (Fig. 1).

The CIA model has been widely studied as a model of RA, largely on the basis of the pathological similarities between the two diseases (Holmdahl et al. 1989). Thus, both RA and CIA exhibit similar patterns of synovitis, pannus formation, erosion of cartilage and bone, fibrosis, and loss of joint mobility (Trentham 1982). Another key similarity between RA and CIA is that susceptibility to both diseases is strongly associated with genes encoding MHC class II molecules, suggesting the involvement of $CD4^+$ T cells in the pathogenesis of both forms of arthritis. Thus, susceptibility to CIA is re-

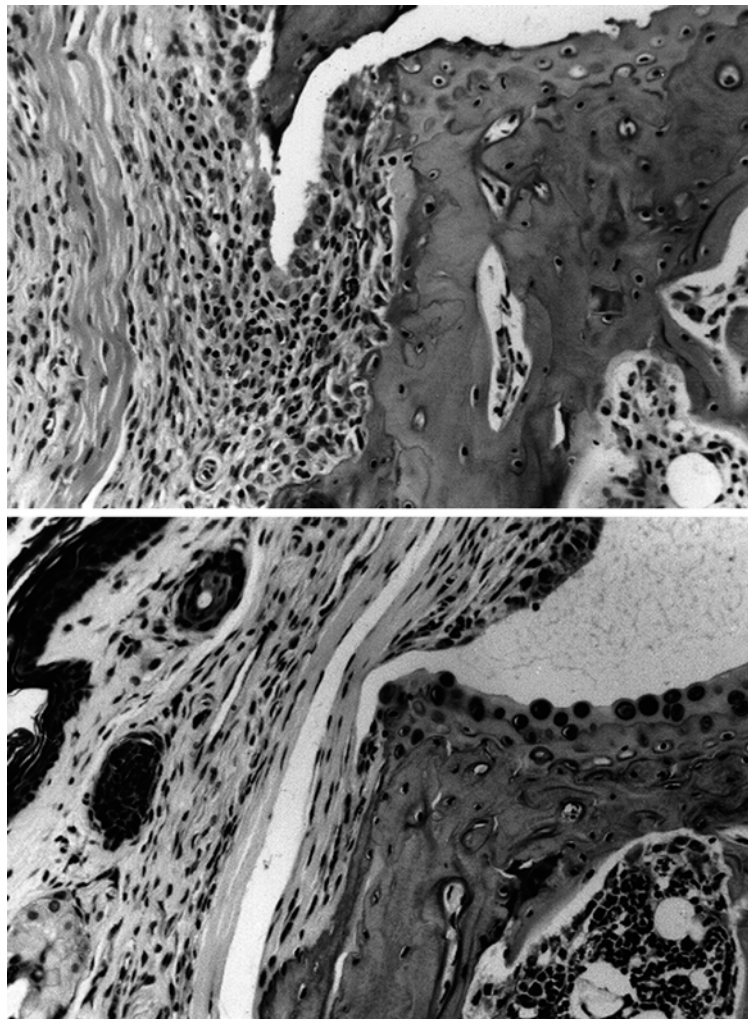

Fig. 1. Erosive changes in CIA. *Top:* Proximal interphalangeal joint of a mouse with CIA showing marginal bone erosion and loss of chondrocytes from the cartilage. *Bottom:* Normal joint. H&E

stricted to mouse strains bearing MHC types I-A^q and I-A^r, and this is analogous to human RA, where certain subtypes of DR4 and DR1 are strongly associated with susceptibility to the disease. In addition to the cellular arm of the immune response, it is also recognised that, as in human RA, humoral responses play a significant role in the pathogenesis of CIA (Holmdahl et al. 1989). However, it should be borne in mind that convincing data have not yet emerged pointing definitively to a role for type II collagen autoimmunity in the bulk of RA patients.

Another important feature of CIA that bears strong similarities with RA is the expression of pro-inflammatory cytokines, including TNFα and IL-1β, in the joints of mice with arthritis (Marinova-Mutafchieva et al. 1997) and the fact that blockade of these molecules results in reductions in both the clinical and histological severity of disease (Geiger et al. 1993; Joosten et al. 1996; Piguet et al. 1992; Thorbecke et al. 1992; Van den Berg et al. 1994; Williams et al. 1992, 1995; Wooley et al. 1993).

Although CIA and RA share many pathological features, there are also important differences between the two diseases, which should be borne in mind when interpreting data from therapeutic studies. For example, CIA induced by immunisation with heterologous (usually bovine, chick, or rat) type II collagen is a relatively acute disease in which arachidonic acid metabolites play an important pathological role in the disease process. This was clearly demonstrated in a study in which cytosolic phospholipase A2α (cPLA2α)-deficient mice were backcrossed on the arthritis-susceptible DBA/1 background. cPLA2α releases arachidonic acid from cell membranes to initiate the production of prostaglandins and leukotrienes. The incidence and clinical severity of CIA was dramatically reduced in cPLA2α-deficient mice compared to wild-type littermates (Hegen et al. 2003). In addition, histological examination revealed that pannus formation, synovial hyperplasia, infiltration of inflammatory cells, and ankylosis were all markedly reduced in cPLA2α-deficient mice compared to wild-type littermates, despite the fact that anti-collagen antibody levels were similar in the two groups (Hegen et al. 2003).

Given the involvement of arachidonic acid metabolites in the pathogenesis of heterologous CIA, it is not surprising that nonselec-

tive cyclooxygenase inhibitors, as well as selective cyclooxygenase-2 inhibitors, are effective in reducing the severity of arthritis (Ochi et al. 2003). In contrast to heterologous CIA, immunisation of male DBA/1 mice with homologous (mouse) collagen results in a more protracted disease course (Boissier et al. 1987; Holmdahl et al. 1986; Malfait et al. 2001), and the chief determinant of chronicity in CIA is likely to be the extent to which the immune response is targeted at self collagen, as opposed to the collagen used for immunisation. We carried out a study of the utility of the chronic relapsing homologous CIA model for testing disease-modifying antiarthritic drugs. For example, we found that, as in human RA, indomethacin was ineffective in preventing joint damage in chronic homologous CIA despite the fact that the same drug was effective in acute heterologous CIA (Malfait et al. 2001). It was also found that inhibitors of TNF were effective in the chronic homologous CIA model, and pulse therapy with anti-CD3 plus anti-TNF was found to induce remission, clinically as well as histologically. In contrast, pulse therapy with either anti-CD4, anti-TNF, or the combination of anti-CD4 plus anti-TNF was less effective in inducing remission. It was concluded that the chronic homologous CIA model was useful for identifying remission-inducing antiarthritic drugs and has predictive value with respect to their joint-protective capacity (Malfait et al. 2001). Another more chronic variant of the classical CIA model is chronic CIA induced in bovine type II collagen-immunised TCRβ transgenic DBA/1 mice, which over-express the TCRβ gene from a T-cell clone that recognises mouse type II collagen (Mauri et al. 1997; Mori et al. 1992).

5.2.5 Proteoglycan (Aggrecan)-Induced Arthritis

As discussed above, convincing data have not yet emerged pointing definitively to a role for collagen autoimmunity in the bulk of RA patients. This has prompted the search for other potential joint antigens that may be the target of the autoimmune response in RA. For example, a T-cell-driven arthritis has been described in BALB/c mice following immunisation with the G1 domain of human proteoglycan aggrecan (Glant et al. 1987, 1990). Histopathological changes include

oedema, proliferative synovitis, infiltration of mononuclear cells, pannus formation, and erosion of cartilage and bone.

In general, it is thought that organ-specific autoimmune diseases are driven by Th1-type responses, characterised by a higher ratio of IFN-γ to IL-4 production. Furthermore, most studies of Th1/Th2 cell activity in animal models as well as human disease suggest that arthritis is a predominantly Th1-driven disease. Hence, it is of interest to observe the development of severe arthritis in BALB/c mice, because this strain has a strong genetic predisposition towards the development of Th2-type responses. Finnegan et al. (1999) addressed the question of whether proteoglycan-induced arthritis in BALB/c mice is associated with a Th2-type response and is therefore distinct from other models of arthritis. It was found that proteoglycan-immunised BALB/c mice developed a higher ratio of IFN-γ production to IL-4 production and that the IFN-γ:IL-4 ratio peaked at the onset of disease (Finnegan et al. 1999). It was also shown that IL-4 treatment prevented arthritis and induced a Th1 to Th2 switch in the immune response. It was concluded that despite the fact that BALB/c mice are predisposed to Th2-type responses, proteoglycan-induced arthritis is a predominantly Th1-cell-driven disease.

5.2.6 Cartilage Oligomeric Matrix Protein-Induced Arthritis

A novel model of arthritis, induced by immunisation with homologous cartilage oligomeric matrix protein (COMP), has been described in rats (Carlsen et al. 1998). Like CIA, susceptibility to COMP-induced arthritis is controlled by genes within the MHC, with the $RT1^u$ and $RT1^l$ haplotypes showing the greatest degree of susceptibility. The arthritis is characterised by synovial hyperplasia and hypertrophy accompanied by pannus and, in some strains, joint erosion. Like type II collagen and aggrecan, COMP is a product of chondrocytes but represents a relatively minor fraction of the extracellular matrix of cartilage. It could be argued that under natural conditions a major cartilage protein, like type II collagen, would probably induce a strong degree of central and/or peripheral tolerance, and therefore would be an unlikely target for the autoimmune response (Carlsen et al. 1998). Thus, in the search for potential auto-

antigens in RA, minor cartilage proteins, such as COMP, should not be overlooked.

5.3 Spontaneous Arthritis in Transgenic Strains of Mice

5.3.1 hTNF Transgenic Mice

The application of transgenic technology to the field of cytokine biology has generated a number of new models of arthritis. For example, mice overexpressing a human TNFα transgene, disregulated by the replacement of the 3′ AU-rich region with the 3′ untranslated region of the human β-globin gene (Fig. 2) were found by Kollias et al. to spontaneously develop arthritis (Keffer et al. 1991). The arthritis could be prevented by continuous treatment with human TNFα-specific monoclonal antibody. Histological examination of the joints of hTNFα transgenic mice revealed that the arthritis bore a number of similarities to human RA and was highly erosive in nature, with subchondral bone being particularly affected (Fig. 3).

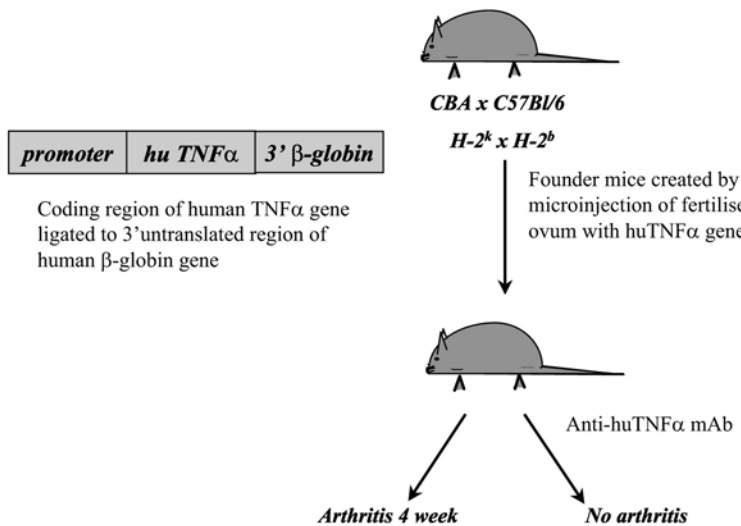

Fig. 2. Generation of hTNFα transgenic mice

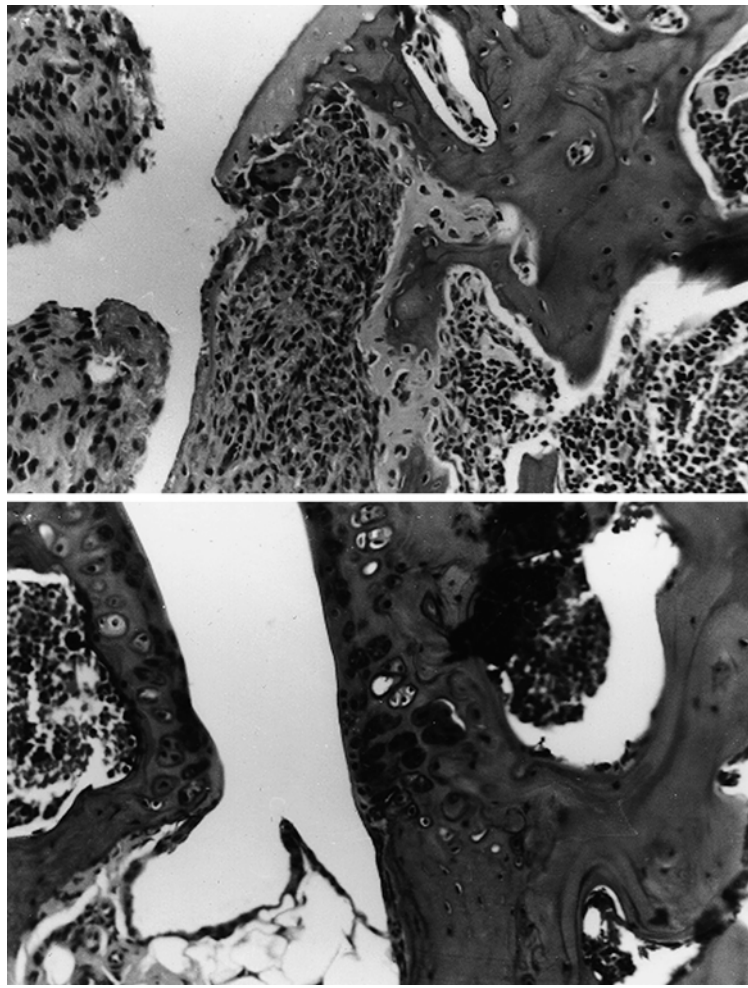

Fig. 3. Joint damage in hTNF*α*-transgenic mice. *Top:* Erosive changes in the cartilage-bone-pannus region of a proximal interphalangeal joint from a hTNF*α*-transgenic mouse with arthritis. Note the focal erosion of subchondral bone. *Bottom:* Normal joint from a nontransgenic littermate. H&E

It was found that TNFα was overexpressed in a number of tissues, including lung, spleen, and the joint, and it is not clear why the joint should be affected to a greater extent than these other tissues. Indeed, a second generation of TNF transgenic mice was generated in which the AU-rich region of the TNFα transgene was deleted by targeted disruption. These $TNF^{\Delta ARE}$ mice developed inflammatory bowel disease, in addition to arthritis (Kontoyiannis et al. 1999).

The occurrence of arthritis in hTNFα-transgenic mice is perhaps not surprising, in the light of what is now known about the role played by TNFα in the pathogenesis of arthritis (Maini et al. 1997). However, a less expected finding was that treatment of hTNFα transgenic mice with an IL-1α/β blocking antibody completely prevented the development of arthritis (Probert et al. 1995). This finding parallels studies in human RA synovial cell cultures where blockade of TNFα was found to diminish IL-1β production (Brennan et al. 1989) and indicates that IL-1 is acting as a major downstream mediator of joint pathology.

hTNFα-transgenic mice were originally derived on a C57BL/6xCBA background (H-2k/H-2b) which is resistant to CIA. However, when back-crossed onto the CIA-susceptible DBA/1 background (H-2q), hTNFα transgenic mice develop a more severe form of arthritis with an earlier time of onset (Butler et al. 1997). This raises the possibility that overexpression of TNFα leads to activation of type II collagen immune responses. To address this question, I compared serum levels of IgG to collagens type II, IX, and IX and to proteoglycan in arthritic hTNFα transgenic mice and nontransgenic littermates. However, no differences in autoantibody levels to any of the cartilage antigens were detected between the two groups. Furthermore, hTNFα-transgenic mice crossed onto a RAG-1 knockout background also develop severe arthritis, indicating that T and B lymphocytes are not required for the development of this form of arthritis (Kollias et al. 1999).

5.3.2 hIL-1α Transgenic Mice

It is known that, in addition to TNFα, IL-1 is a major mediator of joint pathology in arthritis, and IL-1α transgenic mice have been

generated in which human IL-1α is expressed in various organs. IL-1α-transgenic mice were found to develop severe polyarthritis at 4 weeks of age (Niki et al. 2001). Synovitis was observed 2 weeks after birth, and after 8 weeks, synovial lining layer hyperplasia and the formation of pannus were seen. Most importantly, there was severe degradation of cartilage, thereby confirming previous findings regarding the role of IL-1 in cartilage breakdown (Saklatvala et al. 1984, 1985).

In both the hTNFα and hIL-1α transgenic strains of mice, it is clear that the overexpression of pro-inflammatory cytokines leads primarily to the manifestation of arthritis, despite the fact that the overexpression is not confined to the joint. This would suggest that the joints are particularly sensitive to the effects of pro-inflammatory stimuli.

5.3.3 The KRN Model of Arthritis

One of the most intriguing models of arthritis to emerge in recent years is the KRN model of arthritis described by the group of Benoist and Mathis (Kouskoff et al. 1996). The KRN transgenic mouse line expresses a T-cell receptor specific for an epitope of bovine pancreas ribonuclease in the context of I-A^k (Peccoud et al. 1990). However, it was found, serendipitously, that when KRN mice are crossed with NOD mice (I-A^{g7}), the resulting (K/BxN) offspring develop arthritis spontaneously at around 4–5 weeks of age. The arthritis is severe, symmetrical, affects principally distal joints, and resembles human RA in many important respects.

It was subsequently found that the development of arthritis in K/BxN mice was dependent on I-A^{g7} MHC class II molecules and could be blocked by the administration of nondepleting anti-CD4 monoclonal antibody (Korganow et al. 1999; Kouskoff et al. 1996; Mangialaio et al. 1999). The development of arthritis was also found to require the presence of B lymphocytes (Korganow et al. 1999; Kouskoff et al. 1996). Furthermore, arthritis could be transferred, albeit transiently, by injecting naive mice with serum IgG from arthritic mice (Korganow et al. 1999). The molecular target of the autoantibodies was identified as the ubiquitous cytoplasmic enzyme

glucose-6-phosphate isomerase (GPI), and it was found that KRN T cells also recognised GPI, in the context of I-A^{g7} MHC class II molecules (Matsumoto et al. 1999).

The important question concerning the KRN model is how can a joint-specific autoimmune disease arise from autoreactivity to a ubiquitous intracellular enzyme, such as GPI? Immunohistological examination of nonarthritic mouse joints revealed the accumulation of extracellular GPI on the lining of the articular cavity, particularly on the surface of cartilage (Matsumoto et al. 2002). The accumulation of GPI on cartilage was more pronounced in K/BxN mice with arthritis, and colocalised with IgG and the C3 component of complement. It was hypothesised that complexes of GPI and anti-GPI initiate an inflammatory cascade by activation of complement via the alternative pathway (Matsumoto et al. 2002). The important lesson to be learned from the KRN model is that autoimmune arthritis may arise as a result of an immune response to an antigen that is not confined to the joint. As discussed in the previous section, this suggests that the joint is particularly vulnerable to inflammatory stimuli.

5.4 The Use of Animal Models to Investigate the Genetics of Arthritis Susceptibility

As discussed above, susceptibility to human RA is strongly influenced by genes within the MHC class II region. In addition, non-MHC genes contribute to disease susceptibility as well as disease severity. The identification and characterisation of genes contributing to susceptibility and/or severity would provide insights into the aetiopathogenesis of RA and would potentially provide novel targets for therapy. However, environmental variability and genetic heterogeneity make the identification of specific genetic loci determining susceptibility to RA extremely difficult in humans. In animal models, however, environmental differences can be minimised and the genetic heterogeneity can be dramatically reduced. For example, Remmers et al. mapped quantitative trait loci (QTL) controlling susceptibility to CIA in the offspring of resistant and susceptible inbred rat strains. A major susceptibility QTL was identified within the

MHC region (Remmers et al. 1996). This was anticipated as susceptibility to CIA, like RA, is known to be associated with specific MHC class II haplotypes in both rats and mice. However, the authors then went on to compare disease severity only in rats with arthritis-susceptible MHC genotypes. Four QTL were identified outside the MHC class II region, on chromosomes 1, 4, 7, and 10, which were found to contribute to the severity of arthritis (Remmers et al. 1996).

More recently, Olofsson and Holmdahl used the pristane-induced arthritis model to investigate the genetics of susceptibility to this form of arthritis. Fifteen QTL had previously been identified that contribute to susceptibility to pristane-induced arthritis in rats (Nordquist et al. 2000; Olofsson et al. 2002; Vingsbo-Lundberg et al. 1998). Through positional cloning of one of these loci, it was found that a naturally occurring polymorphism of *Ncf1* contributes to the severity of pristane-induced arthritis (Olofsson et al. 2003). *Ncf1* encodes neutrophil cytosolic factor 1, which is a component of the NADPH oxidase complex found in all phagocytic cells. Furthermore, the disease-related allele of *Ncf1* was found to exhibit a reduced level of oxidative burst and to promote the activation of arthritogenic $CD4^+$ T cells. The administration of phytol, an activator of the NADPH oxidase complex, during the induction phase of arthritis was found to reduce the severity of arthritis (Olofsson et al. 2003). Taken together, these findings point to an important role for *Ncf1* in determining the severity of arthritis, and they illustrate the power of this form of genetic analysis in identifying proteins involved in the development of arthritis.

5.5 Therapeutic Studies in CIA

5.5.1 Prevention of Arthritis Versus Therapy of Existing Disease

CIA has come to be the most widely accepted murine model for studies of therapeutic intervention. Normally, such experiments may be divided into those in which treatment is administered before the onset of arthritis and those in which treatment is administered after the onset of arthritis, and these two different regimens may provide

different results. For example, when given before disease onset, a number of anti-T cell therapies (e.g. depleting anti-CD4 mAb, anti-IL-12 mAb, and CTLA4-Ig) have been shown to be capable of blocking the development of arthritis by inhibiting or altering the immune response that precedes the development of the disease. However, such treatments are usually found to be much less effective in reducing the severity of arthritis once the immune response is fully established and the inflammatory response is underway (Hom et al. 1988; Malfait et al. 1998; Ranges et al. 1985; Webb et al. 1996).

5.5.2 Anti-T Cell Therapy

Conflicting reports have emerged relating to the efficacy of T cell-targeted therapeutic strategies in experimental arthritis. For example, a number of early studies showed that polyclonal anti-T cell serum or anti-CD4 mAb could prevent CIA in DBA/1 mice if given around the time of immunisation with type II collagen in adjuvant, but neither treatment was effective when administered after immunisation (Brahn and Trentham 1984; Ranges et al. 1985). In another study in which anti-T cell therapy was evaluated in established CIA, anti-CD4 treatment was shown to have little effect alone, although it was found to inhibit disease progression when given in combination with an antibody to the pan T-cell marker, Thy-1 (Hom et al. 1988). One possible interpretation of this finding is that $CD8^+$ T cells play an important pathological role in the disease process, but this was discounted by the observation that anti-CD8 treatment was not found to modify disease severity, either alone or in combination with anti-CD4. The relative lack of efficacy of anti-CD4 treatment in established arthritis has also been demonstrated in other models of autoimmune disease. Thus, in MRL/*lpr* mice, which develop a lupus-like syndrome that includes a polyarthritis, early anti-CD4 treatment (before the onset of disease) resulted in a significant reduction in subsequent lupus, but had little effect on established disease (Jabs et al. 1994).

Similarly, anti-T-cell receptor α/β (TCRα/β) mAb treatment, like anti-CD4 treatment, has been demonstrated to prevent the induction

of CIA in rats when given around the time of immunisation with type II collagen (Goldschmidt and Holmdahl 1991; Yoshino et al. 1991). In established arthritis, however, conflicting findings have been reported. Anti-TCRα/β treatment in established CIA in rats was shown in one study to reduce disease severity (Goldschmidt and Holmdahl 1991) and in another study to be ineffective (Yoshino and Cleland 1992), whereas anti-TCRα/β treatment actually caused exacerbation of established arthritis in mice (Maeda et al. 1994). Other anti-T cell treatments that have been evaluated in CIA include anti-IL-2R treatment and anti-MHC class II treatment. Administration of anti-IL-2R mAb at the time of immunisation was found to reduce the incidence and the severity of CIA and to decrease circulating levels of type II collagen-specific antibody (Banerjee et al. 1988). The effects of anti-IL-2R treatment in established CIA were not reported, however. Anti-MHC class II treatment was shown to decrease the incidence of CIA and delay its onset, if given at the time of immunisation (Cooper et al. 1988; Wooley et al. 1985) but had no effect on the incidence or time of onset of arthritis when given 2 weeks after immunisation (Cooper et al. 1988).

A major conclusion to be drawn from these reports is that it is much easier to prevent arthritis by applying anti-T cell therapy during the induction phase of CIA than to inhibit the disease during the course of an ongoing inflammatory response. However, this does not necessarily mean that the role of the T cell is confined only to the induction phase of arthritis. Indeed, a number of recent studies provide support for the hypothesis that T cells continue to play an important role in established CIA. It was shown that CTLA4-Ig, which blocks the interaction between CD28 and B7 molecules and therefore modulates T-cell activity, is effective after disease onset (Webb et al. 1996). Similarly, nondepleting anti-CD4 mAb therapy has been shown to be effective in established disease in chronic CIA in TCR-Vβ12 transgenic mice (Mauri et al. 1997). Furthermore, cyclosporin at high dose reduces the clinical severity of established CIA and inhibits the occurrence of joint erosions, in a manner comparable to TNFα blockade (Hom et al. 1988; Williams et al. 1998). Of course, it is now recognised that multiple subsets of regulatory T cells exist, as well as Th2 cells, which could have an anti-inflammatory role in arthritis. Hence, treatments targeted indiscriminately at T cells may

fail to ameliorate arthritis because they do not distinguish between the pro- and anti-inflammatory subsets of T cells.

5.5.3 TNFα Blockade in CIA

The most obvious way of assessing the pathological significance of a particular cytokine in arthritis is to block its activity using specific antibodies, and many studies have focussed on the effect of TNFα blockade in experimental arthritis. These studies showed that treatment of mice with monoclonal or polyclonal anti-TNFα antibodies, or soluble TNF receptors, reduced the severity of arthritis when administered before the onset of clinical arthritis (Piguet et al. 1992; Thorbecke et al. 1992; Williams et al. 1992). Subsequently, we assessed the effect of anti-TNFα treatment in mice with established CIA (Williams et al. 1992). DBA/1 mice with CIA were given twice-weekly injections of TN3–19.12 (anti-TNFα mAb), L2 (isotype control), or PBS over a period of 14 days. The half-life of TN3–19.12 in mice had been previously estimated to be approximately 7 days (Sheehan et al. 1989). It was found that there was a dose-dependent reduction in the severity of arthritis following treatment with anti-TNFα mAb (Fig. 4). At the end of the treatment period, individual joints were graded according to the histopathological severity of arthritis. Anti-TNFα treatment was found to reduce the histological severity of arthritis and to protect joints from erosive changes (Fig. 5).

Soluble TNF receptors are understood to play an important physiological role in regulating the activity of TNFα, and it was subsequently shown that two soluble TNFR constructs were effective in established CIA. In the first study, a p75 TNFR-Fc fusion protein was found to reduce the severity of CIA whether given before or after the onset of the disease (Wooley et al. 1993). In another study, we showed that a p55 TNFR-Ig fusion protein was effective in reducing the clinical severity of established CIA (Williams et al. 1995). Furthermore, when the joints were examined by histology, treatment with TNFR-Ig was found to have exerted a dose-dependent protective effect on joint erosion. The conclusion drawn from these studies was that TNFα is involved in the pathogenesis of CIA.

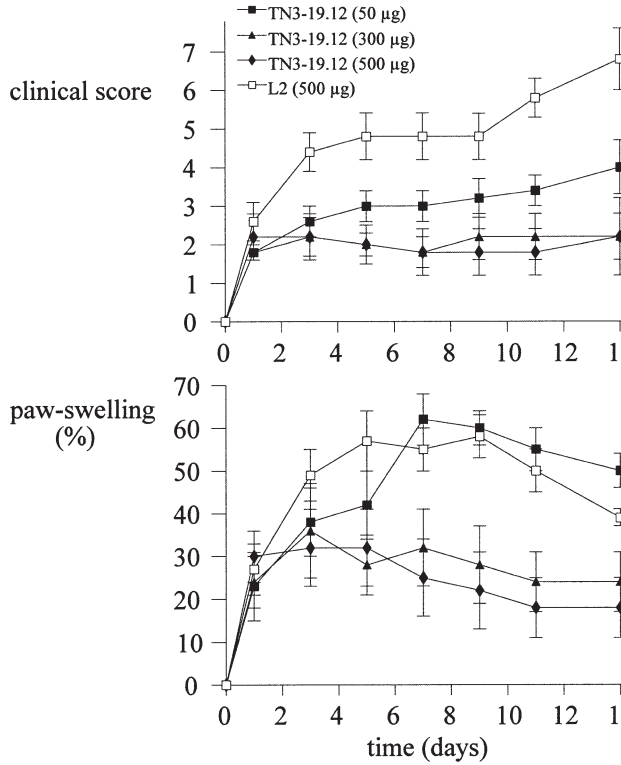

Fig. 4. Effect of anti-TNF*a* mAb (TN3–19.12) on clinical progression of established CIA. L2 is an isotype-matched control mAb. *Arrows* indicate time of injection. *Top:* Clinical score, using a scoring system was based on the following criteria: *0* = normal, *1* = slight swelling and/or erythema, *2* = pronounced oedematous swelling, *3* = ankylosis. Each limb was graded, giving a maximum score of 12 per mouse. *Bottom:* Paw-swelling, expressed as the percentage increment in paw-width relative to the paw-width before the onset of arthritis. (Modified from Williams et al. 1992)

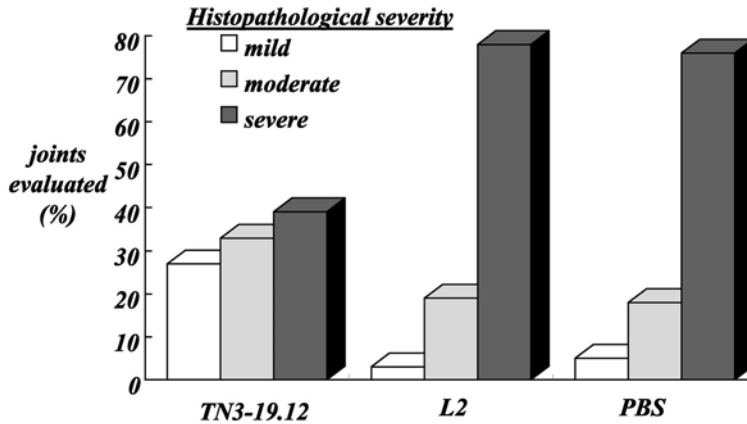

Fig. 5. Histopathological assessment of joints of arthritic DBA/1 mice treated with anti-TNF*α*. The scoring system was as follows. Mild, minimal synovitis, erosions limited to discrete foci, cartilage surface intact. Moderate, synovitis and erosions present but normal joint architecture intact. Severe, extensive erosions, joint architecture disrupted. (Data from Williams et al. 1992)

In addition, the findings provided support for the testing of anti-TNF*α* antibody therapy in human RA.

5.5.4 Combination Therapy: Anti-TNF*α* plus Anti-CD4

The main form of combination therapy tested by our group was anti-TNF*α* mAb plus anti-CD4 mAb. Previously, we and others had shown that anti-TNF*α* therapy was effective in reducing the severity of established CIA (Piguet et al. 1992; Thorbecke et al. 1992; Williams et al. 1992), a finding that was subsequently confirmed in human RA (Elliott et al. 1993, 1994a, b; Rankin et al. 1995). Anti-CD4 therapy, as discussed above, had been shown to be relatively ineffective in established CIA (Hom et al. 1988), although it was effective in preventing the induction of arthritis if given around the time of collagen immunisation (Ranges et al. 1985). From these findings it was concluded that $CD4^+$ T cells played a more promi-

nent role in the induction phase of arthritis, whereas the role of TNF*α* was more prominent in the effector phase of the disease. To test the effect of a combined therapeutic strategy that targets both induction and effector mechanisms, we used the anti-TNF*α* mAb, TN3–19.12 (Sheehan et al. 1989), in combination with a cocktail of two lytic anti-CD4 mAbs, YTS 191.1.2 and YTA 3.1.2 (Cobbold et al. 1984; Galfre et al. 1979; Qin et al. 1987).

DBA/1 mice with established CIA were treated with either an optimal or a suboptimal dose of anti-TNF*α* alone, anti-CD4 alone, or anti-TNF*α* plus anti-CD4. Controls received mAbs of irrelevant specificities. Anti-CD4 mAb alone had a relatively minor impact on the disease compared to the controls, whereas anti-TNF*α* alone was effective at the optimal dose, as shown in previous studies (Williams et al. 1992). However, combined anti-CD4/anti-TNF*α* treatment caused a much more significant decrease in the severity of arthritis than either anti-TNF*α* alone or anti-CD4 alone (Williams et al. 1994). The synergistic effects of anti-TNF*α* and anti-CD4 were particularly apparent at the suboptimal dose of anti-TNF*α*, which on its own was relatively ineffective. For example, it was shown by histology that suboptimal anti-TNF*α* treatment alone reduced the number of erosions in the proximal interphalangeal joints by 20%, and anti-CD4 alone reduced joint erosions by 22%. In contrast, combined anti-TNF*α*/anti-CD4 treatment reduced the number of joint erosions by 72% (Williams et al. 1994). Another finding was that in the mice treated with anti-TNF*α* plus anti-CD4, there was a reduction in the IgM antibody response to the anti-TNF*α* antibody (a hamster IgG1 mAb), a potentially significant finding as the development of anti-globulin responses represents an obstacle to the use of murine mAbs in humans (Waldmann 1991).

5.5.5 Analysis of the Immunomodulatory Effects of Anti-arthritic Drugs in CIA

CIA is a T cell-mediated disease involving a predominantly Th1 response to a defined cartilage-derived antigen (type II collagen). The CIA model therefore offers a powerful research tool for analysing the immunomodulatory properties of potential anti-arthritic drugs.

For example, there is much interest in the possibility of using cAMP-elevating agents for the treatment of RA, and we have used the CIA model to examine how different cAMP-elevating agents influence Th1/Th2 responses.

Spleen or lymph node cells from DBA/1 mice with CIA were cultured in the presence of type II collagen plus one of five different cAMP-elevating agents: rolipram, forskolin, PGE_2, 8-bromo-cAMP, or cholera toxin. Secreted levels of IFN-γ, IL-4, and IL-5 were measured by ELISA.

All of the cAMP-elevating agents tested profoundly suppressed IFN-γ production in a dose-dependent manner. IL-4 and IL-5 production was slightly upregulated at low concentrations of the cAMP-elevating agents, and was modestly suppressed at the highest concentrations of cAMP-elevating agents (Ozegbe et al. 2003). Experiments were then carried out to determine whether T cells were directly affected by cAMP-elevating agents or whether the immunomodulatory effects were mediated via APCs. Pulsing T cells alone for a brief period with cholera toxin produced an almost identical effect to pulsing APCs alone, i.e. downregulation of proliferation, and downregulation of IFN-γ production with little effect on IL-5 production (Ozegbe et al. 2003).

It was concluded that cAMP-elevating agents suppressed Th1 responses to type II collagen to a greater extent than Th2 responses, and that cAMP-elevating agents could directly influence the activity of T cells but, in addition, influenced the ability of APCs to support Th1 responses.

5.5.6 Ethical Considerations

Following immunisation with type II collagen in CFA, mice will develop arthritis of varying degrees of severity and chronicity. In addition, novel treatment regimes may produce unexpected adverse effects. Hence, it is important that the potential benefits are weighed carefully against the cost to the animals.

It is also imperative that animals are monitored on a daily basis for signs of ill health or distress. Clearly defined humane endpoints should be strictly enforced. For example, any mouse showing severe

and sustained paw-swelling should be humanely killed, as should any mouse which has lost 20% or more of its body weight. Any mouse with severe lameness, dyspnea, ruffled fur, weakness, dehydration, or showing a hunched appearance or showing blistering at the injection site should be humanely killed. In addition, the duration of experiments involving arthritic animals and the numbers of mice used should be minimised, compatible with the aims of the study.

In short, the principles of the three 'Rs' should be implemented, i.e. the refinement of scientific procedures, reduction in numbers of animals used, and their replacement wherever possible.

Acknowledgements. This work was supported by the Arthritis Research Campaign of Great Britain.

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6 Animal Models of Inflammatory Bowel Diseases

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Here we discuss animal models of chronic intestinal inflammation and their relevance and implications for inflammatory bowel diseases (IBD) in humans. Accordingly, we will characterize immunological features in human IBD first and then focus on similarities in mouse models of experimental colitis. Special reference will be made to cytokines and cytokine signaling events in intestinal inflammation.

6.1 Inflammatory Bowel Diseases

Alterations of the mucosal immune system have been shown to contribute to the pathogenesis of IBD in humans that are defined as inflammations of the gastrointestinal tract not due to specific patho-

gens (Podolsky 1991; Schunk et al. 2000; Strober and Neurath 1995; Strober et al. 1997; Neurath et al. 1996, 2002a). IBD comprise two major forms: ulcerative colitis (UC) and Crohn's disease (CD). These diseases can be discriminated by clinical and immunological features. Whereas UC is characterized by a more superficial inflammation limited to the large bowel, CD manifests as a transmural granulomatous inflammation that can occur anywhere in the alimentary canal. Recent evidence by various groups suggests a fundamental derangement of mucosal immunoregulation in patients with IBD (Neurath et al. 2001; Elson 2002). One aspect of this altered immunoregulation is a hyperresponsiveness to mucosal antigens caused by a dysregulated immune response to otherwise less immunogenic or harmless products of the intestinal flora. For instance, it was shown that normal mucosal T cells mount far lower in vitro proliferative responses to common microbial antigens than do peripheral T cells, whereas IBD mucosal T-cell responses are equal to that of peripheral T cells (Duchmann and Zeitz 1998). Furthermore, clinical observations show that IBD manifests itself most frequently in the terminal ileum and colon (i.e., those sites with the highest bacterial concentrations in the intestine), that CD relapses are prevented by diversion of fecal stream, and that increases in gut permeability precede acute flares in CD (Duchmann and Zeitz 1998). Therefore, the emerging concept is that bacterial antigens from the commensal flora drive chronic intestinal inflammation in IBD patients.

Bacterial antigens cause an unbalanced activation of the mucosal immune system in IBD patients, thereby leading to chronic intestinal inflammation. In the normal human gut, the intestinal immune system comprises antigen presenting cells (APCs), epithelial cells, intraepithelial lymphocytes, and lamina propria (LP) lymphocytes. The special activation and differentiation status of LP T cells is documented by the finding that proliferative capacities and cytokine profiles of LP lymphocytes differ significantly from those of the peripheral blood. For instance, LP T cells are significantly more responsive to CD2 ligation than peripheral blood lymphocytes but produce less cytokines in response to TCR/CD3 receptor stimulation. Furthermore, with regard to their proliferative capacities it has been shown that LP T cells proliferate poorly in response to TCR/CD3 receptor stimulation compared with peripheral T cells, and somewhat

better but still poorly in response to CD2/CD28 pathway stimulation. In summary, normal human LP T cells are poor proliferators but vigorous lymphokine producers when stimulated via the CD2/CD28 accessory stimulation pathway.

There is now increasing evidence that these LP T cells play a central role in the pathogenesis of IBD (Duchmann and Zeitz 1998). This is underlined by the clinical finding that CD in HIV-infected patients is ameliorated or abrogated when $CD4^+$ T cell numbers in these patients drop. In addition, there is strong evidence that LP $CD4^+$ T cells mediate chronic intestinal inflammation in several animal models of IBD, as discussed below. The key role of LP T cells in IBD is most likely based on their altered activation and differentiation status with consecutive pathologic cytokine responses and macrophage activation. This hypothesis is supported by the clinical effects of monoclonal anti-$CD4^+$ antibodies in patients with CD, and by the observation that reduction of T helper cell numbers by a concomitant HIV infection suppresses disease activity in CD (Duchmann and Zeitz 1998; Emmrich et al. 1991). Consistent with these observations, bone marrow transplantation because of concomitant leukemia can induce long-term remissions in CD, suggesting that the host immune dysregulation plays a role in the perpetuation of this disease that can be corrected by hematopoietic cell transplantation. Finally, antibodies to TNF that are successfully used in treating CD patients induce rapid mucosal T-cell apoptosis (programmed cell death) within 2 days, indicating that the therapeutic efficacy of these antibodies could be due to the elimination of T effector cells in the gut (Hove et al. 2002). Finally, LP T lymphocytes are also a major source for cytokine production in patients with IBD.

6.2 Cytokine Profiles of Lamina Propria T Cells in IBD

Based on the above data there is considerable interest in understanding the function of IBD T cells by determining their capacity to produce various regulatory lymphokines. Since accessory pathway stimulation is important for T-cell activation in mucosal immune responses, it was of interest to determine cytokine production and T-cell proliferation in IBD T lymphocytes under accessory pathway

stimulation. It was found that whereas IBD lamina propria T cells both from UC and CD patients gave lower proliferative responses than did control lamina propria T cells via the TCR/CD3 signaling pathway, proliferative responses via the CD2/CD28 pathway were relatively preserved. The production of IFN-γ, the signature cytokine of Th1 T cells, has also been measured in IBD lamina propria T cells after stimulation with anti-CD2 plus anti-CD28 antibodies. When purified lamina propria T cells from patients with CD were stimulated via accessory signaling pathways, IFN-γ production was significantly increased compared to T cells from control patients, whereas IL-2 production was reduced (Fuss et al. 1996). Thus this lymphokine profile may represent a modified Th1 cytokine profile present in patients with CD. In contrast to CD, few data are available on cytokine production by lamina propria T cells in patients with UC. However, Fuss et al. (1996) showed that purified lamina propria $CD4^+$ T lymphocytes from UC patients produce equal amounts of the Th1 cytokine IFN-γ but large amounts of the Th2 cytokine IL-5 upon stimulation with anti-CD2/CD28 antibodies compared to T cells from control patients. Interestingly, the production of another Th2 cytokine, IL-4, was reduced by UC lamina propria T cells compared to T cells from control patients, suggesting the potential existence of a modified Th2 cytokine profile in UC patients (Fuss et al. 1996). This cytokine profile may at least partially account for the observed activation of B cells and the production of autoantibodies in UC patients, since Th2 cytokines are known to activate humoral immune responses. Taken together, the current data are consistent with a model in which co-stimulated T cells from CD patients produce a modified Th1 cytokine profile (high IFN-γ, low IL-2), whereas T cells from UC patients exhibit a modified Th2 cytokine profile (high IL-5, low IL-4). Consistent with this concept, Fuss et al. recently showed that LP T cells from UC but not CD patients produced large amounts of IL-13.

6.3 Cytokine Signaling in IBD T Cells

The data summarized above suggest that cytokines play a key role in the pathogenesis of IBD in humans. This concept is underlined by

the therapeutic efficacy of several anti-cytokine strategies in IBD patients. Regarding CD, recent studies have focused on understanding the molecular pathways leading to Th1 T-cell differentiation in this disease. The cytokine IL-12 heterodimer (p35/p40) produced by DCs or recently immigrated macrophages induces Th1 T-cell differentiation; this function requires the intracellular activation and nuclear translocation of the transcription factor Stat4 (signal transducer and activator of transcription 4) (Neurath et al. 2002a). However, the importance of the IL-12/Stat4 signaling pathway for CD pathogenesis has been highlighted by recent studies showing that IL-12 p35/p40 heterodimer secretion is increased in active CD, that T cells in this disease express large amounts of IL-12 receptor $\beta 2$ chain, and that Stat4 is activated in mucosal T cells from active CD (Monteleone et al. 1997). Finally, IL-12 has been suggested to cause intestinal tissue injury, suggesting that it may play an active role in tissue destruction in CD patients (Monteleone et al. 1999).

In addition to Stat4, the IFN-γ inducible transcription factor Stat1 has been shown to be involved in Th1 T-cell differentiation. IFN-γ signaling via Stat1 leads to activation of the transcription factor T-bet, a recently cloned member of the T-box protein family expressed in T lymphocytes (Szabo et al. 2000, 2002). Indeed, in CD T-bet is strongly upregulated in LP T cells, showing that these cells exhibit classical features of Th1 T cells (Neurath et al. 2002b). In contrast to CD, few data are available on cytokine signaling and T-cell differentiation in patients with UC. However, it appears that there is no increase in IL-12 production by LP cells in UC patients compared to control patients, while the expression of a potentially IL-12-antagonizing cytokine, denoted EBI3, is increased (Christ et al. 1998).

6.4 Animal Models of IBD

Various animal models of chronic intestinal inflammation have been established recently which will likely provide new insights into the pathogenesis and potential treatment regimens of IBD (Elson et al. 1995; Wirtz and Neurath 2000). Although none of these models truly represents human IBD, these models mimic key clinical, histological, and immunological findings in IBD patients. There have

been more than 70 models described for experimental colitis in rodents, and it is beyond the scope of the present review to discuss all of these models in detail. However, these models generally can be classified into four different groups. The first group of models consists of spontaneously occurring colitis forms such as the cotton top tamarin model and the C_3H Bir mouse (Wirtz and Neurath 2000). The second group consists of inducible models in normal healthy mice such as the DSS colitis model, the TNBS model, and the oxazolone colitis model (Neurath et al. 1995; Boirivant et al. 1998). The third and largest group comprises genetically modified animals such as transgenic mice or rats, mice in which certain genes have been inactivated by homologous recombination, and mice with targeted deletion of genes in certain cell populations only (conditional knockout mice). These models include rats carrying transgenes for HLA-B27 and β2-microglobulin, T-cell reconstituted Tge26 mice transgenic for the human CD3e gene, mice carrying a dominant negative N-cadherin mutant, and mice in which the genes for IL-2, IL-10, Gai2, and the α- or β-chain of the T-cell receptor have been inactivated by homologous recombination (Kuhn et al. 1993; Mizoguchi et al. 1997, 1999). The fourth group consists of adoptive transfer models with either $CD4^+$ or $CD8^+$ T cells (Wirtz and Neurath 2000). The most commonly used model in this group is an adoptive transfer of normal CD45RBhi or $CD62L^+$ T cells from healthy BALB/c donor mice into C.B.-17 SCID or RAG knockout mice (Powrie et al. 1994, 1996; Atreya et al. 2000) that results in colitis in the reconstituted mice within 4–8 weeks (Table 1).

Table 1. Models of experimental colitis in rodents

Group 1	Group 2	Group 3	Group 4
Spontaneous	Inducible	Transfer	Genetically
C_3H Bir mouse	DSS	CD45Rbhigh	IL-2 KO mouse
Cotton top tamarin	TNBS	$CD62L^+$ $CD4^+$	IL-10 KO mouse
	Oxazolone	hsp60 $CD8^+$	STAT4 transgenic

6.5 Lessons from the Animal Models

As discussed above, the IL-12/Stat-4 signaling pathway plays an important role in Th1-mediated intestinal inflammation in humans. This observation has been strongly supported by studies in mice with experimental colitis (Fig. 1). For instance, Th1-mediated models of chronic intestinal inflammation are associated with increased production of IL-12, and neutralizing antibodies to IL-12 p40 prevent the development of Th1-mediated colitis, but they also suppress established Th1-mediated chronic intestinal inflammation (Neurath et al. 1995; Simpson et al. 1998). This effect is mediated by the prevention of Th1 T-cell development and the induction of Fas-mediated T-cell apoptosis in the inflamed gut (Fuss et al. 1999). Furthermore, Stat4-deficient T cells fail to induce Th1-mediated colitis in an adoptive transfer system in RAG knockout mice, whereas

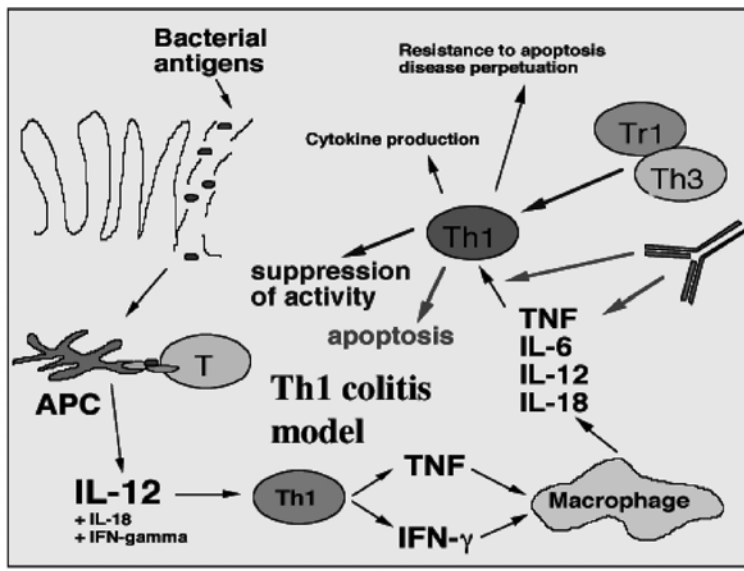

Fig. 1. Model of Th1-mediated experimental colitis driven by bacterial antigens from the lumen and IL-12 production. (Modified from Neurath et al. 2002)

Stat4 transgenic mice (under control of the CMV promoter) develop Th1-mediated colitis upon immunization with DNP-KLH (Simpson et al. 1998; Wirtz et al. 1999). In addition to IL-12, however, the cytokine IL-23 (p19/p40) produced by APCs was shown to activate Stat4 in T lymphocytes (Oppmann et al. 2000), raising the possibility that the observed Stat4 activation in colitis could be at least partially due to IL-23. Furthermore, the neutralizing antibodies to IL-12 used in the above studies on colitis activity appear to suppress the function of both IL-12 and IL-23, indicating that further studies are necessary to delineate the function of these cytokines individually. In this context, high levels of IL-23 production by LP dendritic cells were recently described in the terminal ileum of mice (Oppmann et al. 2000), suggesting that this end-stage effector cytokine might be very important in driving memory T-cell activation in the small bowel.

Additional studies have been done on the role of T-bet in experimental colitis (Neurath et al. 2002b). These studies have shown that T-bet is an important factor for Th1 T-cell development and the regulation of T-cell effector function. Specifically, in murine models of colitis it was found that T-bet-deficient T cells fail to induce Th1-mediated experimental colitis in an adoptive T-cell transfer system. This observation cannot be attributed to the effects of T-bet on IFN-γ production in $CD4^+$ T cells, since $CD4^+$ T cells from IFN-γ knockout mice are fully capable of inducing Th1-mediated colitis in an adoptive transfer system in RAG knockout mice. Instead these results suggest a more general role of T-bet in Th1 T-cell differentiation via regulation of IL-12 receptor $\beta 2$ chain expression. Finally, overexpression of T-bet in T cells was found to augment Th1-mediated colitis, suggesting that T-bet controls Th1-mediated mucosal immune responses.

A potential role for EBI3 in colitis is supported by the recent observation that EBI3-deficient mice show reduced Th2 cytokine responses and are protected from oxazolone-induced colitis, a colitis model mediated by iNK T cells producing IL-4 and IL-13. Furthermore, experimental colitis models support the notion that Th2 cytokines may play a key pathogenic role in regulating colitis activity in vivo. In fact, transfer of Th2 cells has recently been shown to cause colitis in an antigen-specific colitis model. Furthermore, antibodies

to IL-4 attenuate T cell-mediated colitis induced by the hapten reagent oxazolone (Boirivant et al. 1998). In addition, inactivation of the IL-4 but not the IFN-γ gene has been shown to suppress colitis activity in TCR knockout animals that normally display some features of human UC. Furthermore, removal of the cecal tip early in life prevents colitis in TCR knockout mice (Mizoguchi et al. 1996), suggesting that the immune system in this region initiates rather than perpetuates such colitis. This is reminiscent of the observed protective effect of early appendectomy on the development of colitis in UC patients.

There is some recent indication that the role of individual cytokines may vary during the course of experimental colitis. For instance, administration of recombinant IL-4 prevents Th1-mediated colitis in an adoptive transfer model but can augment established Th1-mediated colitis, suggesting that the effects of the Th2-type cytokine IL-4 may be dependent on the stage of the disease in T cell-mediated colitis. Consistently, Spencer and coworkers recently showed that colitis in young IL-10-deficient mice is mediated by IL-12-driven Th1 T cells, whereas later stages of disease appear to be mediated by the Th2-type cytokines IL-4 and IL-13 (Spencer et al. 2002). These findings support the notion that cytokine production and cytokine function may differ during different phases of colitis in vivo.

The above findings on transcriptional polarization of T cells not only give valuable new insights into the immunopathogenesis of IBD but also provide a rationale for selective targeting of signaling cascades in IBD.

6.6 Treatment of Experimental Colitis with Recombinant Cytokines or Anti-cytokine Antibodies

Administration of neutralizing antibodies to cytokines with assumed pathogenic roles is a commonly used approach for treatment of experimental colitis. For instance, IFN-γ and TNF were shown to be involved in the pathogenesis of colitis in CD45Rbhi $CD4^+$ T cell restored SCID mice, as disease was prevented by administration of antibodies to IFN-γ and significantly reduced in severity by treatment

with antibodies to TNF (Powrie et al. 1994). In this experimental model, IFN-γ may either act directly by causing damage to colonic epithelial cells or may act primarily by activating macrophages with subsequent production of inflammatory mediators, such as reactive oxygen and nitrogen intermediates, IL-1, IL-6, IL-8, IL-12, and TNF. Furthermore, it was shown that IL-12 is essential for disease induction in TNBS-induced colitis, as treatment with antibodies to IL-12 prevented disease induction. Furthermore, administration of anti-gp39 (CD40L) antibodies during the induction phase of Th1 responses in TNBS-colitis was associated with decreased IL-12 production, and prevented IFN-γ production by LP T cells and disease activity (Stüber et al. 1996). Conversely, injection of rIL-12 heterodimer reversed the effect of anti-gp39 treatment and resulted in severe disease activity. However, when anti-gp39 was given after disease was established, no effect on disease activity was observed, suggesting that CD40/CD40L interactions are crucial for the in vivo priming of TH1 T cells via the stimulation of IL-12 secretion by APCs in the early phase of colitis. In contrast to anti-gp39, antibodies to IL-12 were also highly effective in treating established chronic colitis in the murine TNBS model.

There is now overwhelming evidence that regulatory T-cell populations may prevent development of colitis by production of cytokines whose functions antagonize those of Th1 T cells. Among the regulatory subsets identified in the gut are Tr1 cells producing IL-10, Foxp3-expressing $CD4^+$ $CD25^+$ T cells, and TGF-β-producing Th3 cells. Thus, a second approach for treatment of intestinal inflammation consists of administration of cells producing anti-inflammatory cytokines or recombinant cytokines with assumed protective roles. For instance, administration of rIL-10 inhibited development of colitis in SCID mice reconstituted with CD45RBhi T cells. In addition to IL-10, TGF-β may also play a pivotal role in the regulation of inflammatory reactions of the intestine. TGF-β is a pluripotent cytokine known to be involved in immune and inflammatory cell processes. Of particular relevance is the fact that TGF-β is a potent inhibitor of T- and B-cell proliferation and down-regulates macrophage cytokine production. The importance of this inhibitory function is underscored by the finding that TGF-β1 null mice show multiorgan inflammation, excessive lymphocytic infiltrations, and early

death. TGF-β has also been shown to be induced upon oral exposure of various antigens and to be an important mediator of oral tolerance. Experimental studies showed that protection from CD45Rbhi $CD4^+$ T cell-induced colitis in SCID mice by co-transfer of CD45RBlo $CD4^+$ T cell population could be reversed by adding antibodies to TGF-β, suggesting a key negative role for this cytokine in disease induction (Powrie et al. 1996; Powrie and Maloy 2003). These data on the functional importance of TGF-β are further supported by an oral tolerance model in mice with TNBS-colitis, induced by oral administration of haptenized colonic proteins (HCPs) prior to rectal administration of TNBS that resulted in suppression of TNBS-induced disease. This suppression (oral tolerance) appears to be due to the generation of mucosal T cells producing TGF-β. These data suggested that regulation of IL-12-driven Th1-responses and TGF-β levels may have relevance for treatment of human inflammatory bowel diseases.

6.7 Therapeutic Modulation of Cytokines in IBD

The above data have encouraged clinical studies to determine whether selective regulation of cytokine levels could be an additional therapeutic option to treat patients with IBD and whether such regulation could be better than treatment with immunosuppressive drugs. Thus, several recent studies have attempted to treat patients with IBD by administration of recombinant cytokines with assumed protective functions or by administration of antibodies to cytokines with assumed pathogenic roles. For instance, systemic administration of antibodies to TNF-α to patients with CD resulted in reduction of disease activity or even remission of disease in about 65% of the patients (Targan et al. 1997). Furthermore, many additional clinical therapies based on modulation of cytokine function are now in phase II trials (e.g., anti-IFN-γ, anti-IL-12, anti-IL6R antibodies) that are based on initial work in experimental models of colitis.

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7 Murine Models of Atopic Dermatitis

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7.1 Introduction

Atopic dermatitis (AD) is an inflammatory skin disease characterized by childhood onset, severe pruritus, and a chronically relapsing course. Epidemiologic data indicate that the incidence of AD has significantly increased over the last few years, today reaching a prevalence of more than 20% in children and 1%–3% in adults (Schultz et al. 1996). Despite its widespread occurrence, the underlying genetic and pathophysiological mechanisms of this complex disease are not precisely understood. During the last few years, however, considerable progress has been made indicating that immunological abnormalities, skin barrier dysfunctions, and environmental factors crucially contribute to the onset as well as exacerbation of

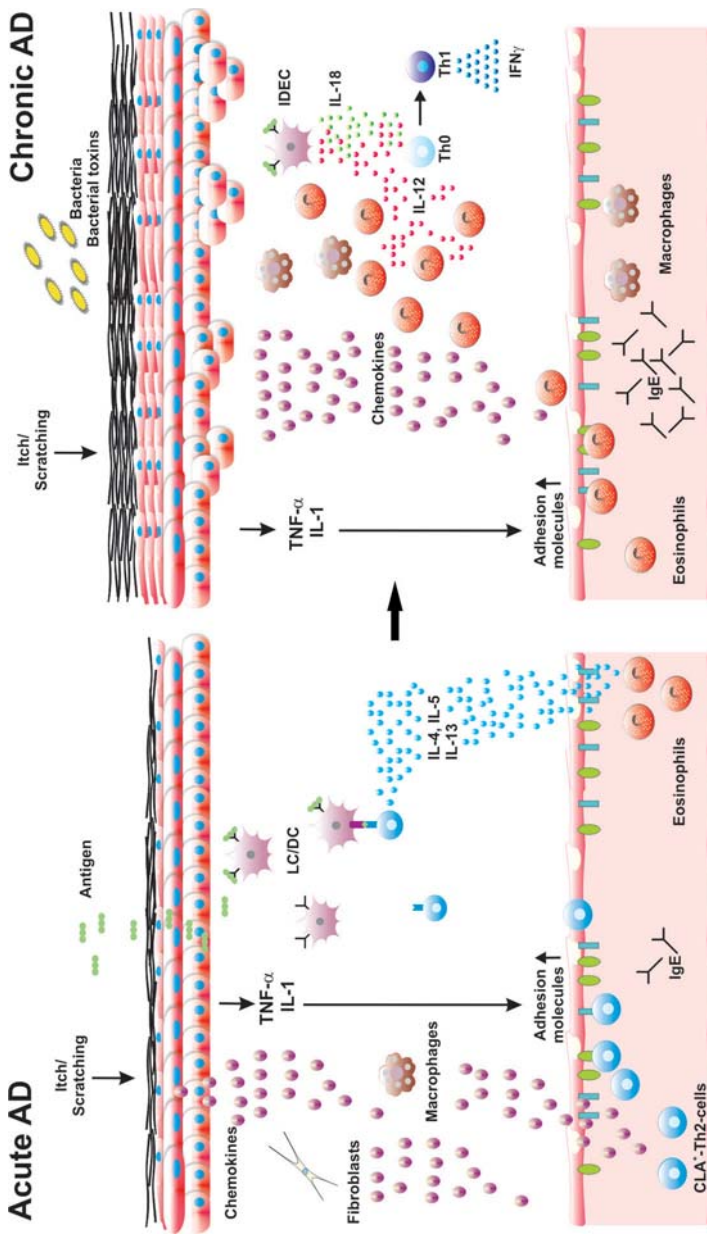

Fig. 1. Major pathways in AD

AD (Leung 2000). Accordingly, different underlying disorders may ultimately result in the development of the characteristic symptoms of AD. Today there is evidence for at least two distinct forms of AD. The "extrinsic" form involves approximately 70% of the patients and is characterized by an immunodeviation with memory T2 lymphocytes expressing the skin homing receptor, cutaneous lymphocyte-associated antigen (CLA) (Akdis and Akdis 2003). The increased production of T2 cytokines such as interleukin (IL)-4, IL-13, and IL-5 (Van Reijsen et al. 1992) subsequently is responsible for the increased production of IgE and eosinophilia. The "intrinsic" form of AD, which involves approximately 30% of the patients, primarily is not associated with an increased IgE production and may result from an epidermal barrier dysfunction (Johansson et al. 2001; Schultz et al. 1996). Moreover, the exacerbation and the course of AD are under the strong influence of several trigger factors such as irritants (Ruzicka 1998), allergens (Ruzicka 1998), inhalants (Scalabrin et al. 1999; Tan et al. 1996; Tupker et al. 1996), food (Eigenmann et al. 1998; Lever et al. 1998; Li et al. 2001; Sampson 1999, 2001; Van Reijsen et al. 1998), autoallergens (Kinaciyan et al. 2002; Valenta et al. 1998, 2000), infectious agents (Breuer et al. 2000; Bunikowski et al. 2000; Cho et al. 2001; Kieffer et al. 1990; Leung et al. 1993, 1998; Leyden et al. 1974; Remitz et al. 2001; Ruzicka 1998), and neuroendocrine factors (Ansel et al. 1996; Ruzicka 1998) (Fig. 1).

Animal models, in particular genetically modified mouse strains, have been very helpful in the past, contributing to a better understanding of pathomechanisms relevant to the development of many inflammatory as well as allergic and autoimmune diseases. Because of the complex and variable mechanisms ultimately being involved in the pathogenesis of AD, it is difficult if not impossible to create an animal model which displays all of the characteristic features of human AD. However, several murine models that exhibit some AD-like symptoms but lack others have been developed and analyzed. In some models such as inbred strains, AD-like features occur spontaneously. In others they develop upon genetic modification or can be induced by sensitization in healthy mice. Therefore, this report briefly will describe and evaluate some of these models concerning their suitability for investigating mechanisms involved in the pathogenesis of AD (Table 1).

Table 1. Major murine models of AD: Advantages and disadvantages

Type of model	Examples	Advantages	Disadvantages
Spontaneous mutation	NC/Nga	Stable and reproducible	Type and number of underlying mutations not known
	NOA	Allow insights in genetic changes	
	DS-Nh	Usually develop typical features of AD	
Genetically modified mice	Transgenics: IL-4, CD40L	Tg animals display typical features of AD	Other (autoimmune-) diseases
	Knockouts: IL-4, IL-5, IL-10; IFNγ, CCR-3, TCR, relB	Investigation of pathogenetic relevance of single parameters	Direct vs. indirect effects
	Mutations: unmodulated	Combinations of parameters	Late onset of symptoms Not all animals affected
Xenotransplantation	Hu-SCID	Specific investigation of immunopathogenetically relevant parameters	Few or no typical clinical features
		Combination of different parameters	Does not reflect complex immunopathology of AD
		Humanized system	
Local or systemic sensitization	Epicutaneous or i.p.: OVA mite antigen, Glu-*S*-transferase	Allows using standard strains of mice	Only local, not "systemic" effects
	Oral: cow milk, peanut	Can be combined with other approaches	
		All animals exhibit the AD phenotype	

Table 1 (continued)

Type of model	Examples	Advantages	Disadvantages
Combined models	Transgenics + sensitization		
	Knockouts + sensitization		
	Genetic modification of inbred strain		
	Sensitization of inbred strain		

7.2 AD-Like Phenotypes in Mice with Spontaneous Mutations

In mice and humans alike, symptoms can arise through spontaneous genetic mutation, for example, due to environmental influences or due to DNA replication or repair defects. Since the genetic basis underlying AD involves a considerable number of genes, there is a good chance for spontaneous mutations to occur that induce AD-like symptoms. Indeed, a few mouse strains have been established which develop human AD-like symptoms. These include the NOA mouse (Natori et al. 1999; Watanabe et al. 1999, 2001), the Ds-Nh mouse (Yoshioka et al. 2003), and the NC/Nga mouse (Suto et al. 1999; Vestergaard et al. 2000), which are the best described and characterized among the mouse models.

The NC/Nga mouse was derived from Japanese fancy mice in 1955 (Matsuda et al. 1997; Tsudzuki et al. 1997), and in addition to having biological characteristics such as high susceptibility to x-irradiation and anaphylactic shock from ovalbumin occasionally display a spontaneous dermatitis shortly before or after weaning. The reason for the development of dermatitis was not investigated until 1997, when a first paper linking spontaneous dermatitis to human AD was published (Matsuda et al. 1997; Tsudzuki et al. 1997). Animals kept under specific pathogen-free (SPF) conditions did not develop dermatitis, whereas animals bred under conventional conditions, i.e., in unfiltered air, from the age of 8 weeks on began to develop signs of dermatitis on ears, face, nose, neck, and dorsal skin which resemble the distribution pattern observed in human AD (Mihm, Jr. et al. 1976; Piloto et al. 1990). All animals developed skin lesions, beginning with itching, erythema, and hemorrhage, followed by superfi-

cial erosion, deep excoriation, scaling, dryness of the skin, and ultimately retarded growth. None of these symptoms occurred in normal Balb/c mice which were kept under identical conditions as the NC/Nga mice, strongly suggesting that an intrinsic factor is the cause of dermatitis. Moreover, upon histopathological examination, striking similarities with human AD were observed. Before the onset of clinical symptoms at the age of 7 weeks in the dermis, an increased number of mildly degranulated mast cells and an infiltration consisting of eosinophils and some mononuclear cells could be detected. Seventeen-week-old mice with severe skin lesions showed thickening of the skin due to hyperplasia with elongated rete ridges and prominent hyperkeratosis as well as parakeratosis. The dermal infiltration consisted of a high number of mast cells, and slightly fewer degranulated eosinophils. Deposits of eosinophilic material, presumably major basic protein and eosinophilic cationic protein, were seen throughout the lesions. With immunohistochemistry, elevated numbers of $CD4^+$ T-cells as well as Mac-1^+ and $F4/80^+$ macrophages were detected, while only a few $CD8^+$ cells were present. As is the case in human AD, plasma IgE and IgG levels were elevated and started to rise between weeks 8 and 12 and further increased until week 17. The rise of Ig levels also was found to correlate with the severity of clinical symptoms. Immunostaining further revealed that in the skin of conventionally raised NC/Nga mice, in contrast to SPF NC/Nga animals, an elevated number of $CD4^+$ T-cells ($\sim$10%) and more than one-third of mast cells stained for IL-4, with more than 25% of mast cells also staining for IL-5. In contrast, almost no $CD4^+$ T cells or mast cells stained for interferon (IFN)γ. These data indicate that the immunopathological findings in the NC/Nga mouse are similar to those found in human AD and apparently also are skewed towards a T2-type immune reaction.

An important chemokine released by Langerhans cells (LCs) is TARC, which is a ligand of CCR4 and a chemoattractant for T2-type T cells (Hashimoto et al. 1999; Imai et al. 1997, 1999; Sallusto et al. 1999). The expression of TARC by LCs is strongly up-regulated during maturation and is increased by tumor necrosis factor (TNF)α and IL-4, but decreased by IFNγ (Xiao et al. 2003). It has been shown that TARC is overexpressed in lesional skin of NC/Nga mice (Vestergaard et al. 1999). Thus TARC may be an amplifier in

AD, since its expression is up-regulated by cytokines released from T2 cells already present and upon release serves as an attractant for even more additional T2 cells.

In order to further prove that the observed spontaneous dermatitis in NC/Nga mice is equivalent to the human disease, the skin barrier function of NC/Nga mice was investigated (Aioi et al. 2001). Accordingly, in 8-week-old NC/Nga mice which were raised under normal conditions, a significantly decreased water retention capacity as well as a higher transepidermal water loss was observed compared to SPF NC/Nga mice or Balb/c mice. Moreover, the ceramide content of the skin was found to be decreased, and predominantly involved a strong reduction of ceramide 1 and 3 levels. These findings were also associated with an increased ceramide-metabolizing enzyme activity and a concurrent decrease in the ceramide-generating enzyme sphingomyelase.

In conclusion, the advantages of this model are that all animals develop human-like AD under normal conditions, and no additional manipulation such as sensitization is required. Moreover, it allows for the investigation of genetic changes in AD and also can easily be combined with other models. One of the disadvantages, however, is that AD-like disease only develops in an "uncontrolled" environment. On the other hand, upon "controlled" epicutaneous or intradermal application of extracts from house dust mites, NC/Nga mice also developed the same AD-like disease (Matsuoka et al. 2003; Sasakawa et al. 2001).

7.3 SCID-hu Mice

Mice with severe combined immunodeficiency (SCID-mice) completely lack mature B and T cells and thus are incapable of mounting an adaptive immune response, while their innate immune response remains intact (Bosma et al. 1983; Kirchgessner et al. 1995). These mice are therefore capable of accepting immunocompetent cells and various tissues from other species, enabling the investigation of a variety of immunological disorders. Chimeric hu-SCID mice are used to study selected aspects of AD and have been evaluated for this purpose in several different models.

Homing of T2 cells to the skin is one of the major features in the pathogenesis of AD. Migration of T2 cells to the skin depends on the one hand on the interaction between CCR4 expressed by T2 cells and CCR4 ligands expressed by LCs and endothelial cells, and on the other hand on the interaction between cutaneous lymphocyte antigen (CLA) on the surface of T cells and E-selectin expressed by endothelial cells. The importance of these interactions during the pathogenesis of AD has been investigated using chimeric SCID-hu mice, transplanted with patches of human skin and reconstituted with Th-cells from human AD skin lesions (Biedermann et al. 2002; Carballido et al. 2003). In these experiments, $CD4^+$ T cells were isolated from skin biopsies of dust mite antigen-challenged patients, and cloned T2 cell lines were established. All isolated $CD4^+$ cells expressed CLA, and the studied clones were selected according to their expression profile of IL-4, IL-5, and IL-10 and the absence of IL-2 and IFNγ expression. They also expressed high levels of CCR4 and migrated in vitro toward CCR4 ligand gradients, with CCL22 eliciting stronger migratory responses than CCL17 (TARC). These data were confirmed in SCID-hu mice in vivo. Two patches of human skin were transplanted onto SCID mice, and after allowing regeneration and rebuilding of vascular structures, the animals were injected with human T2 cells. When CCL22 or CCL17 was injected into the human skin patches, the T2 cells migrated toward the patches. This migration pattern was shown to be selective, since the adoptively transferred cells did not migrate towards skin patches injected with PBS or chemokine ligands for receptors not expressed by T2 cells, such as CXCR3. To further investigate the interaction between CLA and E-selectin on endothelial cells, one of the T2 clones was subcloned into one clone positive for CLA and another one negative for CLA. These subclones in vitro remained equal in their responsiveness to CCL22, but in vivo only the CLA^+ subclone was capable of migrating into the human skin patches. Similar data were obtained using an anti-E-selectin antibody which blocked access of CLA to E-selectin and greatly reduced migration of T2 cells into the human skin patches. Similarly, antibodies to LFA-1, a ligand of ICAM-1, blocked the migration of T2 cells.

The SCID-hu mouse thus is an excellent tool for investigating trafficking patterns in AD and will also serve as a well suited model

to evaluate the anti-inflammatory efficacy of novel compounds designed for the treatment of AD.

7.4 Models Targeting Cytokines and Chemokines

Due to their pro-inflammatory as well as immunomodulating properties and expression patterns in AD skin, several cytokines and chemokines have been related to the generation of an AD-like phenotype. Among these are IL-4, IL-5, IL-13, IL-12, IFNγ, IL-1α, and TNF-α, as well as IL-10, IL-16, and the chemokines RANTES, MCP-4, Eotaxin, TARC, and LARC (Jeong et al. 2003; Nomura et al. 2003 a, b; Schon et al. 2003; Wohlfahrt et al. 2003). Therefore, several mouse models with either a genetic deletion or overexpression of one of these mediators have been investigated for the development of AD-like immunopathological as well as clinical findings.

7.4.1 IL-4

The classical T2 cytokine IL-4 was originally termed B-cell growth factor. It induces the proliferation of activated mature T cells, enhances T-cell cytotoxic properties, but suppresses cytokine formation by T1 cells, and thereby decreases delayed-type hypersensitivity responses. On B cells, IL-4 stimulates major histocompatibility complex (MHC) class II expression, and the expression of the low-affinity IgE receptor (CD23). IgE synthesis is regulated by IL-4 together with IL-13, and is inhibited by IFNγ and transforming growth factor (TGF)-β. Cytokines such as IL-2, IL-5, IL-6, and IL-9 synergize with IL-4 and IL-13 to enhance IgE production. On macrophages, however, IL-4 decreases CD23 expression. Mast-cell growth is stimulated by IL-4, as is the growth of precursor hematopoietic cells, both directly and indirectly by stimulating G-CSF and M-CSF production in monocytes. IL-4 is important for the differentiation towards a T2 T-cell subtype. In contrast, cytokines such as IL-12, IL-18, and IL-23 inhibit the differentiation of IL-4-producing T-cells. Although IL-4 is generated in high amounts by T cells, there is also evidence that IL-4 is released in the skin by keratinocytes or mast

cells (Brown and Hural 1997; Chomarat and Banchereau 1998). The IL-4 receptor was detected on T and B cells, macrophages, dendritic cells, mast cells, fibroblasts, and keratinocytes (Chomarat and Banchereau 1997). Accordingly, IL-4 turned out to be an essential mediator for the differentiation of dendritic cells (Gluckman et al. 1997; Zou and Tam 2002). In vivo, IL-4 was found to mediate immunodeviation towards T2, and therefore in preliminary studies has been used successfully for the treatment of T1-mediated skin diseases such as psoriasis (Martin 2003). Due to its role as mediator in T2-mediated diseases such as AD, several transgenic and knockout mouse strains have been generated to further elucidate the relevance of IL-4 in these diseases.

All of the viable IL-4 transgenic strains exhibit AD-like symptoms, although the array of noticeable alterations differs among the various strains. Accordingly, several mice were generated to express IL-4 under the control of a potent immunoglobulin promoter/enhancer construct (Tepper et al. 1990). One of these transgenic strains highly overexpressing IL-4 turned out to be lethal within 2 weeks after birth, while strains generated using an attenuated construct were viable. Using similar constructs, ubiquitously IL-4-overexpressing mice have been generated (Elbe-Burger et al. 2002). In addition, under the control of a K14 promoter construct, strains were produced which overexpress IL-4 only in keratinocytes (Chan et al. 2001).

Mice which systemically overexpress IL-4 exhibit no obvious clinical symptoms of skin inflammation, but their skin appears dry and scaly (Elbe-Burger et al. 2002). Upon histological examination, acanthosis and hyperkeratosis as well as the deposition of collagenous materials in the dermis were observed. While an increased number of mast cells were found in the dermis, no other inflammatory cells accumulated in the skin. Moreover, in some mice almost no dermal adipocytes were present, and in a number of mice the dermal fat tissue vanished completely, a feature that so far has not been observed in human AD.

The number of epidermal LCs in IL-4 transgenic mice is significantly increased, due to a reduced emigration of LCs, but not because of an increased proliferation or decreased apoptosis. It is not yet clear whether the diminished migratory capacity of LCs is a

direct effect or caused by an altered expression of cell adhesion molecules on endothelial cells. In addition, cytokines which promote Langerhans cell survival, maturation, and migration, such as TNF*α*, GM-CSF, and IL-1, were found to be elevated in the skin of these transgenic mice. The function of LCs was not significantly altered, since in comparison to wild-type LCs they were equally potent stimulators of naïve allogeneic T cells. However, the IL-4 transgenic mice-derived LCs were found to be more effective in processing and presenting antigens.

Mice which overexpress IL-4 only in keratinocytes also display an AD-like skin disease (Chan et al. 2001). Epidermal IL-4 overexpression resulted in the development of dermatitis in almost half of the mice within 12 months. In newborn mice, the appearance of the skin was normal but at the age of 4 months xerosis developed and pruritic, inflammatory skin lesions appeared. They occurred first on the ears and later extended to other areas such as neck, mouth, around the eyes, tail, and legs. Self-inflicted excoriation resulted in bacterial infection with *S. aureus* and *P. aeruginosa* in half of the affected mice. In contrast to the absence of clinical symptoms in the systemically IL-4-overexpressing mice, the skin lesions of the epidermal IL-4-overexpressing animals resembled much more those observed in human AD. Histology of unaffected skin was normal while spongiosis and acanthosis as well as an infiltration of mononuclear cells and degranulating mast cells was observed in early skin lesions. Thus, histopathological features in these mice more closely resemble those observed in human AD skin. T cells were detected in the dermis of early lesions as well as chronic lesions, with $CD4^+$ and $CD8^+$ T cells occurring in roughly the same numbers. Serum IgE and IgG1 levels were found to be elevated, whereas IgG2 amounts in serum were slightly reduced. The onset of dermatitis was linked to a concurrent elevation of IgE and IgG1 levels.

In a set of mouse strains producing different amounts of IL-4 constitutively under the control of an immunoglobulin promoter (Tepper et al. 1990), IL-4 was overexpressed constitutively in the thymus and peripheral T cells, resulting in thymic hypoplasia inversely proportional to the degree of thymic IL-4 expression. The number of $CD4^+$ $CD8^+$ T cells was severely reduced, while $CD8^+$ T cells were significantly elevated. In the strain with the highest thy-

mic expression of IL-4, in addition to the T-cell deficiency, a striking reduction of B cells was found. However, serum analysis revealed an increase in IgE and IgG1 levels. Animals from all strains except that with the lowest IL-4 expression exhibited swelling and erythema of the external eye in association with an inflammatory infiltration consisting of mononuclear cells, eosinophils, and increased numbers of mast cells. Moreover, the number of affected animals correlated to the degree of IL-4 expression in the T-cell compartment.

These findings indicate that inflammatory human AD-like skin lesions associated with a typical inflammatory infiltration can be elicited by the continuous expression of IL-4 in transgenic mice. However, though histological data for all IL-4 transgenic strains appear to be similar, the phenotypes of these mouse strains differ from each other. It may be assumed that the systemic expression of IL-4 in contrast to the expression in keratinocytes results in a number of additional effects at different loci, which in turn could affect the function of lymphocytes, endothelial cells, and other cell types ultimately masking to a certain extent the direct effects of IL-4 on skin physiology.

To further investigate the role of IL-4 in AD, IL-4 knockout mice sensitized locally with ovalbumin were used (Spergel et al. 1999). Upon sensitization the skin thickness in these IL-$4^{-/-}$ mice increased to the same extent as in wild-type animals. The cellular infiltrate in IL-$4^{-/-}$ mice in comparison to wild-type animals clearly was more pronounced, but contained fewer eosinophils, and the number of mast cells was not elevated. Significantly more lymphocytes were detected in the sensitized skin of IL-$4^{-/-}$ animals, with a significant shift towards $CD8^+$-cells. The up-regulation of chemokines such as MIP-2, MIP-1α, and RANTES mRNA in the skin of IL-$4^{-/-}$ mice appears to be responsible for the increased T-cell infiltration. Additionally, IL-2 and IFNγ expression at sites of sensitization in IL-$4^{-/-}$ animals were clearly higher than in wild-type animals, which may account for the more pronounced increase in $CD8^+$ T-cell numbers, at the same time skewing the immune response towards a T1 type. The reduced expression of another chemokine, eotaxin, at the site of sensitization may account for the lower number of eosinophils in the skin. Since IL-4 most importantly controls the antibody isotype

switching towards IgE, a depletion of IgE would be expected in IL-$4^{-/-}$ mice. Accordingly, only very low levels of IgE were detectable in the serum of IL-$4^{-/-}$ mice, with the presence of residual IgE probably due to the activity of IL-13. However, as is the case in human AD, the amounts of total and OVA-specific IgG2a in the serum of IL-$4^{-/-}$ mice were significantly enhanced, as seen in AD. Although IgE levels in IL-$4^{-/-}$ mice are very low, the cellular infiltrate is at least as pronounced as in wild-type animals, indicating that in these animals IgE apparently does not significantly contribute to the AD-like skin inflammation. This assumption was further supported by $IgE^{-/-}$ mice (Spergel et al. 1999), which upon OVA sensitization show a cytokine expression profile for IL-2, IL-4, IL-5, and IFNγ that is comparable to the pattern seen in wild-type mice. Moreover, the cellular composition of the inflammatory skin infiltration of $IgE^{-/-}$ animals is almost identical to the one found in wild-type mice.

The presence of elevated amounts of IL-4 in AD skin is associated with an elevation of serum IgE levels. However, observations made in experiments with OVA-sensitized NC/Nga mice suggest that additional changes in the IL-4 signaling pathway may contribute to the appearance of an AD phenotype (Matsumoto et al. 1999). Accordingly, OVA-immunized NC/Nga mice bred under specific pathogen-free (SPF) conditions produced significantly increased amounts of IgG1 and IgE compared to similarly treated Balb/c mice. Initial IgE serum levels in the two mouse strains were almost identical, but IgE production increased about 12-fold in Balb/c mice, whereas it was raised about 30-fold in NC/Nga mice. B cells of both mouse strains in vitro were capable of producing IgE in the presence of activated $CD4^+$ $CD40L^+$ T cells and IL-4. In these experiments, B cells from NC/Nga mice produced significantly higher levels of IgE, regardless of the source (NC/Nga or Balb/c) of the T cells present. This was attributable neither to an up-regulated CD40L expression by NC/Nga T cells nor to an increased CD40 expression by B cells from NC/Nga mice, but a higher sensitivity of NC/Nga B cells for IL-4. IL-4 signals are transmitted via the IL-4R complex, whose γ-chain is associated with JAK3, a member of the Janus kinase family of non-receptor protein kinases (Hanissian and Geha 1997; Ihle and Kerr 1995; Tortolani et al. 1995; Witthuhn et al. 1994). Upon ligand

binding, JAK3 becomes phosphorylated and thus transmits the incoming IL-4 signal. When the degree of phosphorylation of JAK3 in B cells from SPF NC/Nga mice, Balb/c mice, and NC/Nga mice grown under conventional conditions and showing signs of dermatitis were investigated, it turned out that B cells from NG/Nca mice with dermatitis contained constitutively phosphorylated JAK3, whereas cells from both of the other mice did not. Addition of CD40L and/or IL-4 further enhanced the phosphorylation of JAK3, the combined exposure being additive. Similar data were obtained for B cells from peripheral blood of human donors with or without AD. Thus IgE production in mice with AD-like disease is dependent on the presence of IL-4, and enhanced IgE levels in association with dermatitis may be attributable to alterations in IL-4 signaling. Interestingly, mutations that potentially enhance IL-4 signaling by interfering with SHP-1 binding, an attenuator/inhibitor of IL-4R signaling, have been detected in the human IL-4R α-chain (Hershey et al. 1997). These were more frequent in AD patients than in healthy donors.

The transcription factor STAT6 is crucial for the transduction of IL-4 signals (Quelle et al. 1995; Takeda et al. 1996). Deletion of STAT6 in mice inhibited IL-4-mediated events such as T2-differentiation and class switching towards IgE production (Kaplan et al. 1996; Shimoda et al. 1996). Moreover, STAT6$^{-/-}$ NC/Nga mice express neither IL-4, IL-5, IL-10, nor IgE and almost no IgG1 (Yagi et al. 2002). CD4^{+} T cells in STAT6$^{-/-}$ animals develop predominantly towards the IFNγ-producing T1 type, which was even more pronounced in animals developing dermatitis. Despite the lack of a T2 response and IgE production, these animals when kept under conventional conditions developed skin lesions to the same extent as did wild-type NC/Nga mice, suggesting that neither the T2 response nor IgE are necessary for the appearance of AD-like skin lesions. Skin inflammation in STAT6$^{-/-}$ mice did not differ significantly from those in normal NC/Nga animals, and also was characterized by high numbers of eosinophils and mast cells. Lymph nodes proximal to AD lesions were enlarged, and CD4^{+} T cells isolated from these lymph nodes produced elevated levels of IFNγ but no T2 cytokines. In addition, within the skin lesions neither IL-4 nor IL-5 was detectable, whereas IFNγ was present, as were IL-12 and IL-18, both of

which are inducers of IFNγ production (Grewe et al. 1998; Magram et al. 1996; Stoll et al. 1998; Yoshimoto et al. 1997). The activation of IL-18 is known to be mediated by Caspase-1 (Dinarello and Fantuzzi 2003), which also was found to be expressed in increased amounts in the skin of NC/Nga mice developing dermatitis. Furthermore, Caspase-1 transgenic mice spontaneously develop AD-like dermatitis and exhibit increased serum levels of IL-18 (Yamanaka et al. 2000). Moreover, in skin lesions of these animals, elevated amounts of IFNγ, eotaxin 2 and CCR3 were detected. Therefore, the production of these cytokines appears to be independent of STAT6 and IL-4 signaling, suggesting that the local cytokine environment appears to be responsible for the invasion of IFNγ-producing T cells, which in turn provides the basis for eosinophil infiltration and eczema formation.

7.4.2 IL-5

IL-5, which also belongs to the classic T2 cytokines, is a helper T-cell lymphokine which induces growth and differentiation of activated B cells, and is a key mediator of switching immunoglobulin class synthesis (Zabeau et al. 2003). It is the most important promoter of eosinophil formation and differentiation. Mature eosinophils are activated, and their survival is prolonged in parasitic infestations. IL-5 works synergistic with IL-3 and GM-CSF on eosinophils. IL-5 was found to be elevated in human AD skin as well as in skin of OVA-sensitized mice (Spergel et al. 1998). When the OVA sensitization model was transferred to IL-5$^{-/-}$ mice, no thickening of the epidermis or dermis at the site of sensitization occurred (Spergel et al. 1999). Virtually no eosinophils were present in the skin of sensitized IL-5$^{-/-}$ mice, while the expression of several chemokines in sensitized skin of wild-type and IL-5$^{-/-}$ mice was identical, and the numbers of CD4^{+} CD45^{+} T cells did not show significant differences. However, expression of IL-4 and IFNγ was found to be significantly increased. Deletion of IL-5 had no effect on the expression of IgE or IgG antibodies.

7.4.3 IL-10

IL-10 is synthesized by T1 as well as T2 lymphocytes, cytotoxic T cells, mast cells, B cells, and monocytes; the latter being the major source for IL-10 in humans (Moore et al. 1993, 2001). There is evidence for an IL-10 family of related cytokines, including IL-19, IL-20, IL-22, IL-24, and IL-26. The different receptors for the IL-10 family belong to the cytokine receptor family type 2, which mediate diverse biological effects through the activation of signal transducer and activator of transcription (STAT) factors. IL-10 diminishes IFNγ and IL-2 production by T1 cells, as well as IL-4 and IL-5 generation by T2 cells. Moreover, IL-10 inhibits the release of IL-1β, IL-6, IL-8, IL-12, and TNFα in monocytes, as well as IFNγ and TNFα in NK cells. In monocytes and dendritic cells, IL-10 also down-regulates the expression and release of MHC class II molecules, CD23, ICAM-I, and the accessory B7 molecule.

The anti-inflammatory effect of IL-10 is due to the inhibition of pro-inflammatory cytokines, chemokines, and chemokine receptors such as CXCR2. IL-10 inhibits eosinophil survival and IL-4-induced IgE synthesis, indicating an important role of this cytokine in allergic responses. In conclusion, IL-10 is an important cytokine for B-cell survival and differentiation, the modulation of antigen presentation, and the suppression of T1 as well as T2 cytokine production. It thus contributes to the inhibition of cellular and allergic immune responses while stimulating humoral and cytotoxic immune mechanisms.

The use of IL-10$^{-/-}$ mice revealed the importance of this mediator for the T2 response in allergic dermatitis (Laouini et al. 2003). The role of IL-10 probably is to steer immune reactions towards being protective and to keep them from becoming pathological. When IL10$^{-/-}$ mice were sensitized epicutaneously with OVA, no increase in the production of IL-5 or IL-4 was observed. Consequentially the expression of eotaxin also did not increase and the number of eosinophils in the dermal infiltrate was greatly reduced, while the number of mononuclear cells was elevated. Since IFNγ levels in OVA-sensitized skin of IL-10$^{-/-}$ mice were comparable to levels in wild-type animals, it appears that the lack of IL-10 results in a shift of the immune reaction from T2 to T1. This shift may be due to differ-

ential changes in antigen-presenting cells (APCs), as indicated by data from in vitro stimulation assays in which T cells from OVA TCR transgenic mice were incubated with APCs from either IL-$10^{-/-}$ or wild-type mice presenting their cognate peptide. In these experiments, T cells incubated with APCs originating from IL-$10^{-/-}$ mice produced significantly less IL-4 and significantly more IFNγ compared to T cells which were incubated with APCs from wild-type animals. Thus IL-10 appears to be important for the immune response to antigen and the development of eosinophilia in murine AD-like disease.

7.4.4 IFNγ

IFNγ is produced mostly by activated T cells and NK cells and has complex effects on immune as well as nonimmune cells. It plays important roles in immune surveillance and inflammation, usually in synergy with other cytokines, such as IL-1α and TNFα (Schroder et al. 2004). IFNγ is capable of inducing MHC expression in many tissues, making it particularly relevant to transplantation (Hidalgo and Halloran 2002). IFNγ is also important for the switch to IgG2a, and mice deficient in IFNγ cannot establish an IgG2a response (Bossie and Vitetta 1991; Graham et al. 1993). This also has been observed upon OVA sensitization of IFN$\gamma^{-/-}$ mice (Spergel et al. 1999). These IFN$\gamma^{-/-}$ mice produced significantly less IgG2a but higher levels of IgE. Deletion of IFNγ had no significant effect on skin thickness, chemokine expression, or volume and composition of the cellular infiltrate. Only IL-4 expression was found to be increased twofold at site of antigen sensitization.

NC/Nga mice display defective IFNγ and IL-18 production in response to bacterial toxins, due to a lack of Vβ8$^+$ T-cells and Vβ8$^+$ NK-cells (Habu et al. 2001). This deficiency probably skews the immune response towards the T2 type, which may increase the susceptibility of NC/Nga mice to dermatitis. Indeed, intraperitoneal application of IFNγ, as well as IL-12 or IL-18, which both are potent inducers of IFNγ production, delays the onset of dermatitis, with the effect of IFNγ being less pronounced than that of IL-12 or IL-18. Moreover, all three mediators effectively reduced serum levels of

IgE and IL-4 in NC/Nga mice. Thus the systemic deficiency of Vβ8$^+$-NKT cells ultimately may lead to an abnormal T2-dominant immune response combined with IgE overproduction and AD-like dermatitis. The reduced IFNγ expression in NC/Nga mice may also result in increased levels of the chemokine "thymus and activation-regulated chemokine" (TARC, CCL17) in the skin lesions (Vestergaard et al. 1999). TARC is a ligand for CCR4 (Imai et al. 1997, 1999) and recruits cells expressing these receptors, such as T2 cells, to the skin. Moreover, TARC is expressed by epidermal Langerhans cells and was found to increase upon maturation. An elevation of TARC levels and CCR4 expression in peripheral blood CD4$^+$ T-cells appears to be closely related to AD-like disease activity in NC/Nga mice. TARC expression by LCs is up-regulated by TNFα and IL-4 and down-regulated by IFNγ (Xiao et al. 2003). The local cytokine milieu in AD lesions thus may result in increased TARC production, which in turn results in the migration of additional T2 cells to the skin.

Another potentially crucial protein in AD appears to be the chemokine receptor CCR3, which is expressed by eosinophils (Heath et al. 1997), mast cells, and Th2 cells (Ochi et al. 1999; Sallusto et al. 1997) and whose major ligands are eotaxin, eotaxin-2 and -3, MCP-2, -3, -4, and RANTES (Kaplan 2001). In the skin of OVA-sensitized CCR3$^{-/-}$ mice as well as in their bronchoalveolar fluid, almost no eosinophils were detectable, while the blood eosinophil counts were normal (Ma et al. 2002). Moreover, no major basic protein, which is released by eosinophils and remains detectable even after eosinophil apoptosis, could be found in the sensitized skin of CCR3$^{-/-}$ mice, indicating that neither apoptosis nor reduced release of eosinophils from the bone marrow was responsible for lack of eosinophils in the skin. Mast cell numbers as well as the number of mononuclear cells in the infiltrate in sensitized skin of CCR3$^{-/-}$ animals were comparable to those in wild-type mice. Mononuclear cells predominantly consisted of CD4$^+$ T cells and macrophages. IL-4, IL-5, and IFNγ levels also were not altered in comparison to sensitized wild-type mice. The detection of elevated levels of the T2 cytokine IL-4 indicates that the migration of T2 cells into the skin, which in contrast to T1 cells normally express CCR3, was not affected by the deletion of CCR3. CCR3$^{-/-}$ mice altogether mount a

normal T2 reaction in response to OVA sensitization, which also includes the production of elevated levels of IgE and OVA-specific IgE. Thus CCR3 ultimately appears not to be relevant for the T2 response in AD-like disease, but only for the recruitment of eosinophils to the skin and the lung, thus opening an opportunity for therapeutic intervention by targeting CCR3.

7.5 Models Targeting T and B Cells

Considering the crucial role of both T cells and B cells in the pathogenesis of AD, targeting genes responsible for the generation or function of these lymphocytes appears to be a promising approach to elucidate the underlying mechanisms of this complex disease. Accordingly, $RAG2^{-/-}$ negative mice, which lack B and T cells, were unable to generate a dermal inflammatory infiltration upon epicutaneous sensitization with OVA (Woodward et al. 2001). Neither eosinophils nor mononuclear cells were present in the skin of these mice, and IL-4 as well as IgE expression was within the normal range. In contrast, $IgH^{-/-}$ mice, which lack B cells but have a normal set of T cells, upon sensitization mount a dermal infiltrate and elevated IL-4 production comparable to those of wild-type animals, whereas IgE levels were not increased (Woodward et al. 2001). Thus in this model of OVA sensitization-induced allergic dermatitis, T cells but not B cells appear to be responsible for the development of skin inflammation and elevated IL-4 levels in the skin.

T cells can differentiate into cells expressing the TCR$\alpha\beta$ or TCR$\gamma\delta$ chains, which can both be detected in normal murine skin. Using mice that either were negative for either TCRα or TCRδ revealed that the deletion of TCRδ had no effect on the course of OVA-induced dermatitis, whereas in $TCR\alpha^{-/-}$ mice neither an increased dermal infiltrate of eosinophils or mononuclear cells nor an elevation of IL-4 or IgE levels was apparent (Woodward et al. 2001). These results further limit the number of cell types potentially being responsible for the skin inflammation, IL-4, and IgE induction observed in OVA-induced dermatitis to TCR$\alpha\beta$ T cells.

The interaction between CD40 and its ligand CD40L has a central function in immune-mediating inflammatory responses via the

activation of APCs (Grewal and Flavell 1996; Laman et al. 1996). T cells in the peripheral blood of AD patients were found to exhibit an increased expression of CD40L, HLA-DR, and cutaneous lymphocyte antigen (CLA), which may result in an enhanced activation of epidermal and dermal LCs, macrophages, B cells, epithelial cells, and endothelial cells expressing CD40. CD40-CD40L interaction between $CD4^+$ T cells and dendritic cells is one of the most potent signals for dendritic cell activation and may be essential for the induction of Th reactions. Activation of CD40 expressed by B cells plays an important role in germinal center formation, isotype switching, and survival. Thus the enhanced CD40L expression on T cells in AD in combination with higher numbers of dendritic cells found in lesional skin may be in part responsible for the strong tissue inflammation seen in AD, while the interplay between T cells expressing elevated amounts of CD40L and B cells results in a shift of antibody production towards IgE, as is the case in AD.

Transgenic mice that selectively overexpress CD40L in keratinocytes were found to exhibit several signs of AD-like disease (Mehling et al. 2001). Homozygous transgenics developed massive skin alterations on tails, neck, paws and back within 3–4 weeks. These animals did not breed, were frail, displayed cachexia, and died within 4–5 months. Heterozygous transgenics also developed skin alterations. Within 7 weeks after birth, 80% of the heterozygous transgenic animals developed dermatitis on ears, around snouts, and on the upper thorax. Histology revealed a thickened skin with an acanthotic epidermis and hyperkeratosis, as well as fibrosis in the dermis with elevated numbers of fibroblasts. A massive dermal infiltrate was present, containing large numbers of T cells and macrophages, as well as $CD11c^+$ dendritic cells. The epidermis also contained large numbers of macrophages, but almost no LCs. However, significantly increased numbers of LCs were found in lymph nodes, indicating a higher turnover of antigen-transporting LCs in CD40L transgenic mice. These animals also developed lymph node hyperplasia and splenomegaly, with increased numbers of germinal centers in lymph nodes and spleen, which is consistent with an increased number of B cells. Serum levels for IgM were decreased in transgenic levels, while levels for IgE, IgG1, IgG2a, and IgG2b were increased 2- to 4-fold. Transgenicity for CD40L also introduced a

certain extent of autoimmunity, as antinuclear antibodies and antibodies against double-stranded DNA were found in the transgenic animals. This resembles observations made for human AD, since IgE antibodies directed against intracellular proteins of skin cells also have been detected in sera of human AD patients. This autoimmunity further results in deposition of Igs in the kidney, glomerulonephritis, and proteinuria, indicating impaired renal function. The lungs of several animals also were affected and displayed fibrosis, emphysema, and interstitial inflammation. Injection of serum from transgenic mice did not induce the formation of skin lesions in wild-type mice, indicating a role for autoreactive T cells, which often are present in autoimmune diseases. Indeed, adoptive transfer of $CD8^+$ T-cells resulted in the development of skin lesions within 5–9 days. In order to assess the role of B cells in the induction of the observed dermatitis, CD40L transgenic animals were backcrossed with a B-cell-deficient strain. The resulting B-cell-deficient CD40L transgenic mice developed an inflammatory phenotype similar to that of the transgenics capable of generating B cells.

Mice deficient for CD40, which normally is expressed on endothelial cells, epithelial cells, tissue macrophages, and other resident cells, were used to investigate the role of CD40-CD40L interaction upon epicutaneous sensitization with OVA (Woodward et al. 2001). However, in this model of allergic dermatitis, the level of IL-4 expression in sensitized skin and the amount and composition of the dermal infiltrate were comparable to those found in wild-type mice. Thus in this model, CD40-CD40L interaction appears not to be important for the induction of skin inflammation and IL-4 production. These data again point to the fact that the system in which the role of a selected molecule is investigated must be chosen carefully. While overexpression of CD40L in the skin is closely related to the in vivo situation found in AD, a lack of CD40 clearly is not.

Recently some light has been shed on T- and B-cell signaling pathways involved in the generation of AD using mice with alterations in the CARMA-1 gene (Hara et al. 2003; Jun et al. 2003; Jun and Goodnow 2003). CARMA-1 is a lymphocyte-specific member of the MAGUK (membrane-associated guanylate kinase) protein family, and its task is to interact with phosphorylated Bcl10 upon T-cell receptor cross-linking (Gaide et al. 2002; Pomerantz et al. 2002;

Wang et al. 2002). This interaction is a crucial event in the signaling cascade from the antigen receptor to the activation of the nuclear transcription factor NF-κB. Accordingly, CARMA-1 plays an important role in NF-κB activation and JNK phosphorylation upon antigen contact in T and B cells. Using a genome-wide chemical mutagenesis screening, mice with a point mutation in the CARMA-1 gene were identified and dubbed *unmodulated* (Jun et al. 2003). This point mutation results in the formation of a protein variant with disturbed folding properties, which probably retains some of its function. These mutated mice showed reduced B-cell numbers, deficient B-cell responses to anti-Ig antibodies, polysaccharide and protein antigen, as well as a defective T-cell response to CD28-mediated costimulation. The T1-dependent isotype was selectively reduced, and the reciprocal exaggeration of the T2 response resulted in the occurrence of signs of allergic dermatitis. While IgG1 and IgG2 levels were normal, levels of IgM and IgG3 were significantly decreased. However, after 10 weeks the IgE serum levels started to increase and stayed elevated beyond week 20. This coincides with the appearance of pruritic lesions on the ears and neck. Histological examination revealed hyperkeratosis and an extensive infiltration of mast cells, mononuclear cells, and occasionally eosinophils. Thus, the deficits in NF-κB and JNK activation in *unmodulated* mice induced by a point mutation in CARMA-1 result in a severe dysregulation of the balance between the T1 and T2 responses, suggesting that CARMA-1 is a crucial factor in AD-like diseases.

7.6 Models Targeting Mast Cells

Mast cells are a heterogeneous group of multifunctional cells (Boyce 2003; Galli et al. 2002; Krishnaswamy et al. 2001) with roles in several diseases including allergy, parasite infestation, inflammation, angiogenesis, and tissue remodeling. In addition to several other mediators, mast cells are an important source of cytokines including the NF-κB-dependent cytokines, TNFα, GM-CSF, and IL-8 as well as the T2 cytokines IL-4, IL-5, and IL-13. Thus, not only do mast cells have the capacity to initiate immediate-phase allergic and anaphylactoid reactions and tissue damage, they also have the capacity

to stimulate allergic inflammation, which is characterized by the influx of basophils, eosinophils, and neutrophils. Moreover, the mast-cell mediator histamine has been shown to suppress IL-12 production while inducing IL-10 expression and thus shifting the immune response towards the T2 type (Kung et al. 1995; Nagai et al. 1996; Wershil et al. 1991; Williams and Galli 2000). When mast cell-deficient WBB6F1/J-Kit^{W}/Kit$^{W\text{-}v}$ (W/W^{v}) mice were sensitized with OVA, the numbers of eosinophils, mononuclear cells, CD45^{+}, CD3^{+}, CD4^{+}, and CD8^{+} cells in the skin at sites of sensitization did not differ significantly from the numbers observed for wild-type WBB6F1 mice (Alenius et al. 2002). While IL-4 expression was not altered in the skin of mast cell-deficient W/W^{v} mice, significantly higher levels of IFNγ as well as elevated levels of total IgE were detected. In contrast, OVA-specific IgE and IgG2a levels in W/W^{v} were comparable to those in wild-type mice. However, although these changes can be attributed to the absence of mast cells in W/W^{v} mice, it is still possible that the underlying genetic defect, which involves the c-kit tyrosine kinase receptor and mainly affects mast cells, results in additional changes, as illustrated by the concomitantly decreased numbers of bone marrow granulocytes and megakaryocytes in W/W^{v} mice.

There is also some evidence that the mast-cell mediator chymase, a chymotrypsin-like serine protease, may be involved in the pathogenesis of AD (Watanabe et al. 2002). Accordingly, intradermal injection of chymase induces itching, increases microvascular permeability, and results in the accumulation of inflammatory cells, such as eosinophils and neutrophils (Hagermark et al. 1972; He and Walls 1998 a, b). When NC/Nga mice were treated with a synthetic inhibitor (SUN-C8257) of the murine chymase "mouse mast cell protease 4" (mMCP), the counterpart to human chymase, the AD-like disease significantly improved. This was further confirmed by histological examination showing a decrease of skin thickness and cellular infiltration. Moreover, in SUN-C8257-treated animals, the migration of eosinophils and mast cells to the skin lesions was reduced significantly, while IgE levels were unchanged, as was scratching behavior. Most likely these effects may be explained by the inhibition of mMCP-4 to cleave an as yet unknown target molecule which could function as an inducer of eosinophil migration, as subcutaneous in-

jection of mMCP-4 was shown to enhance eosinophil accumulation. There is evidence that one potential target appears to be stem cell factor (SCF). Accordingly, it has been shown that human, membrane (keratinocyte)-bound SCF is cleaved by chymase and released in a bioactive form (Longley et al. 1997), which induces a pronounced skin mastocytosis (Costa et al. 1996).

7.7 Conclusions

In accordance with the complex immunopathological findings in AD, many different murine models have been established, each focusing on different aspects of the disease. Thus several transgenic and knockout mouse models were helpful to a certain degree in improving our understanding of AD pathophysiology. However, genetic manipulation of a single cytokine obviously cannot mirror the whole picture of a complex disease such as AD. Neither the interaction between selected cytokines nor the entire pathological pathways can be completely evaluated with these models. Yet this type of mouse model has great potential for increasing our knowledge of AD pathology and for investigating the mechanisms of drug activities. Mice with spontaneous mutations resulting in an AD phenotype allow investigations on a broader scale, as do animals treated with antigens in order to induce AD. Combinations of the different approaches increase the size and versatility of the toolkit available, as shown by deletion of genes in NC/Nga mice, thus allowing even deeper insights in the mechanism of AD. Mice with a spontaneous AD phenotype are also suitable for drug testing and development. This also applies to the chimeric SCID-hu mouse, which allows assessment of the interaction between human skin and selected human cell populations. However, models should be chosen carefully and manipulations should be done only after appropriate consideration of side effects or unwanted additional effects.

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8 Epicutaneous Sensitization with Allergens as an Atopic Dermatitis Model

H. Alenius

8.1 Introduction

Atopic dermatitis (AD) is a common pruritic inflammatory skin disease that often begins in infancy and frequently occurs in subjects with personal or family history of atopic disease (Leung 2000). The majority of infants with AD develop asthma and/or allergic rhinitis later in life. Examination of affected skin lesions in AD suggests that skin-homing cutaneous lymphocyte associated antigen-positive (CLA^+) memory T cells and eosinophils play an important role in the pathogenesis of the disease. Immunohistologic analysis reveals a mononuclear cell infiltrate, predominantly in the dermis, consisting of activated $CD4^+$ T cells and macrophages/dendritic cells (Leung 1999). In acute lesions of AD, there is a significant increase of cells expressing interleukin (IL)-4 and IL-5 mRNA and protein, suggesting preferential accumulation of Th2 cells (Hamid et al. 1996; Thepen et al. 1996). In chronic skin lesions of AD, cells containing in-

terferon (IFN)-γ mRNA and protein predominate over those containing IL-4 and IL-5. Products of eosinophils are readily detectable in the skin lesions of AD (Jung et al. 1996; Wakita et al. 1994).

Several observations suggest that allergens play an important role in AD. Approximately 80% of patients with AD have elevated levels of serum IgE and evidence of specific IgE antibodies to a variety of food and environmental allergens. Oral challenge with food allergens exacerbates skin rashes and causes flare-ups in children with AD (Sicherer and Sampson 1999), and a number of studies have shown that avoidance of food and aeroallergens results in clinical improvement (Tan et al. 1996). Finally, allergen specific T cells have been found in skin lesions isolated from patients with AD (Abernathy-Carver et al. 1995). These observations are consistent with the view that epicutaneous exposure to allergens may play a role in AD.

Although much information has been gained regarding the mechanism of allergic asthma, little is known about allergen-induced dermatitis, in part because an appropriate animal model is lacking. A novel mouse model of AD has been developed (Spergel et al. 1998).

8.2 Epicutaneous Allergen Sensitization as a Model of AD

In this murine model, skin inflammation is developed by repeated epicutaneous (EC) sensitization with allergens such as ovalbumin (OVA) (Spergel et al. 1998) or natural rubber latex proteins (NRL) (Lehto et al. 2003).

Mouse skin is first shaved and stripped with transparent i.v. dressing (Lehto et al. 2003; Spergel et al. 1998). Mechanical injury to the skin by tape stripping mimics the trauma induced by intense scratching characteristic of AD (Beltrani 1999). In AD patients, the stratum corneum, the outermost layer of skin responsible for skin's penetration barrier properties, is constitutively disrupted. The disruption makes skin more susceptible to antigen penetration. Skin injury also induces local expression of several cytokines which enhance activation of Langerhans cells (IL-1β) (Wood et al. 1992) and also favors the development of Th2-type immune response (IL-10) (Laouini et al. 2003). After tape stripping, a dose of 100 μg of allergen in PBS or placebo is placed on a patch of sterile gauze (1 cm^2), and then

secured to the skin with transparent i.v. dressing. The patches are placed for a one-week period and then removed. Two weeks later, an identical patch is reapplied to the same skin site. Each mouse gets a total of three one-week exposures to the patch, separated from each other by two-week intervals. Mice are sacrificed and specimens are collected one day after the end of the series of three EC sensitizations.

Repeated EC exposure to allergen elicits a local cutaneous inflammatory response in mice (Lehto et al. 2003; Spergel et al. 1998). This response is characterized by strong epidermal and dermal thickening and increased infiltration of the dermal layer with eosinophils and mononuclear cells including $CD3^+$ and $CD4^+$ cells. The number of degranulated mast cells is also significantly increased in allergen-sensitized skin sites compared to control sites, suggesting that, in addition to $CD4^+$ $CD3^+$ T cells and eosinophils, activated mast cells may be involved in the development and progress of skin dermatitis (Lehto et al. 2003). Examination of cytokine and chemokine milieu in sensitized skin sites demonstrates that EC sensitization induces significant and marked expression of mRNA for IL-1β and IL-4, and to a lesser extent for IFN-γ (Lehto et al. 2003). Furthermore, EC sensitization induces strong expression of several chemokines. Expression of CCL3 and CCL4 mRNA is strongly upregulated in sensitized skin sites, while only low levels of CCL3 and CCL4 are detected in PBS-treated skin sites (Lehto et al. 2003). The expression of CCL2 mRNA is easily detectable in PBS-treated skin sites, but the expression is significantly enhanced after EC sensitization with allergens. The expression of CCL11 mRNA is significantly increased in allergen-sensitized skin sites compared to PBS-treated skin sites (Lehto et al. 2003). EC sensitization with allergens also induces a striking increase in total and allergen-specific IgE levels compared to PBS-treated controls (Lehto et al. 2003; Spergel et al. 1998).

Taken together, EC sensitization with allergens elicits in mice a Th2-type systemic immune response and a local Th2-dominating skin inflammation. The inflammatory cell infiltration to the skin correlates with an induction of proinflammatory and Th2 cytokines as well as chemokines.

8.3 Murine Model of AD in Investigations of Mechanisms of AD

The roles of several cells and cytokines have been assessed in the mouse model of allergen induced skin inflammation.

The functions of T cells, B cells, and CD40L-CD40 interactions were examined in a model of AD (Woodward et al. 2001). RAG-2 knockout mice, which lack both T and B cells, did not exhibit skin dermatitis, induction of dermal IL-4 mRNA, or elevation of serum IgE after EC OVA sensitization. All of these features were, however, present in B-cell-deficient IgH knockout mice. T-cell receptor-α knockout mice did not display skin inflammation, IL-4 mRNA expression, or increased IgE levels after OVA sensitization, but these responses were elicited in T-cell receptor-δ knockout mice after sensitization. Absence of CD40 had no effect on these responses. These results suggest that $\alpha\beta$ T cells, but not $\gamma\delta$ T cells, B cells, or CD40L-CD40 interactions, are critical for skin inflammation and the Th2 response in AD.

Mast cells are important effector cells in IgE-mediated allergic reactions. Mice deficient in mast cells (*W/Wv*) were examined (Alenius et al. 2002). Infiltration of mononuclear cells, T cells, and eosinophils in sensitized skin was equivalent in wild-type (WT) and *W/Wv* mice. Expression of IL-4 mRNA in sensitized skin sites was equivalent in WT and *W/Wv* mice, but IFN-γ mRNA was significantly increased in *W/Wv* mice. This increase was associated with an imbalance in the baseline expression of cytokines that affect the development of Th1 and Th2 cells. IL-4 mRNA was detectable in unsensitized skin of WT mice but not in that of *W/Wv* mice. In contrast, expression of IL-12 mRNA was significantly increased in unsensitized skin of *W/Wv* mice compared to WT controls. Total serum IgE levels were significantly increased in *W/Wv* mice compared to WT controls after EC sensitization. These results suggest that mast cells regulate baseline IL-4 and IL-12 expression and allergen-induced IFN-γ expression in the skin, as well as circulating IgE levels.

Skin lesions in AD are characterized by expression of the cytokines IL-4, IL-5, and IFN-γ. The role of these cytokines was elucidated in the murine model of AD (Spergel et al. 1999). Allergen-sensitized skin from IL-5-deficient mice had no detectable eosino-

phils and exhibited decreased epidermal and dermal thickening. Sensitized skin from IL-4-deficient mice displayed normal thickening of the skin layers but had a drastic reduction in eosinophils and a significant increase in infiltrating T cells. These findings were associated with a reduction in CCL11 mRNA and an increase in mRNA for CCL4 and CCL5. Sensitized skin from IFN-γ-deficient mice was characterized by reduced dermal thickening. These results suggest that both the Th2 cytokines IL-4 and IL-5 and the Th1 cytokine IFN-γ play important roles in the inflammation and hypertrophy of the skin in the model of AD.

It has been demonstrated that mechanical injury to mouse skin, which can be caused by tape stripping, results in rapid induction of IL-10 mRNA (Laouini et al. 2003). IL-10-deficient mice were recently used to examine the role of IL-10 in an AD model (Laouini et al. 2003). Skin infiltration by eosinophils and expression of CCL11, IL-4, and IL-5 mRNA in OVA-sensitized skin sites were severely diminished in IL-10-deficient mice. Following in vitro stimulation with OVA, splenocytes from EC-sensitized IL-10-deficient mice secreted significantly less IL-4, but significantly more IFN-γ, than splenocytes from WT controls. A similar skewing in cytokine secretion profile was observed in the splenocytes of IL-10-deficient mice immunized intraperitoneally with OVA. IL-10-deficient antigen presenting cells (APCs) skewed the in vitro response of OVA T-cell receptor (TCR) transgenic T cells towards Th1. Examination of the Th response of WT and IL-10-deficient mice immunized with OVA-pulsed WT or IL-10-deficient dendritic cells revealed that both dendritic cells and T cells participate in IL-10 skewing of the Th2 response in vivo. These results suggest that IL-10 plays an important role in the Th2 response to antigen and in the development of skin eosinophilia in a murine model of AD.

8.4 Utilization of the Murine Model of AD in Pharmaceutical Studies

The efficacy of novel chemokine antagonist therapies to ameliorate protein-induced skin inflammation was evaluated in a murine model of AD (Homey et al. 2002). A novel skin-associated chemokine

CCL27 and its receptor CCR10 which preferentially mediate chemotactic responses of skin-homing CLA^+ T cells were identified and characterized. Mice that were EC-sensitized with OVA or PBS were simultaneously treated with either neutralizing antibodies against CCL27 or isotype control. Mice treated with neutralizing anti-CCL27 antibody showed markedly decreased leukocyte infiltration into the skin following epicutaneous OVA exposure. Anti-CCL27 administration impaired the recruitment of lymphocytes to the skin of OVA-sensitized mice by more than 90% compared to isotype-treated controls. However, the number of skin-infiltrating eosinophils was not affected, indicating the specificity of anti-CCL27 treatment. Histologically, neutralization of CCL27 resulted in a suppression of inflammation-induced skin thickening and reduced acanthosis. Taken together, these results demonstrate that neutralization of CCL27/CCR10 interactions impairs lymphocyte recruitment to the skin in vivo and leads to the inhibition of allergen-induced skin inflammation.

8.5 Conclusions

Relevant animal models of human diseases are invaluable. The murine model described here incorporates several features that are likely to be important in the pathogenesis of atopic dermatitis in humans. EC sensitization with protein allergens induces dermatitis manifested by epidermal and dermal thickening and characterized by the dermal accumulation of $CD4^+$ T cells, eosinophils, and degranulated mast cells. In addition, allergen-sensitized skin reveals an accumulation of Th2 cytokine mRNA. Finally, EC allergen sensitization in the absence of adjuvant leads to increased levels of total serum IgE and allergen-specific IgE as well.

The availability of this model system should facilitate the investigation of the molecular and cellular mechanisms of allergen-induced skin inflammation. Moreover, the effects of trigger factors of AD (e.g., skin injury and superantigens) on the development of skin inflammation and antibody responses can be examined. Finally, efficacy of novel topical and systemic therapies that might be predicted to have efficacy in humans can be carefully tested.

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9 T-Cell Receptor Transgenic Models of Inflammatory Disorders: Relevance for Atopic Dermatitis?

U. Niesner, F. Hardung, A. Scheffold, A. Radbruch

9.1 Introduction

T-cell receptor transgenic (TCR^{tg}) T lymphocytes offer unique possibilities to study the contribution of T cells to protective and pathological immune reactions. They can be activated and challenged with a defined antigen, their differentiation can be modified at will, and they easily can be traced in vivo, using clonotype-specific antibodies. Although to date TCR^{tg} T cells have not yet been used for the analysis of atopic dermatitis, their role in initiation, maintenance, and flaring of chronic inflammation has been studied in other systems. Many of these studies focussed on the role of T helper 1

(Th1) versus Th2 polarized cells in the initiation phase, and, more recently, on the role of regulatory Th cells. Still, the role of T lymphocytes in the maintenance of chronic tissue-specific inflammation is unclear and remains a major challenge for future research.

9.2 Atopic Dermatitis

Atopic dermatitis (AD) is a pruritic inflammatory skin disease with a prevalence of over 10% among infants and children worldwide that can persist in adulthood (reviewed in Galli et al. 2003; Leung and Bieber 2003). The cause of AD is multifactorial. A broad range of factors such as the environment, genetic predisposition, skin barrier defects, and dysregulation of immune reactivity contribute to the development of AD. The majority of patients suffering from AD show hypersensitivity to environmental and food allergens and develop asthma and/or allergic rhinitis later in life. Fitting into this picture of a typical "Th2-disease", peripheral blood eosinophilia and elevated serum IgE levels, the so-called extrinsic type of AD, can be detected in most of the patients. However, 20% of AD patients display normal serum IgE levels, the so-called intrinsic form of AD. In chronic skin lesions, the epidermis displays hyperplasia and hyperkeratinosis with infiltrates of $CD4^+$ and $CD8^+$ T lymphocytes, dendritic cells, eosinophils, and macrophages. No disease occurs in the absence of $\alpha\beta$ $CD4^+$ T cells in an animal model of AD, indicating the fundamental role of Th cells in controlling the onset of the disease (Woodward et al. 2001).

In AD, an initial Th2 response is followed by a Th1-dominated chronic immune reaction (Thepen et al. 1996). In acute inflammatory skin lesions, primarily Th2 cells are located. Interleukin 4 (IL-4), IL-5, and IL-13, produced by these cells, induce B cells to secrete IgE, further enhance pro-inflammatory chemokine production by resident skin cells (reviewed in Girolomoni et al. 2003), and thereby enhance infiltration of activated immune cells. In particular, the chemokines RANTES, MCP-4, and eotaxin are upregulated in skin lesions and recruit Th2 cells and eosinophils expressing CCR3. In the chronic phase, the influx of dendritic cells, macrophages, and eosinophils leads to the elevation of local IL-12 concentration and

this may contribute to a switch in functional Th-cell polarization, in that in chronic skin lesions interferon-gamma (IFN-γ) mRNA and protein are predominantly expressed by the cutaneous Th cells.

In this descriptive scenario, the decisive molecular switches are still not known. It is not clear how the initial strong Th2 polarization is triggered and directed to the skin insult, nor is it clear how the switch to Th1 is controlled. Finally, little is known about the role of regulatory T cells.

9.3 Animal Models of Atopic Dermatitis

Skin lesions with clinical features of human AD appear spontaneously in cats and dogs, a model of limited relevance (Marsella and Olivry 2003). Several experimental and genetic murine models of AD have been reported which have contributed to some extent to our current understanding of the disease. It should be kept in mind that animal models, how useful they may be, usually just reflect aspects of the actual disease.

Nc/Nga mice (NC mice), when raised under conventional, but not when raised under specific pathogen-free (SPF) conditions, gradually develop chronic relapsing skin lesions from 8 weeks of age that histologically resemble human AD (Suto et al. 1999). Similar composition of cellular infiltrates in the skin lesions, increased expression of IL-4 mRNA, and high serum IgE levels combined with susceptibility to induction of allergic asthma (Iwasaki et al. 2001) make it a suitable model to study AD and other allergic disorders. Although the etiopathogenesis of this murine form of AD in NC mice is not yet clear, this model offers an interesting perspective to identify genes and microbial pathogens involved.

Another mouse model for AD are food-induced skin lesions which can be induced in C_3H/HeJ mice. One-third of female mice develop AD-like skin lesions when orally sensitized with cow's milk or peanut in combination with cholera toxin (Li et al. 2001).

A third model is the transgenic expression of interleukin (IL)-4 in the murine epidermis, leading to spontaneous inflammation of the skin with clinical features of AD (Chan et al. 2001).

Still another AD model is the epicutaneous sensitization of BALB/c mice by means of repeated application of ovalbumin (OVA)-impregnated patches. This treatment results in a local allergic dermatitis, characterized by dermal infiltration with $CD4^+$ T cells, neutrophils, mast cells, and a large number of eosinophils (Spergel et al. 1998). In addition, epicutaneously sensitized mice display antigen-specific airway hyperresponsiveness and increased eosinophils in their bronchoalveolar lavage fluid. OVA-specific IgE and IgG1 serum titers are elevated, as are IL-4 and IL-5 mRNA levels at the site of inflammation, pointing to a Th2-dominated response. However, IFNγ mRNA and OVA-specific IgG2 levels also increase, though to lesser extent. Later studies have confirmed the contribution of IL-4, IL-5, IFNγ (Spergel et al. 1999), and IL-10 (Laouini et al. 2003) to immunopathology in these mice, and extended the model to C57BL/6 and 129SV mouse strains. This model could be easily adapted to studies using TCR^{tg} T cells. TCR^{tg} mice recognizing the model antigen OVA are available on BALB/c background (Murphy et al. 1990) and C57/BL6 background (Barnden et al. 1998), and represent the most widely used TCR^{tg} mouse models.

9.4 TCR Transgenic Mouse Models

$CD4^+$ T helper (Th) cells play a key role in the adaptive immune system and can be classified into regulatory subsets that downregulate immune responses (reviewed in Read and Powrie 2001) and reactive subsets, controlling immune reactions and differentiation of effector cells. A key feature of lymphocytes is their potential longevity and their capacity to memorize their mode of reaction to antigen, i.e., their functional memory (reviewed in Lohning et al. 2002). In this respect, memory Th cells show a considerable functional heterogeneity according to expression of distinct cytokines and chemokines. This spectrum of differentiation can be simplified by classifying Th memory cells as T helper 1 (Th1) versus Th2 cells, according to expression of either T-bet or GATA-3 as "master transcription factors". Upon restimulation, most Th1 cells secrete IFNγ and tumor necrosis factor (TNF)-β, activating macrophages and other killer cells. Most Th2 cells express IL-4, IL-5, and IL-13, thereby support-

ing antibody production by B cells, eosinophil differentiation and survival. By induction of IgE switch transcripts in B lymphocytes, IL-4 targets antibody class switch recombination to IgE at high doses (Jung et al. 1994). Thus, Th2 cells directly induce the allergenic IgE antibodies.

There is growing evidence that Th cells, generally being a part of inflammatory infiltrates, are important for the onset, perpetuation, and control of pathological inflammatory reactions including AD. However, in several inflammatory diseases the causing antigens remain unknown and the role of antigen-specific Th cells cannot be clarified. Even if the antigen is known, maturation and fate of antigen-specific T cells in vivo are difficult to track due to their low frequency and the lack of specific staining reagents, i.e., clonotype-specific antibodies or MHC-peptide multimers. Here, TCR^{tg} mouse models offer a decisive experimental advantage. TCR cassette vectors, into which short PCR products containing the rearranged α and β variable regions can be readily ligated, have facilitated the generation of TCR^{tg} mice (Kouskoff et al. 1995).

The most frequently used transgenic TCRs recognize antigens such as ovalbumin (Murphy et al. 1990), moth cytochrome C (Jorgensen et al. 1992), and influenza virus A/NT/60/68 nucleoprotein (Mamalaki et al. 1992) or are derived from pathogenic T-cell clones of autoimmune diseases with known specificity that are able to transfer the disease. It is not entirely clear whether these particular T-cell clones are actually causally involved in the disease, given the fact that autoreactive T cells can be readily cloned out of the peripheral T-cell pool of healthy individuals. In addition, non-pathogenic T-cell clones may become pathogenic in TCR^{tg} animals, probably just because of their numeric expansion and/or activation by cross-reacting antigens either through the transgenic TCR or through a second endogenous TCR (Waldner et al. 2000).

The frequencies of T cells expressing the transgenic TCR in vivo vary and can reach up to 90%. CD45RB(low) memory Th cells and Th cells secreting effector cytokines which express the transgenic TCR can be detected in the mice prior to exposure to the antigen. These in the absence of the cognitive antigen-activated Th cells are absent in mice with severe combined immunodeficiency (SCID) or inactivated recombinase activating gene ($RAG^{-/-}$) backgrounds, i.e.,

in the absence of functional endogenous TCRs, $CD8^+$ T cells, and B cells. Coexpression of endogenous rearranged TCR chains gives rise to the expression of a second TCR that can activate the transgenic T cells (Lee et al. 1996; Saparov et al. 1999). The rate of spontaneous disease is generally increased among TCR^{tg} animals carrying an autoreactive TCR, e.g., myelin basic protein (MBP)-specific TCR, but often onset of the disease does not occur rapidly after birth, and incidence remains low if mice are kept under SPF conditions. Regulatory mechanisms counteracting the autoimmune disease are still functional in these animals. Crossing the mice onto SCID or $Rag^{-/-}$ background can exacerbate the disease severity and incidence. Here, impaired development of regulatory T cells which depends on the rearrangement of endogenous TCR might be the reason (Olivares-Villagomez et al. 1998).

TCR^{tg} animals contain a high percentage of T cells specific for a particular antigen, and therefore as such are not a good model for any physiological situation. More defined conditions can be generated in models of adoptive transfer, using TCR^{tg} T cells isolated ex vivo, at defined numbers for transfer into syngeneic recipient mice. Clonotype-specific antibodies allow the identification of the transferred cells in the host. Though a minor population, it must be taken into consideration that the transferred cells still comprise an artificial high frequency of mono-specific T cells within the recipient mouse, which could by itself influence the experimental results, as could the choice of the antigen (Zinkernagel and Hengartner 2001).

Activated T cells proliferate and lose the capacity to home to lymphoid tissues, but gain the ability to enter nonlymphoid tissues due to differential expression of selectins, integrins, and chemokine receptors. In this context, using TCR^{tg} cells in an adoptive transfer model, it has been shown that transgenic T cells are preferentially activated in the draining lymph nodes and accumulate in an antigen-specific and E-selectin-dependent fashion at the site of subcutaneous injection of antigen with adjuvant (Reinhardt et al. 2003). In this context, co-transfer of TCR^{tg} cells with different specificities extends possibilities of application and allows a better dissection of unspecific versus antigen-specific effects of transferred cells (Schipf et al. 2003).

To analyze the role of functionally distinct T-cell subsets, TCR^{tg} cells can be sorted or modulated in vitro, and after transfer can be

functionally analyzed in vivo. For instance, differentially polarized Th1 or Th2 cell populations, generated in vitro, can be transferred to test their role in immune reactions. Generally, both subsets mount inflammatory responses at the site of antigen deposition, whether or not the original animal model is Th1- or Th2-dominated. However, histological features and often disease severity are different. Transferred Th2 cells usually cause elevated IgE levels and eosinophilia, while Th1 cells usually cause neutrophilia and massive infiltrates of mononuclear cells. Effective cross-inhibition of Th1 and Th2 immune responses upon cotransfer of both subsets does not occur unless additional regulatory molecules such as transforming growth factor (TGF)-β are introduced (Hansen et al. 2000).

However, it remains unclear how closely the in vivo behavior of in vitro differentiated T cells resembles T-effector cells developing in vivo. In vitro polarized Th1 cells, but not Th2 cells, preferentially express ligands for P- and E-selectin and home to inflamed tissue (Austrup et al. 1997). Ex vivo analyzed Th1 cells as compared to Th2 cells lack this preference (Tietz et al. 1998; Hamann et al. 2003).

9.5 TCR Transgenic Mouse Models of Inflammatory Diseases

TCR^{tg} mouse models have been used for the analysis of a variety of major inflammatory disorders (Table 1), though not for atopic dermatitis. Most closely related would be allergic asthma, which in AD patients does closely correlate. In terms of an initial strong Th2-induced, IgE-triggered, basophil and eosinophil-mediated, type I allergic response, the underlying immune reaction probably is the same for AD and asthma.

9.5.1 Asthma

Asthma is a chronic inflammatory disease of the bronchial airways in which mainly lymphocytes and eosinophils infiltrate the bronchial mucosa. The importance of Th2 cells secreting IL-4, IL-5, and

Table 1. Selected TCR transgenic mouse models of inflammatory diseases

Antigen	Name	Type	Disease model	References
Ovalbumin ($OVA_{323–339}$)	DO11.10	CD4	Asthma	Cohn et al. 1998; Hansen et al. 2000
Islet-specific antigen	BDC2.5	CD4	Diabetes	Katz et al. 1993;
Influenza virus hemagglutinin ($HA_{512–520}$)	CL4	CD8	Diabetes	Morgan et al. 1996
Ovalbumin ($OVA_{257–264}$)	OT-1	CD8	Diabetes	Kurts et al. 1998
Myelin basic protein ($MBP_{1–11}$)	1934.4	CD4	EAE	Liu et al. 1995
Myelin proteolipid protein ($PLP_{139–151}$)	5B6, 4E3	CD4	EAE	Waldner et al. 2000
Myelin oligodendrocyte glycoprotein ($MOG_{35–55}$)	2D2	CD4	EAE	Betelli et al. 2003
Ovalbumin ($OVA_{323–339}$)	DO11.10	CD4	IBD	Strober et al. 1997; Iqbal et al. 2002
Collagen IIa ($CII_{260–267}$)	qCII85.33	CD4	RA	Brand et al. 2002
Ovalbumin ($OVA_{323–339}$)	DO11.10	CD4	RA	Hardung et al. 2001
Glucose-6-phosphate-isomerase	K/BxN	CD4	RA	Kyburz and Corr 2003

IL-13 for the disease (Umetsu et al. 2003) was confirmed using TCR^{tg} cells in an adoptive transfer model. In vitro differentiated OVA-TCR^{tg} Th1 and Th2 cells both localize to the lungs and induce airway inflammation after exposure to inhaled antigen, but only Th2 cells induce eosinophilia, mucus hypersecretion, and airway hyperresponsiveness (Cohn et al. 1998; Hansen et al. 2000), typical clinical features of asthma. In a similar model, the ability of Th1 cells to counteract Th2-driven inflammation upon cotransfer was tested. Here, in vitro differentiated Th1 cells only effectively inhibited airway hyperresponsiveness and inflammation in an antigen-specific

fashion if being engineered to express TGF-β (Hansen et al. 2000). It should be noted that in this experimental model, the generation of allergen-specific plasma cells secreting IgE is the actual pathogenic step. Later stages, such as the recruitment of plasma cells to the pool of long-lived plasma cells or the recruitment of IgE-armed effector cells to the lung have not been tested effectively so far. The switch from a predominant Th2 allergic reaction to a Th1-dominated inflammation has also not yet been analyzed for asthma.

9.5.2 Diabetes

Insulin-dependent diabetes mellitus (IDDM) is a T cell-mediated autoimmune disorder characterized by infiltration of the islets of Langerhans by immune cells, ultimately resulting in the destruction of insulin-producing β-cells. In addition to $CD4^+$ T cells, $CD8^+$ T cells play an important if not major role in the pathogenesis of the disease. Both cell types are able to transfer the disease and recognize self-antigens expressed within the islets. For this reason $CD4^+$ and $CD8^+$ TCR^{tg} models have been used to study disease mechanisms. The diabetes-susceptible NOD mouse strain is a commonly used experimental model of diabetes. NOD mice transgenic for a TCR, derived from the diabetogenic $CD4^+$ T-cell clone BDC2.5, which recognizes a β islet granule antigen, develop spontaneous IDDM with a higher incidence than wild-type animals. Transgenic NOD mice on an SCID background all become diabetic (Kurrer et al. 1997). In accordance with findings in TCR^{tg} models of other inflammatory disorders, in vitro differentiated TCR^{tg} Th1 and Th2 cells are both able to induce IDDM, though diabetes has been correlated with a predominant Th1 phenotype. Moreover, no cross-inhibition of the inflammatory response occurs upon cotransfer of Th1 and Th2 cells (Pakala et al. 1997). Model antigens such as ovalbumin (Kurts et al. 1998) and influenza virus hemagglutinin (Morgan et al. 1996), specifically expressed in β-cells under the rat insulin promoter in combination with adoptive transfer of TCR^{tg} $CD8^+$ cells, have been used to delineate the function of $CD8^+$ T cells in diabetes. Here, double-transgenic mice develop a rapid diabetes and die within 10 days after birth (Morgan et al. 1996). TCR^{tg} autoreac-

tive T cells have proven to be a valuable tool to study the steps required to make an autoreactive T cell autoaggressive (reviewed in Andre et al. 1996), and the destructive potential of both $CD4^+$ and $CD8^+$ T cells has been demonstrated. These results do not have very much relevance to AD, except for showing that both T-cell types can damage tissue expressing their target antigen.

9.5.3 Multiple Sclerosis

Experimental allergic encephalomyelitis (EAE) serves as a model for multiple sclerosis. Susceptible mouse strains, namely SJL and PL/J mice, develop a Th1 cell-driven inflammation in the central nervous system if immunized with components from brain and spinal cord (Kuchroo et al. 2002). Predominantly T cells and macrophages can be identified among infiltrating cells. TCR^{tg} mouse strains recognizing myelin basic protein ($MBP_{1–11}$, Liu et al. 1995), myelin proteolipid protein ($PLP_{139–151}$, Waldner et al. 2000), and myelin oligodendrocyte glycoprotein ($MOG_{35–55}$, Betelli et al. 2003) have been described. Such TCR^{tg} mice develop spontaneous disease. On an immunodeficient SCID genetic background, MBP-specific TCR^{tg} mice develop EAE at increased frequencies, due to the absence of regulatory T cells, the generation of which depends on the rearrangement of endogenous TCRs (Olivares-Villagomez et al. 1998). For TCR^{tg} mice bearing the TCR specific for PLP derived from encephalitogenic and non-encephalitogenic T cell clones, it has been observed that the non-encephalitogenic TCR promotes a more vigorous spontaneous EAE than the encephalitogenic TCR (Waldner et al. 2000). In addition to studies of disease mechanisms, MBP-specific TCR^{tg} mice proved to be a valuable tool for analysis of thymic selection of T cells and induction of peripheral tolerance (Anderton et al. 1999). Triple-transgenic mice bearing a human TCR specific for a conserved MBP peptide, human CD4, and the restricting human histocompatibility leukocyte antigens (HLA) class II region, develop spontaneous EAE on a $RAG^{-/-}$ background (Madsen et al. 1999). This approach could serve as a more general model system for testing strategies to interfere with human TCR-HLA interactions in an animal model.

9.5.4 Inflammatory Bowel Diseases

The inflammatory bowel diseases (IBD) Crohn's disease and ulcerative colitis are chronic inflammatory disorders of the human bowel. A great variety of mouse models for IBD exist, covering different aspects of the disease (Hoffmann et al. 2002). Most of these models show functional dysregulation of T cells and a requirement for the intestinal microflora, most likely reflecting cross-activation of pathogenic T cells. As the specificity of the responding T cells is not well defined, TCR^{tg} models of IBD and the respective model antigens have been used to analyze the possible involvement of TCR-specific signals in etiopathogenesis of the disease versus maintenance of tolerance. Studies in OVA-TCR^{tg} mice fed with ovalbumin revealed a block in the induction of protective, TGF-β-producing T cells by Th1 cells (Strober et al. 1997). Adoptive transfer of OVA TCR^{tg} Th1 and Th2 cells into mice inoculated with OVA-expressing *Escherichia coli* leads to induction of colitis with distinct pathology. Th1 cells induce immigration of neutrophilic granulocytes, while Th2 cells induce immigration of eosinophilic and basophilic granulocytes (Yoshida et al. 2002; Iqbal et al. 2002).

Immune reactions in allergic asthma and IBD share a common tissue, the mucosal surface, but display different T cell-mediated pathology. While animal models of IBD usually reflect a Th1-controlled inflammation, lung inflammation in allergic asthma reflects control by Th2 cells, at least in the initial phase (Neurath et al. 2002). This difference may well reflect differences in the antigenic insult, with gut flora providing Th1-polarizing innate signals, while antigenic challenge of the lung may lack those signals and reflect a default Th2 response. Models of IBD have been widely used to study the effect of regulatory T cells, cells that can downregulate immune responses by virtue of TGF-β (Th3 cells) and/or IL-10 (Tr1 cells) expression, or via as yet undefined mechanisms (T_{reg} cells) (reviewed in Singh et al. 2001). Whether or not these cells need to see the same antigen as the regulated T cells, or an antigen expressed at the same site, remains unclear, since TCR^{tg} regulatory T cells have not yet been applied in these systems.

9.5.5 Rheumatoid Arthritis

Rheumatoid arthritis (RA) is a chronic inflammatory disorder of the joints involving destruction of cartilage and bone. A systemic Th-cell bias has been observed in patients with RA, with predominant Th1 predisposition of peripheral Th cells (Schulze-Koops and Kalden 2001). In RA, the antigens initiating and driving the disease have not yet been identified, although from therapeutic intervention it is clear that T cells are crucial for the induction and maintenance of synovial inflammation. TCR^{tg} models have been used extensively to analyze the question of how inflammation is targeted to the joints. Immunization of mice with collagen-IIa, a cartilage-derived protein, induces an RA-like synovial inflammation and is used as murine model of RA. When collagen IIa-specific TCR^{tg} animals are immunized with collagen and adjuvant, they develop more rapidly a more aggressive arthritis, unless they have been tolerized by previous intravenous injection of soluble antigen (Brand et al. 2002). Tolerance is characterized by antigen-specific IL-4 and IL-10 production of ex vivo derived and antigen-provoked T cells. Antigen-induced arthritis with non-self antigens such as methylated bovine serum albumin (BSA) and cationized OVA injected into the joints has been used in combination with TCR^{tg} cells specific for these antigens. Ovalbumin-induced arthritis can be provoked by a single immunization of mice with ovalbumin and adjuvant, followed by a single injection of antigen in the knee (Hardung et al. 2001). TCR^{tg} cells representing various differentiation states can be adoptively transferred and challenged in the host. Here, preactivated TCR^{tg} Th1 cells are essentially the only pathogenic players, while Th2 cells are much less efficient. OVA-specific T cells retrovirally transduced with IL10 efficiently reduce disease severity, demonstrating the potential of T cells to be used as a vehicle to target beneficial expression of regulatory molecules to the site of inflammation (Setoguchi et al. 2000). In this model the contribution of B cells and antibodies secreted by them is unclear.

In the K/BxNOD (KBN) arthritis model, plasma cells play a crucial role (reviewed in Kyburz and Corr 2003). KBN mice, which are transgenic for a TCR specific for a peptide of bovine pancreas ribonuclease and cross-reactive to the ubiquitously expressed glucose-6-

phosphate isomerase (GPI), develop severe spontaneous arthritis. The role of the TCR^{tg} T cells in this model is to induce GPI-specific B cells to differentiate into antibody-secreting cells. Arthritis in these mice can be transferred with serum or IgG from diseased animals. Immune complexes consisting of GPI and anti-GPI antibodies activate complement, mast cells, neutrophils, and macrophages. It remains unclear and a challenge for future research as to how the resulting inflammation is targeted to the joints, and whether T cells are required to maintain antibody secretion. They control the initial differentiation of specific B cells, but the resulting plasma cells could well enter the pool of long-lived plasma cells, and support pathogenesis independent of continued T-cell help (Manz and Radbruch 2001).

In this respect, the model is related to AD, which also is dependent on T-cell help in the initial phase, as is reflected by the induced B-cell switch to IgE production. Whether or not T-cell help is required later on remains to be shown and will be decisive for the question of which cell types should be targeted for therapy.

Acknowledgements. U. Niesner was supported by a Boehringer Ingelheim Fonds (B.I.F.) PhD Scholarship.

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10 What Must a Model Display for Proof as a Model of Psoriasis?

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10.1 Modelling Psoriasis

10.1.1 What Exactly Should Be Modelled?

In order to develop a model for psoriasis, the disease to be modelled must first be clearly defined. Yet, the superficially easy-to-diagnose "classical" plaque-stage presentation of psoriasis is only one of many clinical variants (Table 1). Although this fact is trivial to trained dermatologists, it is important to keep in mind that any given individual may present with vastly different clinical disease patterns at different time points, underscoring the influence of environmental and modifying genetic factors on disease phenotype. Moreover, both

Table 1. Specificity of clinical features in psoriasis

Erythema	–
Large silvery scales	++
Well-circumscribed plaques	++
Chronic persistence	+
Auspitz phenomenon	+++
Sum of all	++++

Table 2. Specificity of histological features of the psoriatic phenotype

Feature	Specificity
Epidermis	
Acanthosis	No
Altered differentiation	No
Hyperproliferation	No
Acanthosis, papillomatosis	No
Blood vessel loops	No
T-cell infiltrate	No
Injury response	No

clinically and histologically, no single entity defines psoriasis (Table 2). Since only the sum of those aspects listed in Table 2 defines psoriasis, an adequate model should exhibit the same spectrum, rather than featuring only selected elements. Obviously, this is no mean feature to achieve.

10.1.2 Which Genes Should Be Manipulated?

The genetic complexity of psoriasis is notorious. While twin- and epidemiological studies have firmly established a strong genetic contribution, the details are still largely obscure (for a recent review, see Elder et al. 2001). It is clear that a strong genetic determinant resides in the vicinity of the HLA-C locus in the MHC possibly accounting for as much as 50% of the genetic contribution to psoriasis (the so-called PSORS1 locus). However, despite the presence of sev-

eral candidates and several high-density genetic screens, the culprit gene remains elusive (Capon et al. 2002). All other candidate loci, catalogued with a PSORS identifier in the OMIM database, have so far been poorly replicated in independent data sets (The International Psoriasis Consortium 2003). This is at least in part due to the apparent genetic heterogeneity of psoriasis. For the development of an animal model, this scenario precludes manipulating the "right" gene(s) to obtain a disease model. Moreover, since any number of patients are only "phenocopies", any genetically manipulated mouse will, at best, only model a genetic psoriatic subgroup. However, at present we are limited to the obviously over-simplifying type 1/type 2 subgroup classification established by Christophers and Henseler (Henseler and Christophers 1985). Thus, it may turn out that only the identification of psoriasis subgroups based on susceptibility genes will allow a true modelling of the disease.

10.1.3 Which Pathogenetic Feature Is Central?

Even more perplexing than the clinical and genetic uncertainties is the molecular maze presented by the published literature on psoriasis pathogenesis. Inflammatory cytokine signalling, inadequate T-cell activation, aberrant keratinocyte differentiation, and growth factor secretion (among others) have all been observed in psoriatic plaques and described in molecular detail over the past decades (Krueger et al. 1990; Kadunce and Krueger 1995; Prinz 2001). But, corresponding to the clinical and histological disease ambiguities, none of the pathogenetic elements identified so far is specific for psoriasis, and it is unknown why the factors described to date should produce a psoriatic morphology. Thus, fundamental questions such as these remain unanswered: Why are the plaques sharply demarcated? Why do they often remain stationary over extended periods? Why do they conform to (potentially mechanically determined) classical locations at one time but not at another in the same individual? This list of questions could easily be extended. For the time being, the current state of knowledge suggests that focussing on any given pathogenetic aspects, e.g. T-cell activation, may be highly misleading in the development of a disease model.

10.1.4 Can Mice Do the Job?

To make matters even worse, it should be appreciated that the generation of transgenic mice as a model for a disease like psoriasis has serious limitations. First and foremost, mice simply may not get psoriasis even if the "correct" set of genes is manipulated in the right fashion. Second, since we are dealing with a polygenetic disease, currently used approaches of overexpressing or eliminating single genes is inherently flawed. Rather, a true disease model most likely would reflect not a loss but a reduction or partial gain in function of more than one gene. Third, since inbred strains of one or two genetic backgrounds are used, the probably large impact of genetic background for the human disease cannot be modelled. Moreover, while certain environmental factors known to affect disease severity such as concurrent inflammatory processes or exposure to drugs can be modelled, others such as cold climate and psychoemotional stress cannot.

10.1.5 It Still Must Be Attempted!

The myriad uncertainties and potential pitfalls sketched out above may well lead to the notion that modelling psoriasis should be left undone until we know more about the disease and are better at polygenetic manipulation of the germline. However, this would be a misguided conclusion for two reasons. First, and this is a twist not without irony, we will be unable to define the role and relative contribution of disease genes without a genetic disease model. Thus, Juha Kere's group has recently introduced allele-specific transgenes of one of the hot PSORS1 candidates, the HCR gene (Asumalahti et al. 2002), into the mouse germline under the control of the K14 promoter without obtaining any discernible phenotype (Suomela et al. 2003). Although the effect of potentially contributory factors (e.g. interferon treatment, infection, various genetic backgrounds) has not been tested in this model, the role of HCR as the true PSORS1 risk allele (if it is the right gene!) may not even become apparent under specific challenges. Instead, a psoriatic phenotype may only become penetrant within a given genetic susceptibility context. Thus, a mod-

el harbouring a genetic lesion in the same signalling cascade may be required. Since it has been argued that isolation of the PSORS1 mutation cannot be achieved by association-based mapping due to the high linkage disequilibrium present in the HLA (Capon et al. 2002), the phenotype elicited in vivo by manipulating candidate alleles such as HCR will likely represent the only approach to identify the PSORS1 – and other – susceptibility gene(s). Second, new drugs are currently subjected to pre-clinical testing only in the severe combined immunodeficiency (SCID) model (see below) whence they move straight to phase I clinical trials. This limitation precludes large-scale testing of highly innovative, but potentially risky treatment approaches and represents a definite bottleneck in the pipeline for novel therapies. Thus, the hard and rocky road to model a complex polygenetic disease must still be taken.

10.2 Principal Strategies

Psoriasis seems to be a disease specific to humans. Quite a few animal models have been described in mice, either psoriasis-like diseases in strains harbouring spontaneous mutations, or phenotypes generated by specific germ-line modifications. These have been reviewed elsewhere (Schön 1999; Xia et al. 2003) and are covered by other chapters in this volume. The widely used SCID model represents a special approach, as it is not an animal model in the strict sense of the word. Again, current applications of this strategy are covered in-depth in other chapters in this volume and elsewhere (Nickoloff 2000). It would appear that one of the more critical questions to be addressed by the SCID model might be the issue of T-cell receptor specificity or at least TCR variable chain usage in circulating or plaque-resident T cells in recipient mice. This brief chapter simply aims at drawing attention to selected recently established mouse models of psoriasis revealing aspects felt to be relevant by the authors for a general view on psoriasis pathogenesis.

10.3 The IKK2 Model

In 2002, Pasparakis and colleagues reported on the phenotype of Cre-mediated K14-specific ablation of IKK2 in mice on a C57Bl/6 inbred genetic background (Pasparakis et al. 2002). By postnatal day 5, these mice develop a rigid skin with prominent scaling. Several features of this phenotype are reminiscent of psoriasis including a loss of the granular layer, hyperkeratosis, subcorneal pustules, dilated dermal vessels, increased keratinocyte Ki67-staining, overexpression of chemokines MIG, IP-10, and IL-8, and a dermal accumulation of $CD4^+$ cells. Importantly, the phenotype is reversed by genetic ablation of the TNF1-receptor (TNFR1), but not by deletion of the T-cell receptor. Several points can be learned from this experiment. First, a psoriasis-like phenotype can arise from intra-epidermal *down*-regulation of NFκB. Second, local down-regulation of NFκB may, as appears to be the case in these mice, cause a secondary up-regulation of TNF at sites spatially remote from the epidermal pathology. Third, keratinocyte differentiation, which is completely intact in these mice, may represent a pathological feature distinct from the inflammatory cascade in the human disease. All of these points certainly have a bearing on our ideas of psoriasis pathology, even though the murine phenotype as such is only of limited similarity.

10.4 The IRF2 Model

Hida and colleagues (2000) reported the development of a T-cell-mediated skin disease resembling psoriasis in mice deficient for IRF2. Incidentally, this phenotype was reported some 6 years after the initial description of IRF2 knockout mice. IRF2-deficient mice exhibit widespread skin inflammation and hair loss by 8 weeks postnatally. Features reminiscent of psoriasis in these mice include keratinocyte hyperproliferation, chemokine IP10 and MIG activation, and T-cell accumulation in the skin. Features not present in psoriasis include a disorganized muscle layer and prominent fibrosis. $CD8^+$ T cells are hyperresponsive to antigen in these mice; they accumulate as memory cells, and genetic ablation of CD8, but not of CD4, re-

verses the phenotype. Although this phenotype definitely does not model psoriasis closely, two major points are of interest. First, IRF2 physiologically serves as an inhibitor of type 1 IFN signalling. Accordingly, the phenotype of IRF2-deficient mice is mediated by a hypersensitivity toward type 1 IFN. Even though IRF2 is expressed ubiquitously, the phenotype of IFN hypersensitivity in the absence of IRF2 is limited to the skin and associated compartments. In humans, type 1 IFN is known to trigger psoriasis flares, both when injected directly in susceptible individuals and in the course of inflammation. Therefore, IFN hypersensitivity may represent a major disease-modifying mechanism, at least in a subgroup of psoriatics. This is all the more so as we could recently show a genetic association between the IRF2 gene and type 1 psoriasis (Foerster et al. 2004). Second, these mice demonstrate that overactivity of the type 1 IFN system can drive specific $CD8^+$ T cell-dependent inflammatory skin pathology, where these T cells are *not* specific in their TCR repertoire. Therefore, it is entirely possible that no specific set of recurrent antigen specificities underlies the T-cell activation operative in the lesions of plaque-type psoriasis.

10.5 The VEGF Model

Xia and colleagues (2003) recently published a paper on mice expressing VEGF under the control of the K14 promoter. Of all murine phenotypes described to date, these mice exhibit the closest resemblance to human psoriasis. Histological changes in these mice are strikingly similar to the human disease. Furthermore, this phenotype features a later-age onset and a pronounced Koebner phenomenon. The important conclusion of this phenotype is this: mice are able to mount a psoriasiform pattern closely resembling human psoriasis. Hence, genetic manipulation of mice appears to be a viable strategy to model the disease. Obvious experiments now helpful in a further assessment of a potential pathogenetic overlap of these mice with psoriasis include back-crossing to CD4-, CD8-, and TFNR1-backgrounds, clonality assessment of intra-lesional T cells, and treatment with anti-psoriatic drugs. Furthermore, a crossing of these mice with the allele-specific HCR transgenic mice (Kere et al., IID

2003, www.sidnet.org) would be of great interest. Conceptionally, the generation of these mice suggests that hyperactivity of vasculargenic signalling may be a very early event in human psoriasis.

10.6 The Ideal Model

Taking into consideration all aspects sketched out above, an ideal model of psoriasis should allow testing of candidate genes as well as meaningful large-scale screening of candidate drugs. Therefore, the ideal psoriasis model should feature not only phenotypic similarity (i.e. be a phenocopy) but also pathogenetic relatedness to the human disease on the molecular level. Given that this is unachievable at present due to our ignorance of the genetic lesions involved, phenotypes exhibiting the greatest morphological similarity to human psoriasis, such as the VEGF model, should be scrutinized in-depth, and aberrations in the corresponding signalling cascade be specifically sought out in the human disease. At the same time, even the analysis of phenotypes resembling psoriasis to a lesser degree can teach us important lessons. In the case of the K14-IKK2 and IRF2 mice, these lessons are that (1) the culprit genetic lesion may reside outside the skin, and (2) IFN hypersensitivity is sufficient to drive a $CD8^+$ T-cell-dependent skin inflammation of polyclonal nature.

In conclusion, formidable challenges are to yet to be met before psoriasis can be studied in detail aided by a model truly reflecting its complexity. Nevertheless, the experimental efforts already undertaken have advanced our understanding of the disease. Finally, the question remains as to which disease, if not psoriasis, should motivate researchers to overcome technical limits in the quest to tackle complex polygenetic diseases.

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11 From Classical Mouse Models of Psoriasis to a Spontaneous Xenograft Model Featuring Use of AGR Mice

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Psoriasis is chronic inflammatory skin disease (Krueger 2002) with a considerable socioeconomic burden. The quality of life of patients suffering from psoriasis is severely impaired (Ashcroft et al. 1999). The pathogenesis of this common disease is still poorly understood. Furthermore, current treatment options are limited, cumbersome, and time-consuming or have considerable side effects. Progress in defining the cause of psoriasis, as well as developing new treatment approaches, is thus urgently needed. Major progress in these areas critically depends on the availability of a relevant animal model of psoriasis (Schon 1999). This chapter briefly reviews current mouse models of psoriasis, and introduces a new xenotransplantation model, the so-called AGR psoriasis mouse model. The basis of any discussion related to this topic begins with the definition of the ideal psoriasis mouse model.

11.1 What Defines the Ideal Psoriasis Mouse Model?

Psoriasis is characterized clinically by persistent thick, reddish plaques, typically covered with silvery scales. These clinical features are explained by the pathogenic hallmarks of psoriasis:
1. Epidermal hyperproliferation
2. Presence of numerous *inflammatory cells*
3. Increased vascularity

An ideal animal model of psoriasis should reflect these clinical hallmarks, and should also display the typical *histomorphological patterns* of psoriasis such as acanthosis, papillomatosis, and hyperparakeratosis. The overwhelming evidence that *T cells* play a major role in psoriasis should also be a feature of the ideal mouse model. Finally, psoriasis lesions in such a model should be cleared with *known psoriasis drugs*. An ultimate goal would be that the pharmacologically validated animal model reflects the *clinical response in patients*. In the following, we review some of the currently available mouse models, and discuss them according to the proposed criteria of an ideal psoriasis mouse model. A synopsis of the mouse models is given in Table 1.

11.2 Spontaneous Mouse Mutation Models

Certain mutations in mice lead to phenotypes reminiscent of psoriasis with scaling and/or reddening of the skin. Mice homozygous for the *asebia locus* (ab/ab) display scaling of the skin due to epidermal acanthosis and hyperkeratosis (Arundell et al. 1969). The *Flaky skin (fsn) model* is an autosomal recessive mouse mutation that causes epidermal hyperplasia and inflammation, starting at 2 weeks of age (Sundberg et al. 1997). These mice have additional pathological features such as lymphadenopathy, mast cell accumulation, elevated serum IgE levels, and autoimmune glomerulonephritis. The *chronic proliferative dermatitis (cpd) model* displays epidermal hyperproliferation, a mixed inflammatory infiltrate, and enlarged dermal blood vessels. Overall these models seem to be useful to study pathogenic events such as epidermal hyperproliferation, but are limited in their

Table 1. Mouse models of psoriasis

Model	Epidermal hyperproliferation	Increased vascularity	Inflammatory cells	Histomorphological pattern	T cell infiltrate	Antipsoriatic treatments	Reflects human situation
Spontaneous mouse mutations							
Asebia	Yes	Yes	Yes	No	No	Not effective	No
Flaky skin	Yes	Yes	Yes	No	No	Not effective	No
Chronic proliferative dermatitis	Yes	Yes	Yes	No	No	n.d.	No
Transgenic mice							
K14/KGF	Yes	No	Yes	No	No	n.d.	No
K14/IL-6	Yes	No	Yes	No	No	n.d	No
K14/TGF-α	Yes	n.d.	Yes	No	Few animals	n.d	No
K14/IL-1α	Yes	n.d.	Yes	No	Few animals	n.d	No
Inv/IFN-γ	Yes	Yes	Yes	No	Yes	n.d	No
K14/amphiregulin	Yes	Yes	Yes	No	No	n.d	No
K10/BMP-6	Yes	Yes	Yes	No	Yes	n.d	No
K14/VEGF	Yes	Yes	Yes	Yes*	n.d.	n.d	No
Inv/Integrin (α2, α5, β1)	Yes	Yes	Yes	No	Yes	n.d	No
Knock-out mice							
PL/J/CD18	Yes	Yes	Yes	No	Yes	Dexamethasone	No

Table 1 (continued)

Model	Epidermal hyperproliferation	Increased vascularity	Inflammatory cells	Histomorphological pattern	T cell infiltrate	Antipsoriatic treatments	Reflects human situation
hypomorphic RAG2–/–IκBα–/–	Yes	n.d.	Yes	No	Lymphocytes	n.d.	No
Epidermal IKK2 deletion	Yes	Yes	Yes	No	Yes, no functional role	n.d.	No
Graft vs. host model							
CD4+/CD45RB^{hi}	Yes	Yes	Yes	No	Yes	UV-B, CsA	No
Xenotransplantation models							
Nude	Yes	Yes	Yes	Yes	Yes	Yes	Yes
SCID	Yes	Yes	Yes	Yes	Yes	Yes	Yes
AGR129	Yes	Yes	Yes	Yes	Yes	Yes	Yes

Inv, Involucrin; *strain-dependent; n.d., not done; CsA, Cyclosporine A.

value to reflect the pathogenesis of psoriasis. They fail especially with respect to the absence of a relevant T-cell pathogenesis, reflected by the inefficacy of cyclosporine A (CsA) in alleviating skin lesions in these models.

11.3 Transgenic and Knockout Mouse Models

Advances in mouse genetics and molecular biology have enabled the generation of mice with overexpression or absence of certain genes and their gene products. Such transgenic or knockout (KO) mice could be valuable tools to understand the pathogenesis of psoriasis and might provide potential models of psoriasis. Transgenic mouse models mostly overexpress certain genes such as cytokines or adhesion molecules in the epidermis under the control of a keratin promoter. Expression of the gene of interest under the influence of the keratin 14 promoter leads to expression of the gene of interest in the basal layers of the epidermis, while the involucrin and keratin 10 promoters lead to expression in suprabasal keratinocytes. Based on the proposal of the essential role of cytokines/growth factors in the pathogenesis of psoriasis (Nickoloff 1991; Kupper 1989) several groups engineered mice with overexpression of cytokines/growth factors in the epidermis. K14 promoter-driven expression of keratinocyte growth factor (KGF), interleukin 6 (IL-6), and transforming growth factor alpha (TGF-α) led to only marginal alterations of keratinocyte proliferation (Guo et al. 1993; Turksen et al. 1992; Vassar and Fuchs 1991). Overexpression of interleukin 1 alpha (IL-1α) under the keratin 14 promoter led to a macrophage-dominated dermal infiltrate, as well as some acanthosis and parakeratosis in severely affected cases (Groves et al. 1995). Involucrin/IFN-γ transgenic mice showed epidermal hyperproliferation, induction of MHC molecules on keratinocytes, and enlarged dermal vessels in a subset of mice (Carroll et al. 1997). These mice had eczematous features, hair hypopigmentation, and hair loss. K14 promoter-driven amphiregulin mice displayed a macroscopic phenotype suggestive of psoriasis including erythema and scaling. Histology showed hyperkeratosis, parakeratosis, acanthosis, and a mixed inflammatory infiltrate including T cells (Cook et al. 1997). Mice expressing K10/BMP-6 (bone

morphogenetic protein-6, a member of the TGF-β superfamily) showed reduction or increase of keratinocyte proliferation depending on the level of transgene expression (i.e., increased keratinocyte proliferation in low expressors).

Transgenic mice with constitutive expression of K14/VEGF (vascular endothelial growth factor) showed dilated vessels with an increase of mast cells (Detmar et al. 1998). Interestingly, a different line of K14/VEGF transgenic mice showed a more complete picture of psoriasis including epidermal hyperproliferation, T-cell infiltration, typical histology, and vascular changes (Xia et al. 2003). Transgenic mice overexpressing integrins (α2, α5, β1) under the involucrin promoter demonstrated flaking and inflamed skin with epidermal hyperproliferation (Carroll et al. 1995).

Taken together, overexpression of cytokines/growth factors in the epidermis of mice provides interesting insight into the role of cytokines/growth factors in skin, but do not reach the goal of a mouse model of psoriasis (e.g., missing T-cell pathogenesis).

Knockout mouse models also contribute to our knowledge related to psoriasis pathogenesis. PL/J/CD 18 hypomorphic mice developed erythema, hair loss, and scaling. Histopathology revealed epidermal hyperplasia, microabcesses in the stratum corneum, and lymphocyte exocytosis (Bullard et al. 1996). A potential role of altered NFκB expression in skin differentiation was demonstrated by Seitz et al. (1998). IKBα deficiency resulted in widespread dermatitis in mice. RAG2$^{-/-}$IκB$\alpha^{-/-}$ chimeras displayed psoriasiform dermatitis including epidermal hyperplasia and infiltration of lymphocytes (Chen et al. 2000). Epidermis-specific deletion of IKK2 induced a psoriasiform dermatitis dependent on TNF-α (Pasparakis et al. 2002), potentially supporting the role of NFκB and its regulators in epidermal differentiation and proliferation.

Taken together, knockout mouse models provide interesting insights into mechanisms of epidermal hyperproliferation and skin inflammation but do not display the key features required for a psoriasis mouse model such as proven T-cell immunopathogenesis and clear histopathological patterns. A recent interesting model using reconstitution of scid/scid mice with major histocompatibility (MHC)-matched, but minor histocompatibility mismatched CD4^{+}/CD45RB^{hi} T cells demonstrated many features of psoriasis and provided a

proof of concept of T-cell immunopathogenesis in this model. However, its graft vs. host disease (GvHD) character does not allow this model to be completely representative of a psoriasis lesion, since GvHD in humans does not lead to psoriasiform lesions.

11.4 Xenotransplantation Models

One of the main drawbacks of many "mouse models" of psoriasis is the absence of the specific microenvironment of human skin. Xenotransplantation models take advantage of the possibility to transplant human skin onto immunosuppressed mice without rejection of the transplanted tissue. The first such xenotransplantation model involved the transplantation of human skin onto nude mice. Human psoriasis skin could be maintained for more than 2 months on nude mice (Fraki et al. 1983). In addition, scid/scid recipients were used as excellent acceptors of human grafts. The severe combined immunodeficiency (SCID) mouse model was used to demonstrate that injection of autologous psoriatic T cells into xenotransplanted normal-appearing skin from the same patient was able to induce psoriasis (Wrone-Smith and Nickoloff 1996). These experiments established for the first time a strong link between T cells and the induction of psoriasis in a disease-relevant mouse model. The SCID mouse model was also validated pharmacologically, as several agents known to clear psoriasis such as vitamin D3 and cyclosporin A also improved psoriatic lesions using SCID mice (Dam et al. 1999). However, one major drawback of this model was the necessity to inject immunocytes or T cells into the graft to start the conversion from uninvolved to diseased skin. To study early pathogenetic events of psoriasis in immunosuppressed mice in the absence of any necessity for graft manipulation, a new mouse model of psoriasis was developed, as described next.

11.5 The AGR Mouse Model

SCID mice lack T and B cells, but show mature natural killer (NK) cells with normal NK cell activity. As NK cells are involved in rejection of xenogeneic tissue, SCID mice are not ideal acceptors of

human skin grafts. To improve acceptance of human grafts, the AGR129 mouse model of psoriasis was developed. AGR129 mice show NK cells with severely impaired cytotoxic activity in vitro and in vivo due to a deficiency in type I (A) and type II (G) IFN receptors, in addition to lacking T and B cells (RAG-$2^{-/-}$) (Grob et al. 1999). We used AGR129 mice as excellent acceptors of human prepsoriatic skin grafts to show spontaneous onset of psoriatic lesions (Boyman et al. 2004). Development of psoriasis did not require any exogenous cells or factor except for those contained within the engrafted skin itself. Our experiments demonstrated that proliferation of local T cells, dependent on TNF-α production, is an essential element of the development of psoriatic lesions (Boyman et al. 2004). The first experimental evidence derived from this new psoriasis mouse model points to a major importance of resident immune cells. The model has implications for new therapeutic strategies for psoriasis and other T cell-mediated diseases.

11.6 Conclusions

Psoriasis is one of the most prevalent T cell-mediated chronic inflammatory diseases. However, there is still a poor understanding of this disease's pathogenesis, as well as an array of therapeutic agents with ill-defined mechanisms of action. Newer xenotransplantation mouse models such as the AGR129 mouse model hopefully will provide the necessary scientific insights to connect new therapeutic measures such as biological agents with disease pathogenesis in this enigmatic disease.

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12 The Psoriasis SCID Mouse Model: A Tool for Drug Discovery?

W.-H. Boehncke

12.1 Modeling Psoriasis

12.1.1 Definition of the Disease

Psoriasis is a common inflammatory skin disease affecting about 2% of the population in the Western world. Its prevalence along with a chronic recurrent course turns it into a significant socioeconomic problem. The basic features of its pathogenesis have long been recognized and are readily reflected by the characteristic clinical phenotype of the most common variant, chronic plaque-stage psoriasis: Affected skin areas are erythematous and elevated because of a dense inflammatory infiltrate. The resulting plaques are covered by a silv-

ery scaling reflecting the excessive hyperproliferation of the keratinocytes. To date, there is broad consensus that inflammation precedes the epidermal changes, but the occurrence of inflammation and epidermal hyperproliferation are the hallmarks of psoriasis.

Histologically, both processes can be described in more detail. Elongated and dilated capillary loops in the dermal papillae mirror a state of increased angiogenesis, with the endothelial cells in the affected areas expressing adhesion molecules of the selectin class. The latter initiate the multistep process of leukocyte extravasation, a prerequisite in the formation of an inflammatory infiltrate, which is located in the upper dermis; but cells also invade the epidermis' neighboring areas of papillary dermis with the enlarged capillary loops. Lymphocytes contribute significantly to this infiltrate with T-helper cells being evenly distributed, whereas T-suppressor cells show a bias towards epidermal localization. Epidermal hyperproliferation results in profound epidermal thickening (acanthosis) and expanded epidermal rete pegs into papillary dermal space (papillomatosis). The turnover rate of epidermal cells is dramatically accelerated, resulting in faulty keratinocyte differentiation: The granular layer is constantly absent over the tips of the dermal papillae, and persisting nuclei of keratinocytes sometimes occur in the corneal layer (parakeratosis).

The complex phenotype of psoriasis is influenced by multiple factors, and there is ample evidence for several genes contributing to its genetic background.

By definition, a model system is a simplified representation of reality allowing more controlled manipulations of defined parameters. From a scientific point of view, a model is intriguing, if it is simple. Therefore, studies in cell culture systems seem more informative than those in complex systems, e.g., animal models. The opposite is often true in the field of applied biomedical research, e.g., drug discovery. A complex model might allow more reliable "extrapolations" towards the biological relevance of the effects observed in the real (patho-) physiological setting, e.g., a disease (Table 1).

Animal models are widely used in basic and applied biomedical research. Although complex, they still often meet the criteria of controlled manipulations of the parameters of interest. In this regard, the introduction of knockout and transgenic technologies allowed the

Table 1. Checklist for animal models representing psoriasis

Inflammation
Mononuclear infiltrate
Rich in lymphocytes
Accentuation in papillary dermis
Dilated capillary loops
$CD4^+$ T lymphocytes in dermis and epidermis
$CD8^+$ T lymphocytes primarily in the epidermis
Epidermal hyperproliferation
Acanthosis
Papillomatosis
Parakeratosis

generation of animal models as powerful tools for defining the biological role of genes, gene products, and cell populations. This approach, however, has two shortcomings: (1) frequently, manipulation of a single gene results in a highly complex phenotype, and (2) by definition, polygenetic diseases cannot truly be modeled by the manipulation of a single gene. Two other strategies involve studies of animals with psoriasis-like diseases and xenogeneic transplantation models.

12.1.2 Animal Models of Psoriasis

12.1.2.1 Psoriasis-Like Diseases in Animals

There are reports of psoriasis-like diseases in monkeys (Jayo et al. 1988), dogs (Mason et al. 1996), and pigs (Dunstan and Rosser 1986). A rhesus monkey described by Lowe et al. (1981) showed striking parallels with psoriasis, in as much as it suffered from a chronic dermatosis with erythematosquamous plaques localized at the extensor sites of the extremities (Table 2). The histology was similar to psoriasis, and intralesional injections of steroids improved the condition. Because these cases are sporadic and seemingly rare, they are not suitable for systematic investigations. Sometimes, spontaneously occurring mutations give rise to a psoriasis-like phenotype. Several of these mutations have been established as phenotypically fairly stable inbred strains, e.g., the *ic* mouse exhibiting epider-

mal hyperplasia, or a mouse strain with inducible psoriasis-like dermatitis (*hr*).

The mutation most vigorously studied is the "flaky skin mouse" (*fsn*) (Table 2). *Fsn* is a spontaneous autosomal recessive mutation mapped to chromosome 17 and characterized by multiorgan abnormalities including prominent erythematosquamous skin lesions (Beamer et al. 1995). Interestingly, a direct impact of the genetic background on the phenotype can be demonstrated: A7J mice with the *fsn* mutation develop an inflammatory infiltrate rich in neutrophils, whereas those cells are absent in the infiltrate of BALB/cByJ mice bearing the same mutation. This interdependence of genotype and phenotype is also seen in the human disease, where type I and type II psoriasis can be differentiated, both characterized by distinct HLA associations (Henseler and Christophers 1985). Bone marrow-derived cells represent the crucial effector cell population in the *fsn* mice. Transplantation experiments as well as cross-breeding clearly demonstrate that the respective cells are not lymphocytes (Sundberg et al. 1993). Still, although the *fsn* mutation does not share the proposed immunopathogenesis with psoriasis, it appears to be a useful model for studying local events resulting in hyperproliferative inflammatory alterations of the skin (Schon et al. 2001).

Although inbred mouse strains allow systematic investigations more readily than sporadic cases, they are still hampered by the disadvantage of the genetic defect not being exactly defined. This disadvantage can be overcome by transgenic animals.

12.1.2.2 Psoriasis into the Animal: Transgenic Models

Transgenic animals are characterized by a stable manipulation of their genome by integrating additional genes. Using this approach, a variety of animal models was generated exhibiting at least certain aspects of the complex phenotype of psoriasis (Sellheyer 1995; Boehncke 1997). Four of these approaches are particularly telling (Table 2).

Reasoning that cytokines such as TGF-α play a central role in epidermal hyperproliferation by acting as autocrine growth factors, two groups generated mice overexpressing TGF-α in the epidermis (Vass and Fuchs 1991; Dominey et al. 1993). The resulting phenotype was characterized by a psoriasis-like epidermal hyperplasia and

Table 2. Strategies for the development of animal models for psoriasis

Strategy	Phenotype	Comment	Reference
Sporadic cases			
Rhesus monkey	Typical clinical picture Leukocytic infiltrate	Single case	Lowe et al. 1981
Spontaneous mutation			
Flaky skin mouse	Epidermal hyperproliferation	Genetic background influences phenotype	Beamer et al. 1995; Sundberg et al. 1993; Schön et al. 2001
	Leukocytic infiltrate Additional features (anemia)	Lymphocytes key effector cells	
Transgenic animal			
TGF-α	Psoriasis-like epidermis	No inflammation	Vassar and Fuchs 1991
Integrins	Chronic recurrent course Typical histology	Central role for keratinocyte dysregulation	Dominey et al. 1993; Carroll et al. 1995
BNLF-1	Psoriasis-like epidermis Aberrant keratin 6 expression Inflammatory infiltrate	"Spontaneous" carcinomas	Wilson et al. 1990
HLA-B27, β2m	Psoriasis-like epidermis Inflammatory infiltrate Additional organs affected	Clinical picture of spondyarthropathy	Hammer et al. 1990
Xenotransplantation			
SCID-hu	Preservation of the full complexity of lesional skin	Possibility to analyze each component of the "human system"	Boehncke et al. 1994, 1996; Nickoloff 2000; Gilhar et al. 2002

the development of benign papillomas, but absence of an inflammation.

Besides cytokine overexpression, a second approach also targeted keratinocytes: Whereas under physiological conditions only basal keratinocytes express adhesion molecules of the integrin family, their suprabasal expression is restricted to certain pathological conditions including wound healing and psoriasis. Consequently, transgenic mice were produced expressing β1-integrins under the control of the involucrin promoter, thus limiting their expression to suprabasal keratinocyte layers. The resulting phenotype showed striking similarities to psoriasis, namely typical epidermal alterations along with a profound inflammatory infiltrate rich in lymphocytes with T-suppressor cells being restricted to the epidermis; the inflammation had a chronic recurrent course (Carroll et al. 1995).

Originally designed for elucidation of Epstein-Barr virus (EBV)-mediated carcinogenesis, a mouse expressing the EBV oncogene BNLF-1 turned out to exhibit a psoriasis-like epidermal phenotype characterized by neo-expression of keratin 6, a feature highly characteristic (but not specific) for psoriasis (Wilson et al. 1990).

Finally, it is worthwhile to mention a rat model with a highly complex phenotype. Having failed to generate a phenotype in mice, HLA-B27 and human β2-microglobulin (which is important for the function of HLA class I molecules) were expressed in rats. These animals developed inflammatory diseases of several organs including the skin and nails – resembling psoriasis – and joint involvement (Hammer et al. 1990). This model differs from all other approaches mentioned inasmuch as the systemic expression of the functional HLA-B27 molecule triggers a multi-organ disease comprising a psoriasis-like dermatosis.

In summary, transgenic animals allow clear-cut genotype-phenotype analyses. It is noteworthy, however, that the phenotype of single transgenic animals may turn out to be surprisingly complex, and still they mirror only certain aspects of psoriasis. Finally, as pointed out already, a polygenetic disease cannot completely be represented by a single transgenic animal by definition. To overcome these obstacles, xenogeneic transplantation models have been explored.

12.1.2.3 Psoriasis onto the Animal: Transplantation Models

Xenogeneic transplantation models in which human skin is grafted onto animals with an immune deficiency preventing graft rejection seem to be the obvious links between well defined animal models and patients. Theoretically, nude mice (*nu*) should be suitable recipients for xenografts. These mice are athymic, thus lacking thymus-dependent immune responses including the ability of graft rejection (Reed and Manning 1973). Several groups explored the possibility to transplant lesional and nonlesional skin from patients with psoriasis onto nude mice, but observed resolution of the characteristics of lesional skin as well as psoriasis-like transformation of normal skin (Haftek et al. 1981; Krueger et al. 1975). This approach is therefore not suitable for studies on psoriasis pathogenesis or drug development.

Nonspecific alterations of grafts using nude mice as recipients might be due to the still-active B-cell system in those mice. Consequently, similar experiments were repeated in mice characterized by a combined deficiency of the T- as well as the B-cell system. In 1983, Bosma et al. described the autosomal recessive *scid* mutation on chromosome 16 (Bosma et al. 1983). This mutation affects V(D)J rearrangement and double-strand break repair (Schuler et al. 1986; Foulop and Phillips 1990; Roth et al. 1992). V(D)J rearrangement in SCID mice is characterized by defective coding joint formation, which prevents the development of mature B and T lymphocytes, hence the designation SCID for severe combined immunodeficiency. These mice are even better recipients for xenografts than nude mice. Consequently, SCID-hu xenotransplantation systems have been used in many fields of research investigating basic principles in biology as well as complex pathomechanisms of defined diseases (for review, see Boehncke 1999). The fact that human skin grafted onto SCID mice persists largely unaltered throughout the life span of the recipient (about 2 years) (Kaufmann et al. 1993) paved the way for the broad application of this model in the field of dermatology. Since then, this approach has proven extremely powerful, e.g., to model leukocyte recruitment to the skin (Yan et al. 1994), and regarding allergic and autoimmune diseases (Petzelbauer et al. 1996; Herz et al. 1997; Zillikens et al. 2001).

With regard to research on psoriasis, the major breakthrough came when it was demonstrated that lesional as well as nonlesional psoriatic skin could be grafted onto SCID mice, and both phenotypes were preserved at least for a period of several months (Boehncke et al. 1994) (Table 2). This finding enabled several groups to perform highly informative experiments on the pathogenesis of psoriasis. Using this approach, direct experimental evidence was generated that bacterial superantigens trigger psoriasis (Boehncke et al. 1996), and that T lymphocytes play a central role in the onset of this disease (Nickoloff 2000).

Given the contribution of the psoriasis SCID mouse model to elucidation of its pathogenesis, it was only logical to evaluate its usefulness in drug discovery.

12.2 The Psoriasis SCID Mouse Model as a Tool in Drug Discovery

12.2.1 Limitations

As described so far, a considerable number of animal models have been established reflecting at least certain aspects of the complex clinical appearance of psoriasis. Some of these models have been informative with regard to its pathogenesis, or at least in unraveling the molecular basis for different phenomena contributing to the establishment of a psoriatic lesion, e.g., epidermal hyperproliferation. They have not been particularly helpful as tools in drug discovery, however. This is not too surprising given that none of the animal models really preserves the full complexity of the human disease. And sometimes, application of the identical animal model may yield different and sometimes even contradicting results in the hands of different groups. This holds true even for the vigorously studied flaky skin mouse, where some groups see a therapeutic efficacy of steroids, while others do not.

The psoriasis SCID mouse model seems to be the perfect tool for testing innovative therapeutic strategies before introducing them into the clinical phase of development. One has to admit, however, that

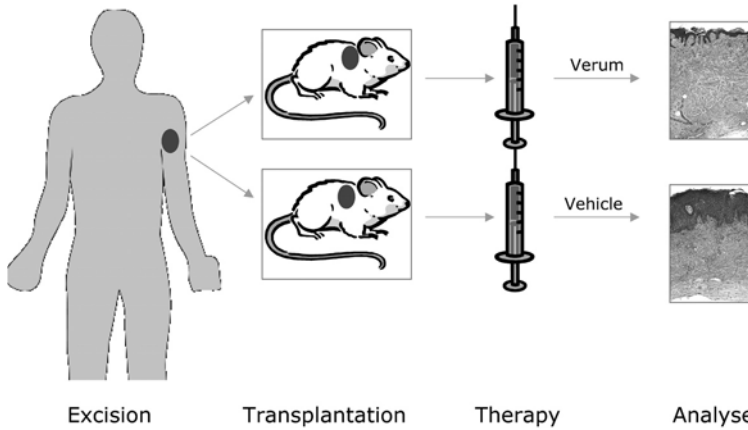

Fig. 1. The psoriasis SCID mouse model represents a xenotransplantation of lesional psoriatic skin from a patient/donor to a mouse with severe combined immunodeficiency (*SCID*). Following excision of the skin, grafts are derived and placed onto the back of SCID mice. After a healing phase of several weeks, the treatment protocol is initiated. Upon completion of the therapy the mice are sacrificed, and grafts are analyzed

from a certain point of view this is not really a model. It is still the original disease with the lesion being separated from the patient prior to manipulations/treatment (Fig. 1).

This implies that factors initially active in the patient (donor) may also influence the graft. Drugs used to treat the patient may well influence the results obtained. Hence, application of the psoriasis SCID mouse model should be planned as carefully as a clinical study. Criteria to select suitable donors should include a sufficient "washout" phase from previous treatments before skin is excised and grafted. Additionally, the nature of the disease should be defined as specifically as for the purpose of a clinical study: Confirmation of the correct diagnosis, duration of the disease, type of lesion, and severity are relevant parameters in this regard.

Use of the psoriasis SCID mouse model is labor-intensive. The absolute number of grafts generated and analyzed in any given project is therefore limited. Further restrictions of this model's power lie in the fact that usually several grafts are derived from one lesion

of a single donor. Thus, the data generated from these grafts represent only one experiment and are comparable to repeated measures of the same condition in an in vitro experiment. A typical protocol asking for nine grafts from one donor, with three being treated alike (e.g., positive and negative control plus test drug) is like performing one reaction of an in vitro experiment in triplicate. If this experiment was repeated three times, it would equal a clinical trial focusing on one marker lesion in only three patients. Therefore, the psoriasis SCID mouse model is suitable to generate a proof of principle in vivo, but can hardly be used to screen large numbers of drugs or to establish dose-response curves.

As mentioned above, use of the psoriasis SCID mouse model implies the removal of skin from a donor. Although this does not necessarily have to be full-thickness skin (Boehncke et al. 1994, 1996), and split-skin obtained with a dermatome has been shown to be equally useful (Gilhar et al. 2002), one still has to convince a patient to become a donor. It is unlikely that a patient (or an ethics committee!) will allow the researcher to excise as much skin as he wants. Consequently, the number of grafts that can be obtained from one donor is limited. Results obtained with the grafts from the same donor usually are fairly consistent, but there is ample inter-individual variability making it difficult to pool the data from different series of transplantations (Boehncke et al. 1999). This can be controlled to a certain degree by specifically defining the type and the location of the lesion from which the grafts must be derived. But the variability in the clinical course among patients is also expected to influence the fate of these patients' grafts. It would therefore not come as a surprise if a test drug exhibited efficacy in grafts from one donor but failed to do so in another, given that most established antipsoriatic therapies show response rates not higher than around 80%.

Obtaining skin from a donor involves risks and adverse effects. The skin must be excised with local anesthesia, and patients need to be informed about all risks in the context of a minor surgical procedure such as this, including scar formation, although wounds within lesional psoriatic skin are usually characterized by an almost perfect wound healing process. Given the risk of scar formation, a clinician must answer the question why separation of the lesion from the patient is so crucial for the respective purpose.

12.2.2 Alternatives and Advantages

Psoriasis can be treated either topically or systemically. Innovative developments are under way for both routes of application. Although the psoriasis SCID mouse model is generally applicable to screen any type of drug and application route, its true domain is testing the efficacy of innovative antipsoriatic drugs applied systemically.

Human skin grafted onto SCID mice is known to preserve many of its characteristics (see Sect. 12.2.3). But this does not necessarily hold true for all parameters. One particularly sensitive point is the barrier function of the skin. Although not yet formally proven, one can postulate that human split-skin grafts will exhibit deficits in this regard. Even when using full-thickness skin, one must consider that the graft is surrounded by murine skin exhibiting a high density of hair follicles and a much thinner epidermis. In either setting, inflammatory processes going along with wound healing after transplantation might also have an impact on this parameter. Therefore, results of studies with topically applied drugs must be interpreted with care. Effects observed may well be due to systemic rather than topical effects of the test drug, which should penetrate the graft or surrounding murine skin more readily compared to lesional skin in situ. Even if systemic effects did not occur, one is left with the possibility that a test drug exhibits effects only because a disturbed epidermal barrier allows penetration at least into the skin compartment, whereas this might not occur on lesional skin in situ.

Considering safety aspects, one necessary side effect of the psoriasis SCID mouse model is scar formation at the site of excision. In the context of topical drug application, this represents one of the most severe adverse effects expected. Therefore, the alternative of the psoriasis plaque test is worth considering. Here, the treatment is limited to a marker lesion on the patient, and the effects can be monitored clinically (Bangha and Elsner 1996). When biopsies are taken, a comprehensive set of data can be generated in this setting. Since the original disease is treated, the results reflect clinical reality. Also, the time course of the effects observed can be established more readily compared to the SCID model. Additionally, dose-finding studies are easy when assigning different lesions (or segments of a major lesion) to distinct drug concentrations. Scar formation would

Table 3. Synopsis of the psoriasis plaque test

Advantages	Disadvantages
Dealing with the "real" disease	Uncertain safety if compound is poorly characterized
Well established for screening of topically applied drugs	
Time course studies possible	
Dose-response studies possible	

Table 4. Synopsis of the psoriasis SCID mouse model

Advantages	Disadvantages
Preserved complexity of the psoriatic lesion	Labor-intensive
Good consistency with clinical observations	Qualitative answers
Safe for the patient	No time course
	Small grafts (topical application!?)

only occur in case of toxic effects. Among the disadvantages of this approach is the possibility to sensitize the patient to the drug. In order to properly inform the patient, one also needs to have at least some preliminary data on pharmacokinetics and mutagenicity of the test drug. In conclusion, the psoriasis plaque test is a suitable tool to test topically applied drugs once a fundamental set of data on toxicology, pharmacokinetics, and mutagenicity is available (Table 3). Among the drugs for which published data obtained with the psoriasis plaque test are available are steroids, retinoic acid, cyclosporine A, vitamin D derivatives, anthralin, mycophenolate mufetil, calcineurin inhibitors, and hydroxyl urea; all drugs were applied topically.

When it comes to systemic treatments, the psoriasis plaque test is still an option. And it has been used with great success for different compounds. The most common route of application is subcutaneous injection. Examples comprise the anti-CD11a antibody efalizumab and cytokines (Asadullah et al. 1998; Gottlieb et al. 2003). On the other hand, drugs that have not yet been characterized sufficiently with regard to their safety profile and potential hazards can readily

be used in the psoriasis SCID mouse model. This might be particularly important when a proof of principle is needed in order to decide on the commitment to a drug development program at the doorstep to clinical phases (Table 4).

In this context it is important to evaluate the consistency of results obtained in the psoriasis SCID mouse model and clinical experiences.

12.2.3 Correlation of the Psoriasis SCID Mouse Model with Clinical Experiences

Published observations on the efficacy of established antipsoriatic treatments in the psoriasis SCID mouse model date back to the year 1999.

The first such publication (Boehncke et al. 1999) adopted a protocol used by the same group for pathogenesis studies (Boehncke et al. 1996): Full-thickness lesional skin was excised from three patients with chronic plaque-stage psoriasis. The skin of each donor was used to derive 12 grafts, each transferred onto an SCID mouse. Three mice formed one group undergoing an identical protocol: Daily oral application of dexamethasone (0.2 mg/kg body weight) over a period of 4 weeks served as positive control, daily oral applications of PBS was used as negative control, and the leukotriene synthesis inhibitor BAY X 1005 at doses of 1 and 5 mg/kg body weight given orally twice daily was the test drug in this particular project. After the mice were sacrificed, the grafts were analyzed histologically, using a semi-quantitative classification for papillomatosis, parakeratosis, and Munro's microabscesses. Additionally, epidermal thickness was measured quantitatively using an ocular micrometer. The results obtained were highly consistent within the treatment groups, but considerable inter-individual variability occurred. Overall, dexamethasone exhibited a robust antipsoriatic efficacy mirrored by profound reduction of acanthosis and papillomatosis as well as parakeratosis. Moreover, the inflammatory infiltrate was substantially reduced, and Munro's microabscesses could rarely be detected. This is in line with clinical observations documenting the anti-inflammatory and antipsoriatic efficacy of steroids. Interestingly, the lack of

efficacy seen in the case of BAY X 1005 also parallels clinical experience (R. Müller-Peddinghaus, personal communication) (Table 5).

In the same year, Dam and co-workers published a study with 1a,25-dihydroxycholecalciferol, cyclosporine A, and interleukin-10 (Dam et al. 1999). Here, split-skin obtained using a keratome from six patients was used. The publication makes it impossible, however, to exactly delineate treatment protocols with regard to group sizes and number of individuals from which the grafts were derived. The drugs were injected intradermally twice weekly for 3 weeks at doses of 63 ng (dihydroxycholecalciferol), 0.15 mg (cyclosporine A), and 990U (interleukin-10). Because the grafts were relatively large (1.7×2.2 cm), the authors were able to evaluate their clinical appearance throughout the study phase using a scoring system for the parameters scaliness, induration, and erythema. Additionally, the grafts were analyzed histologically after completion of the treatment phase; this included measuring epidermal thickness. Based on these criteria, resolution of the psoriatic phenotype was observed under the treatment protocols utilizing dihydroxycholecalciferol and cyclosporine A. Notably, interleukin-10 failed to show efficacy in this study, according to the authors. As in the study by Boehncke et al. (1999), the results for dihydroxycholecalciferol and cyclosporine A correspond well to the clinical experiences: Both drugs are well-established treatment modalities for psoriasis (Morimoto et al. 1986; Wong et al. 1993). The failure of interleukin-10 to show efficacy in this model, however, comes to a surprise and contradicts published observations of clinical effects in a pilot phase I/II study (Asadullah et al. 1998) (Table 5).

Efalizumab is a monoclonal antibody to human CD11a, thus blocking the function of LFA-1. This molecule is involved in T-cell activation as a co-stimulatory molecule, interacting with ICAM-1 on antigen-presenting cells. Additionally, interactions between LFA-1 and ICAM-1 play a role in lymphocyte extravasation, a crucial process for cutaneous inflammation. Efalizumab (Remicade) has proven antipsoriatic efficacy in clinical phase III studies and awaits approval for the treatment of psoriasis (Gottlieb et al. 2003; Boehncke 2003). This biologic has been used in the psoriasis SCID mouse as well; the transplanted mice were treated over a period of 2 weeks by daily intraperitoneal injections (6 mg/kg body weight). Additional

Table 5. Correlation between efficacy of established antipsoriatic drugs in the psoriasis SCID mouse model and clinical experience

Drug	Treatment protocol (SCID)	Outcome SCID model	Clinical experience
Dexamethasone	4 weeks orally 0.2 mg/kg	Good efficacy	Rarely used
Clobetasol proprionate	3 weeks topically	Moderate efficacy	Well-established Mild to moderate psoriasis
Cyclosporine A	2 weeks i.p., 20 mg/kg 4 weeks i.c., 0.15 mg	Good efficacy	Well-established moderate to severe psoriasis
1α,25-Dihydroxy-cholecalciferol	4 weeks i.c., 63 ng	Good efficacy	Usually used topically mild to moderate psoriasis
Efalizumab (Raptiva)	2 weeks i.p., 6 mg/kg	Good efficacy	Effective, moderate to severe psoriasis

groups received cyclosporine A (daily intraperitoneal injections, 20 mg/kg body weight), clobetasol proprionate (topically), or PBS (Ziegler et al. 2001). Full-thickness grafts were derived from 13 patients with psoriasis, and the readout was a computerized evaluation of the epidermal area along with classic histology and immunohistochemistry. The authors found a reduction in epidermal area under the active treatments, although the density of infiltrating T lymphocytes seemed to be unaltered (Table 5).

Taken together, the observations published to date document consistency regarding the efficacy of established antipsoriatic drugs in the psoriatic SCID mouse model and clinical practise. This has so far been demonstrated for steroids, dihydroxycholecalciferol, and cyclosporine A. With the exception of clobetasol proprionate, all drugs were given systemically. On the other hand, a compound that failed to show sufficient clinical efficacy to validate further development also showed only minimal effects in the psoriasis SCID mouse model. The sole compound where contradicting published data exist is

interleukin-10. Still, interleukin-10 is not among those candidates close to be filed for approval as an antipsoriatic treatment. This may indicate that, although principally effective, its potential does not seem to be as good as that of many other biologics.

12.2.4 Perspectives: Preclinical Evaluation of Innovative Therapeutic Strategies

Extrapolation to the clinical setting is a key requirement for models used in order to identify promising candidates for innovative therapies. This criterion is met by the psoriasis SCID mouse model, which has particular advantages over alternative approaches when it comes to evaluation of systemic therapies (see Sect. 12.2.2). Besides investing numerous drugs which are already well established in the treatment of psoriasis (see Sect. 12.2.3), the psoriasis SCID mouse model is now widely used to identify candidate compounds suitable for clinical evaluation. Several publications document the application of this model to provide proof of principle for novel therapeutic strategies (Table 6).

Table 6. Innovative (not yet established) therapies tested in the psoriasis SCID mouse model for which published data are available

Drug	Treatment protocol	Outcome SCID model	Comment
Troglitazone	6 weeks orally, 200 µg	Significant reduction of epidermal hyperplasia	Five patients treated successfully; drug sale discontinued (liver toxicity)
PS519 (proteasome inhibitor)	4 weeks i.p., 1 mg/kg	Good efficacy	Other proteasome inhibitors in phase III (not psoriasis)
Efomycine M	4 weeks s.c., 5 mg/kg	Good efficacy	Bimosiamose effective in pilot phase I/II

Psoriasis is often treated with agents that activate nuclear hormone receptors of steroids, retinoids, and vitamin D. A related nuclear hormone receptor is the peroxisome proliferators-activated receptor-γ (PPARgamma) that can be activated by its ligands, including the thiazolidinediones (Henry 1997; Spiegelman 1998). Ellis and co-workers used troglitazone, a currently available thiazolidinedione, for the treatment of diabetes mellitus in the psoriasis SCID mouse model (Ellis et al. 2000). Lesional skin from three patients was obtained, and a total of 22 grafts were transplanted onto 11 SCID mice (two per mouse). The mice were treated orally with 200 μg of troglitazone daily over a period of 6 weeks before grafts were harvested and the area of the epidermis was determined. This treatment protocol resulted in a significant reduction of the epidermal area, and a normalized pattern of epidermal differentiation occurred. Additionally, troglitazone was given to five patients with moderate to severe psoriasis at doses ranging from 200 to 600 μg/day over a period of 12 weeks. The authors noticed a "substantial improvement" of all patients, but did not give additional objective measures.

Troglitazone can therefore be added to the list of drugs for which consistent results have been obtained in the psoriasis SCID mouse model and in patients. This example also highlights that efficacy of a therapy can readily be tested in the psoriasis SCID mouse model, whereas safety cannot: Shortly after submission of the manuscript by Ellis and co-workers in September 1999, the manufacturer of troglitazone discontinued its sale because of reports of liver toxicity (March 2000).

Adhesive interactions, regulated by cytokines and chemokines, are crucial for leukocyte localization at sites of inflammation. The first steps of leukocyte recruitment include rolling on the vessel wall mediated by selectins and glycoproteins bearing the sialyl Lewis^x moiety. Adhesive interactions involved in leukocyte extravazation represent attractive targets for the treatment of inflammatory processes (Boehncke and Schon 2003). Two complementary approaches to interfere with leukocyte rolling were recently published where in vivo proof of principle was generated using the psoriasis SCID mouse model:

Nuclear factor κB (NF-κB) is a pivotal transcription factor in chronic inflammation. Given that it is involved in the control of the

genes that encode E-selectin and the homing receptor for skin, cutaneous lymphocyte-associated antigen (CLA), it is thought to be important for leukocyte rolling. To function, NF-κB must be released from the inhibitory protein IκB, a task performed by a proteolytic particle, the proteasome. Proteasome inhibition is a means to prevent NF-κB activation and is therefore predicted to result in anti-inflammatory effects (Elliot et al. 2003). In a series of very elegant experiments, Zollner et al. were able to demonstrate that a proteasome inhibitor, PS519, indeed suppresses NF-κB binding to DNA and consequently downregulates expression of NF-κB-dependent expression of E-selectin ligands, such as CLA. This results in reduced T-cell rolling in vivo as demonstrated by intravital microscopy (Zollner et al. 2003). Subsequently, SCID mice bearing full-thickness grafts from three patients with chronic plaque-stage psoriasis were treated with daily intraperitoneal injections of PS519 at a dose of 1 mg/kg over a period of 4 weeks. Dexamethasone (0.2 mg/kg) and PBS served as positive and negative controls, respectively. Each treatment group consisted of nine mice. Treatment with PS519 as well as dexamethasone resulted in a highly significant reduction of epidermal thickness ($p<0.001$), paralleled by a reduction in the cutaneous infiltrate also reaching significance ($p<0.05$). Although PS519 has not yet been used in patients, the general feasibility of this approach is underlined by the fact that some proteasome inhibitors are currently in phase III trials for other indications (Elliot et al. 2003).

Since interference with the expression of CLA results in reduced leukocyte rolling and anti-inflammatory effects, this may also be true for the complementary strategy of blocking the ligand for CLA, E-selectin. This concept was tested in the psoriasis SCID mouse model utilizing efomycines, a new family of small molecules that structurally mimic the binding sites of selectin ligands, thus functioning as sialyl Lewis^x mimetics and competitive inhibitors of CLA (Schon et al. 2002). Having demonstrated that efomycine M interferes with selectin-mediated adhesion in vitro and in vivo, a similar experiment as in the case of PS519 was performed. Full-thickness skin from three patients with chronic plaque-stage psoriasis was dissected into nine grafts each and transplanted onto SCID mice. The treatment consisted of a 4-week course with daily subcutaneous injections of efomycine M (5 mg/kg). Oral application of dexametha-

sone (0.2 mg/kg) and subcutaneous injections of PBS were the positive and negative controls, respectively. This experiment convincingly documented the efficacy of efomycine M, mirrored by a significant reduction in epidermal thickness ($p<0.01$) and substantial reduction of the inflammatory infiltrate compared to PBS.

As is the case for PS519, efomycine M has not yet been used to treat patients with psoriasis. However, a pilot phase I/II trial has been initiated with another small molecule selectin inhibitor, bimosiamose, after its efficacy was demonstrated in the psoriasis SCID mouse model (M. Friedrich, personal communication).

In summary, tests with established anti-psoriatic drugs in the psoriasis SCID mouse model generated results which were fairly consistent with clinical experiences. This validates its application for screening novel therapeutic strategies. And so far, extrapolation from the results obtained in the psoriasis SCID mouse model has also proved to be reliable with regard to the efficacy of the respective therapy. Currently, numerous companies use this model to evaluate promising candidates before they are introduced into the clinical phase of development.

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13 Pro- and Anti-inflammatory Effects of IL-4: From Studies in Mice to Therapy of Autoimmune Diseases in Humans

T. Biedermann, M. Röcken

13.1 IL-4: A Pro- or Anti-Inflammatory Cytokine?

Interleukin (IL)-4 is largely known for its anti-inflammatory effects due to its capacity to suppress Th1 responses and protective immunity against intracellular pathogens. This was first demonstrated in 1990 in infections of mice with *Leishmania major* (Sadick et al. 1990). This report showed that early and complete neutralization of IL-4 for 6 weeks with anti-IL-4 mAb redirects Th2 into Th1 immunity and provides protective immunity against *L. major*. This conflicts with a 1989 study, where IL-4 transfection of tumor cells induced potent anti-tumor immunity (Tepper et al. 1989). Based on this first report with cytokine-transfected tumor cells, several clinical trials with IL-4 in humans were conducted in tumor patients but failed. The understanding of IL-4 was further complicated by data

showing that IL-4-producing Th2 cells directly mediate tissue destruction and lead to autoimmune diseases if transferred to immunodeficient hosts (Pakala et al. 1997; Lafaille et al. 1997). These data indicated that the anti-inflammatory role of IL-4 depends on several co-factors that influence the outcome of immune responses but remained to be identified.

13.2 Differential Role of IL-4 During the Initiation Versus Established Immune Response

In leishmaniasis, Th1 cells mediate delayed-type hypersensitivity reactions (DTHRs), which provide protective immunity. In contrast, Th2 responses to *L. major* fail to establish protective DTHRs and consequently, Th2-dominated immune reactions to *L. major* provide only insufficient protection. In the case of leishmaniasis, Th2 cells lead to fatal disease courses. DTHRs are important for the integrity of the host organism in case of infection with intracellular pathogens, but Th1 cells can also mediate harmful DTHRs that cause inflammatory autoimmune diseases. In cases of such harmful DTHRs, antigen-specific deviation of Th1 immunity into Th2 immunity may be a new approach possibly devoid of the side effects associated with the current immunosuppressive treatment regimens. Whether it is possible to induce anti-inflammatory Th2 cells in order to protect against Th1-mediated, harmful autoimmune and inflammatory diseases was first tested in experimental allergic encephalomyelitis (EAE), an animal model for multiple sclerosis. The results showed that antigen-specific Th2 cells did not induce EAE and that a Th2-inducing regimen prevented the development of EAE (Racke et al. 1994; Nicholson et al. 1995; Falcone et al. 1998). These studies showed that deviation of a Th1 immune response by IL-4 into an IL-4-producing Th2 phenotype can attenuate DTHRs. Based on these and other studies in mice, Th-cell differentiation in patients suffering from organ-specific autoimmune diseases such as multiple sclerosis, inflammatory bowel diseases, rheumatoid arthritis, and psoriasis were analyzed. The data showed that all these diseases were associated with Th1 responses. For human diseases, treatment of ongoing inflammatory processes as opposed to prevention is

needed. Therefore, studies were designed to investigate the therapeutic potential of anti-inflammatory Th2 cells in established DTHRs.

Among the various model diseases for inflammatory autoimmune diseases, contact hypersensitivity responses of the skin (CHS) to exogenous haptens in sensitized animals are of special interest. CHS and skin inflammation can be more rapidly and easily elicited, monitored, and correlated with functional ex vivo immune assays than organ-specific autoimmune diseases. Studies with such a CHS model allowed us to show that therapeutic interventions with IL-4 can even be effective in the therapy of established CHS (Biedermann et al. 2001 a). IL-4 therapy during ongoing CHS-reaction inhibited skin inflammation and significantly reduced the ratio of IFN-γ to IL-4 during subsequent CHS challenges. This therapeutic effect lasted for at least 8 weeks. Over the past 10 years, inhibition of Th1 cell-mediated development and DTHRs by IL-4 or Th2 cells was demonstrated in a variety of disease models. Thus, IL-4 and IL-4-producing Th2 cells are generally considered to be important antagonists of Th1-induced inflammatory immune responses (Racke et al. 1994; Röcken et al. 1996; Liblau et al. 1995). Importantly, adoptive transfer of antigen-specific Th2 cells together with the respective antigen can also inhibit CHS. Yet this inhibition occurs not directly, but only after repeated antigen application (Biedermann et al. 2001a). This delayed therapeutic effect of IL-4-producing Th2 cells indicated that Th2 cells may interfere with Th1-mediated DTHRs not directly but indirectly by deviating newly primed naïve Th cells or even Th1 cells towards a Th2 phenotype. Whether and how Th2 cells can deviate the development of naïve Th cells prone to become a Th1 phenotype toward a Th2 phenotype was investigated with T cells from transgenic mice, each expressing a different TCR. One TCR-transgenic Th cell was a Th2 cell line and the other TCR-transgenic Th cell was a naïve Th cell population. Both were activated with a Th1-driving adjuvant (FA) and the respective peptides, in vivo. With the use of clonotypic antibodies, we were able to show that Th2 cells can co-localize with naïve Th cells in one lymph node area. If, and only if, the Th2 cells were activated simultaneously and in the same lymph node as the naïve Th cells, the Th2 cells determined the differentiation of the naïve Th cells and deviated from a Th1 toward a Th2 phenotype, even in the presence of the Th1-inducing adjuvant CFA (Schipf et al. 2003).

13.3 IL-4 in the Treatment of the Th1-Dominated Disease Psoriasis

Thus, data from animal models demonstrated that in vivo deviation of immune responses by IL-4 or IL-4-producing Th2 cells could be an effective and promising approach to treat inflammatory autoimmune diseases in humans. To address this question, a dose-escalation study was set up to investigate the therapeutic effects of IL-4 in psoriasis, a Th1-associated inflammatory autoimmune disease of the skin and joints (Ghoreschi et al. 2003). Patients were trained for continuous IL-4 application 5 days a week over a 6-week period. This regimen strongly reduced the clinical disease score in psoriasis patients at concentrations known to induce Th2-cell development in vitro. Analysis of skin biopsies revealed that this regimen reduced pro-inflammatory Th1-related cytokines such as IFN-γ, IL-8, and IL-19, a cytokine that is thought to promote keratinocyte proliferation. This was not only due to Th lymphocyte deletion, as Th lymphocytes were still detectable in psoriatic skin. These Th cells no longer expressed the Th1-marker chemokine receptor CCR5, and IL-4 became detectable in skin samples. Thus, IL-4 was capable of inducing IL-4 in humans, to inhibit the expression of prototypic pro-inflammatory Th1 cytokines and to improve human inflammatory autoimmune disease.

13.4 Role of IL-4 in Dendritic Cell Maturation

Besides its capacity to induce in Th cells a Th2 phenotype and in B cells the IgE switch, other effects of IL-4 have been discovered in early experiments. The early report that IL-4 transfection of tumor cells established protective immunity lead to phase I and II trials testing IL-4 in cancer immunotherapy (Tepper et al. 1989). These trials failed. Yet experiments with either IL-4-transgenic or IL-4-deficient mice or neutralizing IL-4 antibodies suggested that, under certain conditions, IL-4 may paradoxically promote Th1 differentiation and DTHRs (Salerno et al. 1995; Erb et al. 1997; Noben-Trauth et al. 1996; Mencacci et al. 1998; Schuler et al. 1999; Bagley et al. 2000). For instance, genetically IL-4-deficient mice have defects in

developing severe EAE or Th1 responses when infected with certain strains of *L. major* or *Candida albicans (C. albicans)* (Noben-Trauth et al. 1996; Mencacci et al. 1998). Similarly, a critical role for IL-4 was demonstrated in the development of efficient Th1 responses against haptens (Salerno et al. 1995; Traidl et al. 1999), and auto-, allo- and even tumor-antigens (Tepper et al. 1989; Schuler et al. 1999; Bagley et al. 2000; Radu et al. 2000; Golumbek et al. 1991). This phenomenon remained unexplained until recent research led to a better understanding of dendritic cell (DC) maturation. The description of distinct DC phenotypes that induce either Th1 or Th2 cells led to the search for factors that were involved in the development of the Th1-inducing DC1 or the Th2-inducing DC2. A first hint came from in vitro studies showing that IL-4 influences DC differentiation into a DC1 phenotype that produces IL-12 (Hochrein et al. 2000; Kalinski et al. 2000; Ebner et al. 2001).

Based on these findings, the apparent paradox that IL-4 may on the one side induce DC1 and on the other side deviate Th-cell differentiation towards a Th2 phenotype could be analyzed in vivo. This was first done in the model of *L. major* infection, which represents the best-established model to study Th2 differentiation in vivo. Prior data had shown that the presence of IL-4 during the period of T-cell activation leads to Th2 differentiation and disease progression (Launois et al. 2002; Himmelrich et al. 2000).

In order to investigate the role of IL-4 in DC1 development in vivo, IL-4 was given to Balb/c mice during the first 6 h of infection, the period when DC became activated by *Leishmania promastigotes* (Biedermann et al. 2001 b). This regimen of IL-4 application induced DC1 within 12 h, Th1 development, and established protective immunity against *L. major*. Importantly, if IL-4 application was extended to the period of Th-cell activation, IL-4 again induced a Th2 phenotype and abrogated protective immunity against *L. major*. This confirmed that IL-4 early during DC activation is effective in inducing a DC1 phenotype in the activated DC and induces protective Th1 responses, whereas some cytokines drive Th cells towards a Th2 phenotype and abrogate protective immunity against *L. major* when given during T-cell activation.

13.5 Conclusion

Together the detailed analyses of the actions of IL-4 in various disease and immunization protocols indicated that one single cytokine may exert opposing effects on the development of immune responses. The biological effect of IL-4 depends on the primary target cell of this cytokine during an immune reaction, and factors such as the time of cytokine application and concentration during various phases of immune responses ultimately determine whether this cytokine has pro- or anti-inflammatory effects. Similar findings have been reported for other "paradoxical" cytokines, including IFN-γ, IL-10, and IL-12. Therefore the biological significance of cytokines and especially their use in clinical trials requires a critical review of the underlying animal experiments, including factors such as time, dose, and frequency of application.

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14 What Must a Model Display for Proof as a Model of Contact Dermatitis?

C. Hauser

14.1 Introduction

Contact dermatitis is an inflammatory response of the skin to compounds brought into contact with skin. While proteins can induce such a response in some situations (e.g., protein contact dermatitis), cutaneous inflammatory reactions to small molecules are more prevalent and more frequently a medical problem. This is probably due to the fact that small molecules diffuse better across the stratum corneum, the major molecular barrier of the skin. The other major factor that determines diffusion of molecules across the stratum corneum is the degree of lipophilia.

14.2 General Notions

Contact dermatitis was originally recognized as a medical condition. Allergic contact dermatitis can be induced by small molecules, usually small reactive chemicals or metal ions, in susceptible individuals who were previously in contact with the same compound. Skin contact with such a compound in susceptible individuals elicits a greater (“exaggerated”) inflammatory response at the site of application than in previously nonexposed individuals. Thus, the attribute “allergic” or the term “hypersensitivity” was used to more closely describe this type of skin reaction. A compound that induces in the majority of individuals the same reaction (including in previously nonexposed individuals) is designated an irritant and its reaction “irritant contact dermatitis.” It is thought that the adaptive immune system plays a central role in contact hypersensitivity but not irritant contact reactions. Methods to elicit these reactions experimentally in humans were developed in the beginning of the last century and are now known as skin patch tests or epicutaneous tests. It was recognized very early with the use of patch testing suspected compounds that it is sometimes difficult to distinguish between an allergic and an irritant skin reaction. This has not significantly changed since then. There are no hard criteria recognizable by the naked eye or by conventional light microscopy that allow a reliable separation of the two types of skin reaction.

14.3 Principles of the Murine Contact Hypersensitivity Models

The understanding of the mechanisms involved in allergic contact dermatitis has increased dramatically in the last 100 years. The progress in understanding the mechanisms of action involved in irritant contact dermatitis, however, has been very modest and will not be further considered here. The reason for the rapid development of knowledge regarding contact hypersensitivity is due to the application of the principle of patch testing in small animals. While guinea pigs were used initially, the mouse has become the preferred species in the last 30–40 years. Oxazolone, dinitro-fluorobenzene (DNFB), and trinitro-

chlorobenzene (TNCB) were the most frequently employed contact sensitizers used in animal experiments, the latter being eventually abandoned because if lacked commercial availability. Fluorescein isothiocyanate (FITC) has been used as a contact sensitizer, particularly when migration of dendritic cells to draining lymph nodes was studied. The reason why these highly reactive chemicals were and are continued to be used is their potency to induce contact hypersensitivity. One or two applications are sufficient to render most of the animals hypersensitive. The use of proteins to elicit contact hypersensitivity reactions in mice has become more common in the last few years (Wang et al. 1996; Spergel et al. 1999; Herrick et al. 2000). It is, however, not clear whether contact hypersensitivity to proteins imitates human allergic contact dermatitis, atopic dermatitis, or other forms of dermatitis. Atopic dermatitis is probably best imitated by this model. A smaller concentration of sensitizer is used to elicit a contact hypersensitivity skin reaction in sensitized animals. Contact hypersensitivity reactions in mice is usually elicited 5–7 days after sensitization. The reaction is also positive relative to controls at later time points but decreases in magnitude. The contact hypersensitivity reaction in mice is normally determined 24 h after application of the eliciting dose. Because the site of sensitization (usually the abdomen in mice) exhibits an inflammatory response at the time of challenge (primary allergic contact dermatitis), another site, usually the ear in mice, has been chosen to elicit contact hypersensitivity reactions (secondary allergic contact dermatitis).

The cutaneous hypersensitivity reaction elicited in mice can be measured by various methods. The most popular assay is to measure ear thickness. Ear thickness (edema) is usually measured by a caliper. Ear thickness measurement should always be conducted in a blinded fashion. Other assays include the determination of ear weight or myeloperoxidase activity in the ear homogenate. The histological analysis in mice exhibits a mixed mononuclear and neutrophilic cell infiltrate, edema, and various degrees of epidermal damage but no spongiosis. The latter, a hallmark of eczematous dermatitis including allergic contact dermatitis in humans, may not be recognizable in mice because the ear epidermis consists of only 2–3 cell layers. The inflammatory cell infiltrate in human contact hypersensitivity reactions is rich in mononuclear cells and much poorer in neutrophils than in mice.

14.4 Differences Between Human Allergic Contact Dermatitis and Murine Contact Hypersensitivity Reactions

At this point it may be useful to remember the differences from human contact hypersensitivity reactions. First, the time between the sensitization and the elicitation reaction in humans is usually unknown. Although reexposure after sensitization without clinical manifestations in humans cannot be excluded, the time between sensitization and elicitation may be much longer than in mice. While initially it was thought that the "memory" for contact sensitizers lasts for years, it can vanish over longer periods of time (months/years) in a significant portion of individuals (Nielsen et al. 2001). The reason for this is unknown. Loss of memory due to lack of exposure or induction of tolerance may be explanations. Murine contact hypersensitivity ear swelling responses may not be elicited beyond 10–12 days after sensitization. The second difference is that the kinetics of the elicitation reaction are different. Most human patch test reactions become apparent between 48 and 96 h, some rare reactions becoming visible only after 7–10 days. The third significant difference is that humans are not exposed to experimental contact sensitizers such as oxazolone, DNFB, and TNCB and thus do not develop contact hypersensitivity to these compounds, unless they are exposed experimentally (Friedmann 1990) or accidentally. In these situations, however, they appear to be potent contact sensitizers. Thus, the vast majority of human contact sensitizers differ from those routinely used on mice. Only a limited number of human contact sensitizers has been tested in mice, most of them in the murine local lymph node assay (Ryan et al. 2000), the guinea pig maximization assay becoming less commonly used. The local lymph node assay is currently in use to predict contact hypersensitivity in humans. Most human contact sensitizers appear to be less potent than the compounds used experimentally. Repeated, prolonged, or very intense skin contact is probably required for most human sensitizers to render them allergic. Fourth, murine contact hypersensitivity reactions do not exhibit spongiosis as in humans and has a neutrophilic cell infiltrate that is more prominent than in human allergic contact dermatitis reactions.

The most significant morbidity in humans from contact dermatitis is caused by chronic forms of this hypersensitivity reaction. Murine

models of chronic hapten-induced contact hypersensitivity have recently been developed using repeated TNCB application (Kitagaki et al. 1995). This model has not yet found wide application, probably because of the long assay duration (3–4 weeks). The cellular infiltrate in this model consists of mononuclear cells and eosinophils. Th2 cells appear to be the predominant T-cell subset in this model, explaining induction of specific IgE and an immediate reaction in addition to the late-phase reaction (Kitagaki et al. 1997). In this model, cutaneous mast cell hyperplasia is found responsible for the readily detectable immediate swelling reaction. A similar model was recently described using oxazolone. In contrast to the DNFB model, repeated skin painting with oxazolone resulted in increased interferon (IFN)-γ but not IL-4 accumulation in the ear (Fujii et al. 2002).

14.5 Mechanisms Underlying Murine Contact Hypersensitivity

For drug development, an animal model of contact hypersensitivity should ideally be based on the same mechanisms as the human counterpart. All our functional information, however, is derived from murine models. Only correlative but very rare functional information comes from human contact dermatitis. Most of the correlative data, however, appears to agree generally with information obtained in mice. The most important tools used to explore the mechanisms of contact hypersensitivity are antibodies, pharmacological inhibitors, and genetically modified mice (transgenic and knockout mice).

It is impossible to comprehensively review the mechanisms of contact hypersensitivity here; three areas in mechanism research that have yielded conflicting results will be described. One is the role of proinflammatory cytokines in the initiation of contact hypersensitivity in mice. The increase in IL-1β has been reported to be the first cytokine upregulated in epidermal Langerhans cells after application of hapten (Enk et al. 1992). Thus, once mice deficient in this cytokine were available, contact hypersensitivity was assessed. In one report the contact hypersensitivity was diminished (Shornick et al. 1996), but not in other studies (Nakae et al. 2001; Zheng et al. 1995). It is still not clear whether the genetic background of the ani-

mals or subtle differences in the methods explain the divergent results. The increasing complexity of the IL-1 family of cytokines with additional members such as IL-18 and more recently discovered genes (Dunn et al. 2002) may be responsible. In any case, the published results suggest that the function of IL-1β can be compensated in many situations, and clarified that IL-1β was not a suitable target for drug development regarding contact hypersensitivity.

Askenase et al. reported that contact hypersensitivity reactions were reduced in mast cell-deficient mice (van Loveren et al. 1983). A contradicting report appeared soon after theirs (Galli and Hammel 1984). It was more recently shown that contact hypersensitivity is indeed reduced in mast cell-deficient mice and can be reconstituted by mast cell substitution (Biedermann et al. 2000). Mast cells appear to be a source in skin for MIP-2 and tumor necrosis factor (TNF)-α in contact hypersensitivity. It was shown that these cytokines were responsible for the attraction of the neutrophils into challenged skin. The blocking of neutrophil migration to challenged skin diminished the ear swelling reaction, indicating that the recruited neutrophils were responsible for a large part of the ear swelling reaction. Other reports suggest that neutrophil infiltration is required for subsequent homing of T cells into skin (Miyachi et al. 1981; Dilulio et al. 1999). How are mast cells activated by hapten application in sensitized mice? Askenase reported some years ago that a soluble antigen-specific factor was required for the transfer of contact hypersensitivity reactions (van Loveren et al. 1983). Today, it has become clear that very-early (within 1 day)-produced antibody from B-1 cells and even light chains alone that retain some binding capacity to hapten may induce mediator release from mast cell and complement activation (Tsuji et al. 2002; Paliwal et al. 2002; Redegeld et al. 2002). This in turn is then responsible for subsequent cellular infiltration of the hapten-challenged site. Dissection of these steps make it clear that IgE-dependent protein-induced hypersensitivity in skin and respiratory airways is very analogous: The initial step, i.e., mast cell degranulation, is mediated by IgE, permitting cellular infiltration observed in the so-called late phase reaction. While INF-γ, perforin, and Fas are important effector molecules in hapten-induced contact hypersensitivity (Kehren et al. 1999), IL-4, IL-5, and IL-13 represent critical effectors in Th2-dominant protein contact hypersensitivity (Spergel et al. 1999; Herrick et al. 2000, 2003).

The discovery of adhesion molecules and their ligands on leukocytes and T cells fostered the hope that these molecules may increase our understanding of T-cell homing in cutaneous hypersensitivity, and that these molecules may become targets for drug development. Interference with integrins or their ligands involved in homing of T cells into non-lymphoid organs remains promising. Drug development of such inhibitors will most likely generate drugs with systemic immune suppressive action, although they may be used in T cell-dependent skin diseases. Initial experiments with selectin knockout mice were disappointing. Contact hypersensitivity reactions were marginally or not at all inhibited. Likewise, an E-selectin inhibitor did not show any efficacy in psoriasis (Bhushan et al. 2002). When selectin ligands were targeted, the situation changed radically. Fucosyltransferase VII (Fuc VII) controls E-, P-, and E-selectin ligands on T cells. When this gene was deleted, contact hypersensitivity reaction was inhibited by about 70% (Smithson et al. 2001). When fucosyltransferase IV, which controls mainly P-selectin ligands on T cells was deleted, contact hypersensitivity was only slightly reduced. In double knockout mice, however, contact hypersensitivity was reduced to background. In adoptive transfer experiments it was shown that migration of labeled T cells to hapten-challenged skin was reduced by about 80% when Fuc VII knockout donor cells instead of wild-type cells were used. These experiments established the crucial role of these two glycosyltransferases with a predominant role of Fuc VII in contact hypersensitivity and skin migration of T cells. It was recently shown that a pharmacologic inhibitor of P-selectin and E-selectin ligand formation in T cells inhibits almost completely contact hypersensitivity and T-cell homing to challenged skin (Dimitroff et al. 2003). These experiments suggest that Fuc VII is an excellent target for drug development with potential application to numerous T cell-dependent skin diseases. Fuc VII is particularly interesting because its inhibition may not affect immunity in internal organs. Although cutaneous delayed-type hypersensitivity was abrogated in P-, E-, and L-selectin triple knockout mice, the animals cleared lymphocytic choriomeningitis virus from internal organs, as did wild-type animals (Erdmann et al. 2002). The improvement or prevention of a psoriatic phenotype of skin transplanted to immunodeficient mice with efomycine M (Schon et al. 2002), a P- and E-selectin blocker, demonstrates that targeting of

selectins involved in skin migration of T cells may be a treatment concept that may be applicable to T cell-dependent skin diseases other than contact hypersensitivity.

The three-step model of adhesive interaction between leukocytes and activated endothelium postulated that ligands of seven transmembrane domain receptors, in particular chemokine receptors in addition to integrins and selectins may play a role. Chemokine ligands upon binding to their receptors on T cells induce a conformational change in integrins, conferring on them higher affinity and thus increased adhesion to the underlying endothelium, a prerequisite for extravasation. Campbell et al. reported that CCR4-expressing T cells accumulate in inflamed skin, CCR4 is preferentially expressed and functional in circulating cutaneous lymphocyte associated antigen (CLA)-positive T cells, and that TARC (CCL17), a CCR4 ligand, can be found on inflamed cutaneous endothelium (Campbell et al. 1999). The same group showed later in adoptive transfer experiments that lack of CCR4 was not sufficient to block contact hypersensitivity and skin homing of T cells, but that additional blocking of CTACK (CCL-27), a chemokine exclusively produced by keratinocytes, was required to inhibit in both assays (Reiss et al. 2001). Anti-CTACK alone was not inhibitory in contact hypersensitivity. This result was compatible with functional redundancy among chemokines. This view was challenged by Homey and co-workers, who found significant inhibition of ear swelling in a DNFB-driven contact hypersensitivity reaction and an ovalbumin-induced contact hypersensitivity model by administration of anti-CTACK antibody alone (Homey et al. 2002). The factors explaining the discrepancy between the results of the two groups remain unknown.

In conclusion, it is difficult to postulate features of an animal model for contact hypersensitivity aside from the model being simple and reproducible. The main reason for this is that the vast majority of functional knowledge stems from mouse models of contact hypersensitivity but not the human disease. Nonetheless, this model has also proved its usefulness for testing new drugs. I think it makes no sense to ask too much from such models, even if they may not completely reflect their human counterpart. Good activity of an experimental compound in murine contact hypersensitivity should remain one of the prerequisites for phase I studies.

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15 Acute and Chronic Models of Allergic Contact Dermatitis: Advantages and Limitations

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15.1 Introduction

Pharmaceutical companies are spending increasing amounts of money for drug discovery. The costs of bringing new drugs to the market have risen from about $ 120 million (US) in the late 1970s to currently about $ 850 million. Attrition rates in clinical development are still very high, and up to 90% of new compounds fail in clinical phases I–III. Late-stage clinical failure is to a considerable extent due to lack of clinical efficacy, indicating that there is a strong need for highly predictive in vitro and in vivo models for the respective indications. In this article, recent developments in animal models of acute and

chronic allergic contact dermatitis (ACD) will be reviewed. Such models are of major importance not only for ACD, a frequent inflammatory skin disease with a high need for better therapy, but even beyond dermatology. Therefore, ACD models are considered to be telling to a certain extent for immune diseases characterized by a type 1-cytokine pattern in general such as rheumatoid arthritis, graft rejection, multiple sclerosis, psoriasis, and other diseases.

15.2 Ideal or Ideology: Features of Ideal Animal Models of ACD

Allergic contact dermatitis in both humans and rodents is a T cell-mediated disorder (Askenase 2001; Gorbachev and Fairchild 2001; Kalish and Askenase 1999; Wang et al. 2001). This has been shown using CD4-, CD8-, TCR α-, MHC class I- and class II-deficient mice (Wang et al. 2001). Substances targeting T cells such as anti-T-cell antibodies (Gocinski and Tigelaar 1990; Bouloc et al. 1998) and IL-2-diphtheria toxin fusion proteins (Pullerits et al. 1999) also block contact hypersensitivity reactions, indicating that blocking T cells is sufficient to reduce inflammatory responses. On the other hand, this does not mean that all other approaches leaving T-cell numbers or activity untouched are ineffective, which is discussed in more detail below. The mechanism of this disease can be separated into two distinct phases, sensitization and elicitation (Grabbe and Schwarz 1998). In the *sensitization phase*, Langerhans cells take up haptenated proteins, get activated, mature, and migrate from the epidermis to the skin draining lymph nodes. Upon encounter with an antigen-specific naive T cell, Langerhans cells present antigenic peptides to these lymphocytes, which become activated, clonally proliferate, and express a set of adhesion molecules which allows them to recirculate to the organ from which the antigen has been derived. In the *elicitation phase* of contact dermatitis (challenge), the organism is reexposed with the same hapten to the skin. As haptens are also irritants, proinflammatory cytokines are produced by resident skin cells and adhesion molecules are upregulated, finally leading to the recruitment and activation of several leukocyte subpopulations including T cells. Figure 1 summarizes the two phases of ACD. The

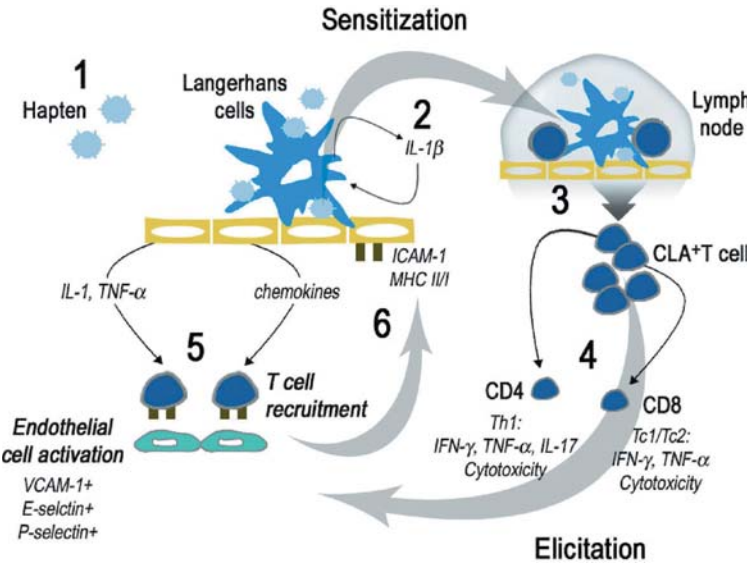

Fig. 1. Sensitization (*1–3*) and elicitation phase (*4–6*) of allergic contact dermatitis. Haptens penetrate the skin (*1*) and are taken up by epidermal Langerhans cells leading to Langerhans cell activation (*2*) and migration to skin-associated lymph nodes (*3*). They present antigens to naive T lymphocytes in a class I or class II-dependent manner to $CD8^+$ or $CD4^+$ T cells, leading to lymphocyte activation and clonal proliferation. Naive T cells activated in the skin-associated lymph nodes by epidermal Langerhans cells also acquire a certain set of a adhesion molecules such as CLA and chemokine receptors allowing them to circulate to the skin. The second encounter with the haptenic antigen at the side of skin contact induces T-cell activation and cytokine production by T cells (*4*). At the same time, haptens induce keratinocyte production of TNF-*a* and IL-1*a*, leading to activation of endothelial cells and expression of adhesion molecules such as the CLA counterreceptors E- and P-selectin and the VLA-4 ligand VCAM-1 (*5*). These adhesion molecules together with induced expression of chemokines such as CCL17, CCL22, or CCL27 attract skin-seeking CLA^+ T cells which promote eczema via induction of apoptosis of inflamed (e.g., ICAM-1 and MHC class II^+) keratinocytes (*6*)

relative role of $CD4^+$ versus $CD8^+$ cells in the elicitation phase remains controversial (Gorbachev and Fairchild 2001), but it has been shown in C57BL/6 mice using the model allergens 2,4-dinitrofluorobenzene (DNFB) and oxazolone that both $CD4^+$ T cells and $CD8^+$ T cells contribute to the full development of contact hypersensitivity (Wang et al. 2001). Although the possibilities for comparable experimental approaches in humans are more limited than in rodents, it has been observed that $CD4^+$ and $CD8^+$ T-cell clones obtained from nickel-allergic patients exhibit strong cytotoxicity against IFNγ-treated keratinocytes (Traidl et al. 2000). The cytokine IFNγ is of key importance for contact hypersensitivity, as it increases Fas expression by keratinocytes, leading to keratinocyte apoptosis mediated by soluble or membrane-bound FasL produced by activated T cells (Trautmann et al. 2001).

Table 1 summarizes the most important features which should be fulfilled by an "ideal" animal model of ACD. Besides mimicking the essential aspects of the human disease as closely as possible, these requirements also include technical and ethical considerations. The complexity of scientific needs suggests that it is unlikely that one single model will fulfill all requirements, and indicates the need for multiple models.

Table 1. Features of an "ideal" model of allergic contact dermatitis

T cell-dependent
Induced by environmental factors (haptens)
Involvement of type 1 or type 2 cytokines dependent on hapten
Typical macroscopic and microscopic morphology
Interventive responses reflect human situation
Acute and chronic models available
Molecular changes reflect human situation
Compatible with ethical considerations
Technical aspects:
Inexpensive, fast, easy to handle, allow "medium throughput", reproducible

15.3 Rodent Models of Acute ACD

The choice of the hapten determines – among other factors – the immunological reaction. DNFB, trinitrochlorobenzene (TNCB) or picryl chloride, and oxazolone are the most widely used haptens and result in a predominantly type I cytokine response when acutely applied (Girolomoni et al. 2001; Grabbe and Schwarz 1998; Kimber and Dearman 2002). Repetitive allergen challenge results in a chronic-intermittent disease; however, the cytokine pattern in skin and draining lymph nodes is shifted toward a type 2-dominated response (Kitagaki et al. 1995, 1997, 1999). In contrast to the abovementioned haptens, topical application of respiratory allergens such as trimellitic anhydride (TMA) or of FITC will result in specific IgE antibody production and an inflammatory skin reaction with a marked increase in IL-4 production by $CD4^+$ T cells indicative for a predominantly type 2-cytokine pattern (Betts et al. 2002; Dearman et al. 1996, 2002; Schottelius et al. 2002; Ulrich et al. 2001).

For *sensitization*, these compounds are applied onto the shaved flanks of the animals. Several days later (e.g., 5 days for DNFB, TNCB, and TMA or 14 days for oxazolone), the animals are reexposed with the same hapten at a different site of the body – typically the ears – which will result in an inflammatory response (*elicitation* or *challenge*). A clear advantage of rodent ACD models is the high degree of model standardization and the huge experience with these models, leading to considerable reproducibility. Standard readouts of these models are determination of ear thickness or ear weight as an objective measure of skin inflammation/edema, in situ myeloperoxidase and neutrophil elastase activity as a measure of granulocyte and neutrophil skin infiltration, cytokine concentrations in situ, and histological examinations (e.g., epidermal or total skin thickness, lymphocyte infiltration, etc.) which are rather fast to perform and easy to handle. Finally, gene expression analyses will allow further insight into the understanding of allergic contact dermatitis and of the mechanisms of action of compounds of interest (Fig. 2). A huge number of reagents (e.g., monoclonal antibodies for immunohistochemistry) are available especially for mice but also for rats. Moreover, rodents and in particular mice are small animals which require only limited amounts of test compounds for in vivo

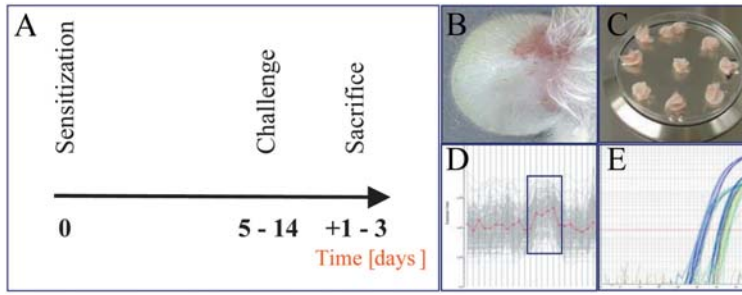

Fig. 2. **A** Typical experimental protocol and examples for read-outs performed in allergic contact dermatitis models. **B** Inflamed mouse ear 24 h after DNFB challenge. **C** Measurement of ear weight. **D** Measurement of gene expression in skin using gene arrays. **E** Quantitative mRNA determination for genes of interest to verify gene array data

studies. This aspect is of critical importance for the characterization of newly synthesized compounds when only limited amounts of test compounds are available. Ethical aspects also contribute to the preferred use of rodents compared to higher animals such as dogs or monkeys. An exception are minipigs: As the skin of these animals more closely resembles human skin, minipigs are of specific importance for testing topical drug development candidates (Vana and Meingassner 2000). This review focusses on rodents, as they are most widely used.

15.4 From an Ideal Model to Reality: Challenges of Rodent ACD Models

Although rodent ACD models are extremely helpful and widely used in dermatological research, there are several drawbacks of these models. In humans, one of the typical clinical hallmarks of acute eczema are *vesicles* which are not observed in rodents. Vesicles are the macromorphological correlate of an intercellular edema or *spongiosis*, which is also absent in rodents (Fig. 3). These differences in the macro- and micromorphological picture are thought to result from differences in the skin architecture. Whereas in humans the epider-

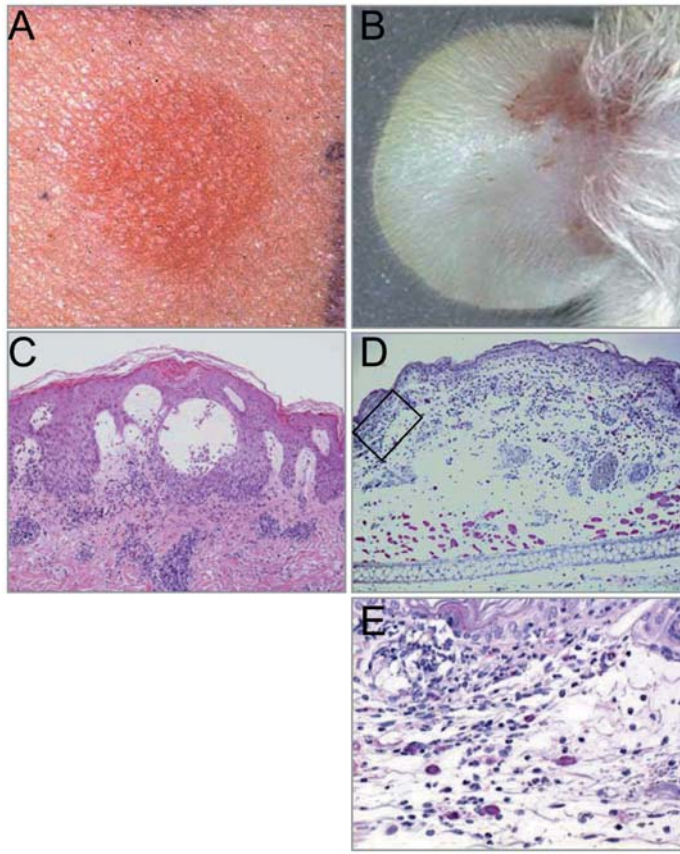

Fig. 3. Clinical and histological appearance of acute allergic contact dermatitis in man and mouse. **A** Allergic patch test reaction in a patient allergic to nickel sulfate 72 h after challenge. **B** Murine allergic contact dermatitis in response to DNFB 24 h after challenge. **C**, **D** Corresponding histology of **A** and **B**, respectively. Original magnification ×10. **E** Greater magnification (×40) of **D** showing only few $CD3^+$ (*purple*) lymphocytes among many granulocytes

mis is a very robust and tight, multi-layered epithelium, in rodents – especially in mice – the epidermis contains only very few layers of keratinocytes. The latter may not be sufficient for generation or retention of intercellular edema.

The *cellular composition of the skin infiltrate* is also quite different in humans compared to rodents. Lymphocytes are quite frequent in human acute contact dermatitis (Fig. 3C), whereas neutrophils are by far the most frequent cell type in murine ACD (Fig. 3D, E). The earliest histopathological findings during the response in rodents are mast cell degranulation, vasodilatation, and influx of neutrophils. Marked T-cell infiltration is not observed before 6–8 h after challenge. There are at least two reasons for this important difference: (a) It is well known that only memory T cells can express the set of necessary adhesion molecules, enabling them to invade inflamed organs such as the skin, whereas naive cells predominantly migrate to lymphoid organs such as the lymph nodes. Memory cell numbers in healthy, young mice are, however, remarkably lower compared to those in diseased humans (<10% in mice vs. approx. 50% in adult humans). Therefore, *bystander, allergen-unspecific T-cell infiltration* in rodents can hardly occur, whereas infiltration of bystander T cells in addition to allergen-specific T cells are typically observed in humans. The consequence of the expected reduced numbers of bystander T cells in mice compared to humans for the validity of the model needs further clarification. (b) There might be a different role of resident *mast cells* between mice and humans. It has been shown that murine mast cells in ACD produce TNF-α and MIP-2. Reconstitution of mast cells in mast cell-deficient mice in the presence or absence of a neutralizing MIP-2 antibody clearly demonstrated that mast cell-derived MIP-2 is involved in both the regulation of neutrophil trafficking and murine ACD responses (Biedermann et al. 2000). Whether this mechanism is also active in humans is unclear. Mast cells are present in human ACD and become activated, which can be concluded from their degranulation (Dvorak et al. 1976; Rantuccio et al. 1978). However, mast cell activation in human ACD may be somehow incomplete (Zweiman et al. 1998), which may result in the markedly diminished influx of neutrophils.

Given the differential role of mast cells and neutrophils in mice compared to humans, it is expected that compounds targeting these

two leukocyte subsets and their activation may result in *different responses in murine versus human allergic contact dermatitis*. Indeed, leukotriene receptor antagonists have been shown to be active in animal models of contact dermatitis (Ekerdt et al. 1991) but later failed in clinical studies. Non-steroidal anti-inflammatory drugs (NSAIDs) such as indomethacin have been shown to be active in rodent models of contact hypersensitivity (Tarayre et al. 1990), but are not effective (Queille-Roussel et al. 1990) and therefore not used in humans. Indomethacin, although effective in rodents, is well known to aggravate psoriasis, another type 1 cytokine-mediated inflammatory skin disease. The reason for this discrepancy between the response in humans and rodents remains unclear. In addition, clinical trials in dermatological indications using fully humanized, neutralizing antibodies against IL-8 have been disappointing, although this approach has been successful in different skin inflammation models in animals; this antibody has not been tested in ACD (Harada et al. 1996; Larsen et al. 1995; Yang et al. 1999). It can be speculated that this discrepancy may be again due to targeting a leukocyte subset which plays a differential role in rodent versus human ACD. The best example of an incomplete predictivity of the rodent ACD models are NSAIDs which showed some activity in ACD models (Tarayre et al. 1990) but are not used and/or are ineffective in the human disease (Queille-Roussel et al. 1990). Again, these compounds are considered to be predominantly targeting unspecific and not T cell-mediated immune responses. It may therefore be speculated that rodent models of ACD are less predictive when targeting pathways which are of differential importance in the models as compared to humans. In contrast, T cell-targeting compounds appear to be active in both rodent models and in the human situation. This has been shown for a broad number of approaches including glucocorticosteroids, anti-T cell approaches (e.g., mycophenolate mofetil, Mehling et al. 2000; azathioprine), and calcineurin inhibitors (cyclosporine A, FK506/tacrolimus, pimecrolimus), which are summarized in Table 2. Other approaches directed against T cells have only been tested in rodents and not in humans for the indication of ACD, and therefore exclude any conclusions regarding predictivity of these models. Among these therapeutic modalities are anti-T-cell antibodies, IL-2-diphtheria toxin fusion protein, and IL-10 (Sobel et al. 1987;

Table 2. Allergic contact dermatitis in animal models partially correlates with responses in humans

Compound class	Generic name	Trade name [a]	Response (model) [b]	References	Response (human)	References
GCs						
	Methylprednisolone aceponate [c]	Advantan	+	Zaumseil et al. 1992	+	Brazzini and Pimpinelli 2002
	Diflucortolone valerate [c]	Nerisona	+	Kapp et al. 1976	+	Wendt et al. 1978
	Clobetasol propionate [c]	Dermoxin	+	Yawalkar et al. 1991	+	Wendt et al. 1978
NSAID						
	Indomethacin [c]	Diclofenac	+	Tarayre et al. 1990	0	Queille-Roussel et al. 1990
	Bufexamac [c]	Parfenac	0	Boyera et al. 1992	0	Christiansen et al. 1977; Queille-Roussel et al. 1990
			+	T.M. Zollner, unpublished observations	+	Achten et al. 1973
Calcineurin antagonists						
	Cyclosporine A [d]	Sandimmun	+	Rullan et al. 1984	+	Higgins et al. 1991
	Tacrolimus [c]	Protopic	+	Meingassner et al. 2003	+	Lauerma et al. 1992; Schnopp et al. 2002
	Pimecrolimus [c]	Elidel	+	Meingassner et al. 2003	+	Queille-Roussel et al. 2000

Vit D_3 and derivatives						
	Calcitriol [d]		+[e]	Zugel et al. 2002	Not used	
	Calipotriol [c]	Psorcutan	0/–[e]	Fujii et al. 2002; Garrigue et al. 1993; Tani et al. 1989	Not used	
Others						
	Anti IL-8 mAbs [d]	n.a.	+	Harada et al. 1996; Larsen et al. 1995	Program discontinued	
	Anti-T cell antibodies [d]		+		Not known [f]	
	IL-2 toxin [d]		+		Not known [f]	
	IL-10 [d]		+		Not known [f]	
	LTB4 receptor antagonists [d]	n.a.	+	Ekerdt et al. 1991	Program stopped	
	Mycophenolate mofetil [d]	CellCept	+	Mehling et al. 2000	+/?	Pickenacker et al. 1998
	Azathioprine [d]	Imurek	+	Tarayre et al. 1990	+/?	Morrison and Schulz 1978; Verma et al. 2000

[a] Examples are given for marketed compounds.
[b] Response to the elicitation is mentioned: +, efficacious; 0, ineffective; –, worsening in models or human situation.
[c] Topical application.
[d] Systemic administration.
[e] Depending on time point of application.
[f] Active in psoriasis (see text).

Schwarz et al. 1994; Pullerits et al. 1999; Xu et al. 1997), which were active in psoriasis (Prinz et al. 1991; Gottlieb et al. 1995; Asadullah et al. 1998).

15.5 Models of Chronic ACD

The most widely used ACD models are acute models, whereas in humans ACD is most often a chronic disease. In the acute models inflammation reaches a maximum 24–48 h after challenge. Therefore, therapeutic interventions are difficult and test compounds are applied either prior to or simultaneous with the allergen challenge, which is in clear contrast to the human situation, where an established disease needs to be treated. Furthermore, in chronic inflammation, protein expression might be different compared to acute lesions. Therefore, therapeutic interventions that are effective in acute models may show different results in models of chronic disease. This is exemplified by different functional roles for E- and P-selectin versus ICAM-1 in acute versus chronic lesions regarding neutrophil emigration to murine skin (Mizgerd et al. 1999). Differential expression of E-selectin versus ICAM-1 and VCAM-1 in acute versus chronic inflammation has also been reported in a different species, namely rabbits, which may indicate that this is a more general observation (Abe et al. 1996). Therefore, although more laborious, chronic ACD models may mirror the clinical situation better.

15.5.1 Repeated Allergen Challenges in Wild-Type Mice

Models of chronic ACD have been reported for several allergens (TNCB, DNFB, oxazolone) both in mice and rats by repeated application of the haptens (Fujii et al. 2002; Kitagaki et al. 1995, 1997, 1999; Nagai et al. 1997; Natsuaki et al. 2000; Shimada et al. 2003; Webb et al. 1998). Unexpectedly, the time-course of antigen-specific hypersensitivity responses shifted from a delayed-type hypersensitivity to an immediate-type response followed by a late-phase reaction, when epicutaneous applications were repeated. This was not observed in mast cell-deficient mice (Natsuaki et al. 2000). An altered

cytokine balance with a shift toward Th2-dominated responses has been shown, which would represent the natural evolution processes directed toward reducing a more deleterious Th1 response (Kitagaki et al. 1997). In addition, hapten-specific IgE production has been observed. Repeated oxazolone challenge in rats has been shown to be associated with a continuous Th1 cytokine response (Fujii et al. 2002), and thus, the shift in time-course and cytokine production does not occur in response to all of the above-mentioned haptens in all rodents. As ACD in humans is thought to be a type 1-dominated response, repeated allergen challenges in rodents using haptens such as DNFB or TNCB might not represent a useful model for chronic contact dermatitis. It rather mirrors certain aspect of humoral, IgE-mediated immune responses which rather may have potential for indications such as atopic dermatitis.

15.5.2 Chronic Inflammation Following Single Allergen Challenges in Gene-Modified Mice

Approaches using gene-modified mice may be more promising. Chronic, long-lasting inflammatory responses have been shown following a single hapten challenge in mice overexpressing costimulatory molecules, cytokines, and growth factors.

Mice which overexpress IFNγ under the control of an involucrin promoter have been shown to suffer from hair loss and chronic eczema after single allergen challenge. Histologically, the lesions show several hallmarks of eczema and are characterized by increased ICAM-1 and MHC class II expression by keratinocytes. The skin of severely affected mice was characterized by a dermal infiltrate of T lymphocytes (Carroll et al. 1997). This model is discussed in more detail in Chapter 16.

Costimulatory molecules such as CD80 (B7-1), CD40, and CD40L are important for the optimal activation of T cells by antigen-presenting cells. For a better understanding of these molecules with regard to skin diseases, these molecules have been overexpressed in the skin using a keratin 14 promoter. With regard to CD80 overexpression, the skin of such transgenic mice was grossly and histologically normal, with normal numbers of Langerhans cells

and dendritic epidermal T cells. Immunologic challenge of transgenic mice with epicutaneous haptens such as FITC revealed enhanced and persistent delayed-type hypersensitivity responses, with prolonged kinetics of resolution compared with nontransgenic controls (Nasir et al. 1994; Williams et al. 1994). It was concluded that expression of CD80 was not sufficient for induction of an inflammatory response by itself but that it amplifies the immune response after allergen/antigen exposure. Overexpression of CD40 by keratinocytes caused keratinocyte TNF*a* secretion and dendritic cell emigration when this molecule was selectively engaged using anti-CD40 antibodies. In addition, exclusive CD40 engagement on keratinocytes during a contact hypersensitivity response displayed exacerbated and prolonged cutaneous immune reactions relative to control mice (Fuller et al. 2002). Overexpression of CD40L resulted in spontaneous skin inflammation and massive regional lymphadenopathy, and an increase in IgG1/IgG2a/IgG2b/IgE serum concentrations was detectable with positive titers for antinuclear and anti-dsDNA antibodies. Furthermore, renal immunoglobulin deposits, proteinuria, and lung fibrosis were observed which resemble mixed-connective-tissue-like autoimmune disorders, possibly by breaking immune tolerance against the skin (Mehling et al. 2001).

Overexpression of VEGF/VPF has been shown in several skin diseases including psoriasis and allergic contact dermatitis (Brown et al. 1995). Therefore, it has been speculated whether the balance of angiogenic and anti-angiogenic factors such as thrombospondins might be involved in the pathophysiology of these diseases. To test this hypothesis, VEGF transgenic and thrombospondin-2 (TSP-2)-deficient mice have been generated. Lack of TSP-2 resulted in a significantly enhanced inflammatory response with increased angiogenesis, edema formation, and inflammatory infiltration. Ear swelling and inflammation persisted for more than 2 weeks in TSP-2-deficient mice. Moreover, the fraction of rolling leukocytes was significantly increased in the untreated skin of TSP-2-deficient mice (Lange-Asschenfeldt et al. 2002). Overexpression of VEGF under a keratin 14 promoter results in a spontaneous, psoriasiform dermatitis in aged mice. Following mechanical irritation (Koebner phenomenon), such lesions can also be observed earlier in life. Histological characterization of the lesions revealed excellent correlation with the

human situation including rete ridge formation (Xia et al. 2003). As observed in thrombospondin-2-deficient mice, intravital fluorescence microscopy revealed highly increased leukocyte rolling and adhesion in postcapillary skin venules (Detmar et al. 1998). Furthermore, single allergen challenge results in a prolonged and exaggerated immune response (M. Detmar et al., submitted). The results obtained from VEGF transgenic and thrombospondin-2-deficient mice reveal an important role of angiogenic factors in regulating the extent and the duration of edema formation, angiogenesis, and inflammatory cell infiltration during acute and chronic inflammation.

Taken together, mice overexpressing IFNγ, CD80, and VEGF and thrombospondin-deficient animals show prolonged and exaggerated immune responses after allergen challenge which may be interesting for both basic research and intervention studies of chronic eczema.

15.6 Conclusion

Basic immunological research has not only provided a deeper insight into the pathophysiology of ACD but has also generated a plethora of appealing animal models for both acute and chronic ACD. Some of the more established models are already extensively characterized and have proven to be T cell-dependent with an involvement of Th1 and Tc1 cells. They display at least some of the classical histological hallmarks of human ACD, and responses to experimental therapies in rodents do to a large extent reflect the human situation. However, the established models are mostly acute, self-limited inflammation models. More recent developments such as mice overexpressing costimulatory or angiogenic molecules need further characterization. Gene and protein expression profiling would allow comparisons of expression profiles in human disease with those obtained from different animal models. More systematic investigations regarding the effects of well established effective therapeutic approaches in humans and in model situations compared to drugs effective only in contact dermatitis models, not in humans would be desirable. In the future, this may help to select relevant models for specific needs such as functional target validation and compound characterization and may finally provide the desired, more predictive in vivo models.

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16 Transgenic Mice Expressing IFN-γ in the Epidermis Are a Model of Inflammatory Skin Disease and Systemic Lupus Erythematosus

F. M. Watt

16.1 Introduction

Over the years several gene promoters have been characterised that enable transgene expression to be targeted to the epidermis. These have facilitated the development of many different experimental mouse models of skin disease. A range of cytokines and growth factors have been expressed in the epidermis of transgenic mice, providing valuable information about their role in normal and damaged skin (Schon 1999).

Cytokine release by keratinocytes is believed to play an important role in contact dermatitis and other inflammatory conditions (Barker et al. 1991). To test the potential involvement of IFN-γ, my laboratory generated transgenic mice in which a murine IFN-γ transgene was expressed under the control of the involucrin promoter (Carroll et al. 1997). The involucrin promoter is expressed in the suprabasal layers of the interfollicular epidermis and also in the inner root sheath of the hair follicle. While the IFN-γ transgenic mice do indeed have a profound inflammatory skin condition, they also produce autoantibodies, which has led us to explore them as a model for systemic lupus erythematosus (SLE).

In this chapter I describe the phenotype of the mice, from the initial characterisation of the macroscopic skin phenotype, through the realisation that the mice were producing autoantibodies and had renal disease, to our attempts to define the mechanisms underlying the disease process, and finally our experimental treatment strategy with an anti-apoptotic drug.

16.2 Macroscopic Phenotype

Three independent founder lines of mice were generated, with the transgene copy number ranging from two to 32. The skin and autoimmune phenotypes of all three lines are similar, although the highest copy number line is the most severely affected. IFN-γ is detectable by ELISA in protein extracts of the skin (Carroll et al. 1997). Most animals have undetectable levels of IFN-γ in the serum, indicating that the phenotype is due to local production of IFN-γ in the epidermis (Carroll et al. 1997; Seery et al. 1997).

Although the mice appear normal at birth, by day 8 many of them are smaller than their nontransgenic littermates and have retarded hair growth (Carroll et al. 1997). By 2 weeks after birth, all of the transgenics display hypopigmentation of the hair. By 3 weeks, the most severely affected transgenics show severe growth retardation, hair loss, skin reddening, and flaky skin lesions. The mice also have enlarged spleens and smaller thymuses than controls.

The hair hypopigmentation correlates with a marked reduction in the number of tyrosinase-positive melanocytes in the epidermis. It is

interesting that there are some human conditions in which inflammatory skin disease is associated with skin depigmentation (von den Driesch et al. 1992; Arata and Abe-Matsuura 1994).

16.3 Skin Phenotype

16.3.1 Spontaneous Skin Lesions

The loss of melanocytes is only one aspect of the changes in the skin of IFN-γ transgenics (Carroll et al. 1997). In the most severely affected mice there is epidermal hyperplasia, reflecting an increase in the numbers of both viable cell layers and cornified layers (hyperkeratosis) (Fig. 1). There are regions in which the cornified layers are parakeratotic, and there are occasional microabscesses in the cornified layers (Fig. 1). Electron microscopy revealed severe spongiosis in all layers of the epidermis and an increase in the number of granular layers and number of keratohyalin granules per cell, except in the parakeratotic regions.

In some severely affected mice, there are regions of the skin in which the epidermis becomes separated from the dermis at the level of the basement membrane (Fig. 1). There is an infiltration of lymphocytes, macrophages, and monocytes into the space between the

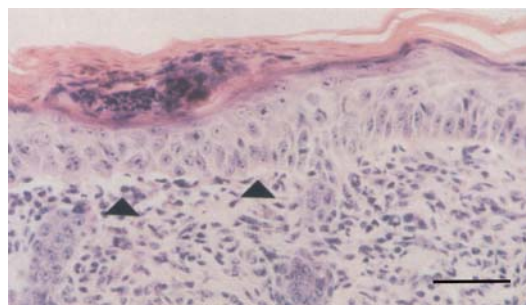

Fig. 1. Skin phenotype of IFN-γ transgenic mice. Haematoxylin and eosin-stained section of back skin from transgenic mouse. *Arrowheads* show split at dermo-epidermal junction. Scale bar, 70 μm. (Reproduced from Carroll et al. 1997, with permission)

epidermis and the dermis. Dilation of the blood vessels in the dermis is also observed.

We examined expression of a number of markers in the skin (Carroll et al. 1997). The proportion of Ki67-positive keratinocytes is markedly increased, although proliferation remains largely confined to the basal layer. There is suprabasal expression of β1 integrins, and keratins 6 and 17 are expressed in the interfollicular epidermis, all of which are markers of hyperproliferative epidermis (Hertle et al. 1992; McGowan and Coulombe 1998). Keratinocytes in transgenic epidermis express ICAM-1 and MHC class II molecules, proteins that are known to be expressed on the surface of keratinocytes treated with IFN-γ (Dustin et al. 1988; Griffiths et al. 1989).

In inflamed transgenic skin there are reduced numbers of Langerhans cells in the epidermis, and this correlates with increased numbers of Langerhans cells in the lymph nodes (Carroll et al. 1997). Langerhans cells are the major antigen-presenting cells of the skin, and our data suggest that in the transgenics the Langerhans cells may be moving to the lymph nodes to present antigen to resident T cells. The number of CD3-positive lymphocytes is reduced in the epidermis but increased in the dermis (Carroll et al. 1997). There is an influx of macrophages and monocytes into the dermis, which is to be expected, given the well established role of IFN-γ as an inducer of myeloid cell differentiation and activation (Schultz and Kleinschmidt 1983).

16.3.2 Contact Dermatitis

In contact dermatitis, an inflammatory stimulus is provided by exposure of the skin to environmental agents such as poison ivy and nickel. When skin has become sensitised to the irritant, subsequent exposure can lead to an MHC-restricted, hapten-specific T-cell response known as allergic contact dermatitis (Enk and Katz 1995). The epidermis becomes hyperproliferative and thickened, with characteristic spongiosis as a result of accumulation of fluid in the intercellular spaces. There is infiltration of lymphocytes and monocytes into the dermis, and the blood vessels become dilated.

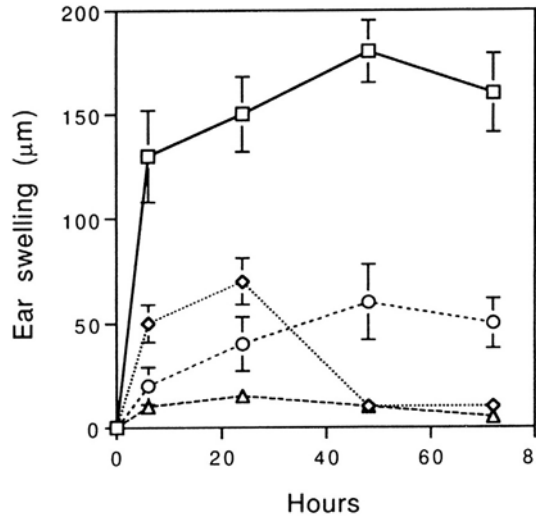

Fig. 2. Contact hypersensitivity response of IFN-γ transgenic and wild-type littermate mice. *Diamonds*, *triangles*, wild-type; *squares*, *circles*, transgenic. Ear thickness was averaged from values obtained at three different sites on each ear at intervals after application of DNFB. As a control, some mice (*triangles*, *circles*) did not receive a sensitising dose of DNFB prior to application to the ear. Each measurement represents the mean ± standard deviation from four mice. (Reproduced from Carroll et al. 1997, with permission)

Application of contact sensitisers to the epidermis stimulates a T lymphocyte-mediated immune response that can be assessed by subsequent challenge with the same hapten. The abdomen of IFN-γ transgenic mice exhibits an acute local inflammatory response to a sensitising dose of 2,4-dinitrofluorobenzene (DNFB), whereas that of control mice does not (Carroll et al. 1997). Compared to transgene-negative littermates, transgenic mice show an increased hypersensitivity response to DNFB challenge 6 h after application to the ear. In control mice, ear thickness returns to normal after 48 h, but in transgenics swelling does not decline significantly after 72 h (Fig. 2).

These findings lend weight to the evidence that there is a primary role for IFN-γ in mediating contact hypersensitivity reactions. For

example, IFN-γ expression is increased in contact hypersensitivity (Kondo et al. 1994), and mice lacking the IFN-γ receptor have a reduced contact hypersensitivity response (Saulnier et al. 1995).

16.4 Autoantibody Production

Since IFN-γ is known to induce autoantibody production in mice (Gu et al. 1995; Lee et al. 1995), we tested the serum from the transgenic mice for autoantibodies. We found that serum from all transgenic strains stains the nuclei of every cell type tested (Carroll et al. 1997; Seery et al. 1997). Antibodies to dsDNA, histones, and nucleosomes were found (Seery et al. 1997, 1999; Fig. 3).

Sera from male and female transgenics were compared with negative control littermates and, as a positive control, sera from MRL/lpr mice. MRL/lpr-lpr mice are a murine lupus model and are characterised by a genetic defect in the Fas/FasL system (Takahashi et al. 1994). Compared to the negative littermates, both male and female IFN-γ transgenics show evidence of anti-dsDNA production; however, levels are significantly higher in female animals. Anti-histone autoantibody levels in serum from male transgenics do not differ significantly from those of negative littermates. Female transgenics pro-

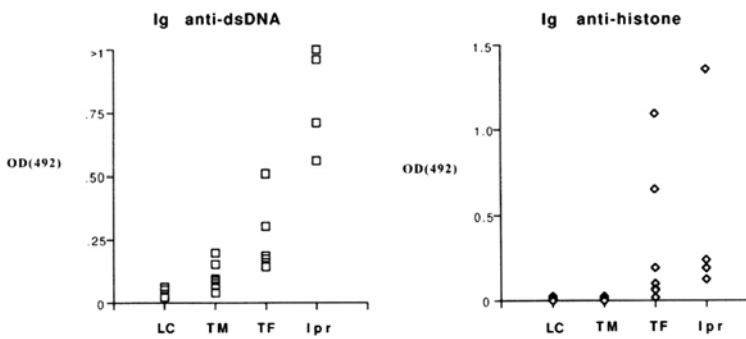

Fig. 3. ELISA assays of serum anti-dsDNA and histone autoantibody levels. *TM*, transgenic males; *TF*, transgenic females; *LC*, wild-type littermates. As a positive control MRL/lpr mice were also examined. (Reproduced from Seery et al. 1997, with permission)

duce anti-histone antibody titres that are at levels comparable to those of MRL/lpr mice (Fig. 3).

16.5 Renal Disease

Systemic lupus erythematosus (SLE) is a non-organ-specific autoimmune disease with a prevalence that is similar to that of multiple sclerosis (Kotzin 1996). Patients with SLE are predominantly female. They have characteristic inflammatory skin lesions and autoantibodies to nuclear antigens. Most SLE patients have renal damage as a result of accumulation of autoantibodies in the kidney mesangium and capillary walls, resulting in glomerulonephritis. This led us to examine the kidneys of the IFN-γ transgenic mice.

Anti-dsDNA antibodies are known to deposit in the kidneys of 60%–70% of SLE patients. When we examined the kidneys of IFN-γ transgenics we found deposition of IgG in the kidney glomeruli of all female mice (Seery et al. 1997). Five out of eight male transgenics tested also had evidence of IgG deposition.

Histological examination of the kidneys demonstrated clear evidence of glomerulonephritis in female mice only (Seery et al. 1997). The severity of the lesions varied from mild mesangial nephritis to severe diffuse proliferative glomerulonephritis. Subendothelial-mesangial deposits were confirmed by electron microscopy. The mice with the highest levels of anti-dsDNA had the most severe proliferative glomerulonephritis (Seery et al. 1997).

Having established that the transgenics had autoantibodies to dsDNA and histones, immune deposits in the kidneys and renal disease, it was apparent that the mice had several features of SLE. This led us to re-evaluate the skin phenotype. The skin of IFN-γ transgenic mice displays many abnormalities that are characteristic of acute cutaneous lupus erythematosus in humans, such as the induction of MHC and ICAM-1 expression on keratinocytes, loss of epidermal dendritic cells, the dermal mononuclear infiltrate, and alopecia (David-Bajar and David 1997). The occasional separation of the epidermis from the dermis with the infiltrate of haemopoietic cells (Fig. 1) is reminiscent of the hydropic degeneration of basal cells that is a characteristic of SLE (Lever and Lever 1983).

16.6 Role of *αβ* T Cells

Affinity maturation of IgG antinuclear antibodies implies a central role for autoantigen-specific CD4-positive T cells in the pathogenesis of human SLE (Radic and Weigert 1994; Desai-Mehta et al. 1995). To determine whether T cell-dependent processes are involved in the pathogenesis of the IFN-γ transgenics, we generated IFN-γ transgenic mice deficient in either $\alpha\beta$ or $\gamma\delta$ T cells (Seery et al. 1999). TCR $\delta^{-/-}$ transgenics continue to produce anti-nuclear autoantibodies and to develop severe kidney lesions. In contrast, TCR $\beta^{-/-}$ IFN-γ transgenic mice do not produce anti-nucleosome, anti-dsDNA or anti-histone autoantibodies, and kidney disease is abolished (Fig. 4).

$\alpha\beta$- And $\gamma\delta$-deficient IFN-γ transgenic mice continue to develop IFN-γ-associated skin disease, lymphadenopathy, and splenomegaly, showing that these features are separable from the production of autoantibodies. Interestingly, the skin phenotype in human lupus patients has occasionally been observed in the absence of systemic autoimmune disease (David-Bajar and David 1997).

We conclude that the autoantibody-mediated pathology of IFN-γ transgenics is completely dependent on $\alpha\beta$ T cells. This emphasises the relevance of the model to SLE, as there is strong evidence for a central role of this T-cell subset in the human disease (Kotzin 1996). In contrast to our mice, MRL/lpr mice develop features of lupus in the absence of $\alpha\beta$ T cells, and it has been postulated that $\gamma\delta$ T cells can substitute in this model (Peng et al. 1996).

16.7 Contribution of Apoptotic Keratinocytes

The dependence of anti-nuclear autoantibody production on $\alpha\beta$ T cell function suggested that the generation of antibodies is an antigen-driven process and raised the question of the source of antigen. Exposure of the skin of SLE patients to UV light can exacerbate systemic disease, implicating the epidermis as the source of self antigens in the generation of pathogenic anti-nuclear antibodies (Norris 1993). Apoptotic keratinocytes have been suggested to be the source of self (Casciola-Rosen and Rosen 1997).

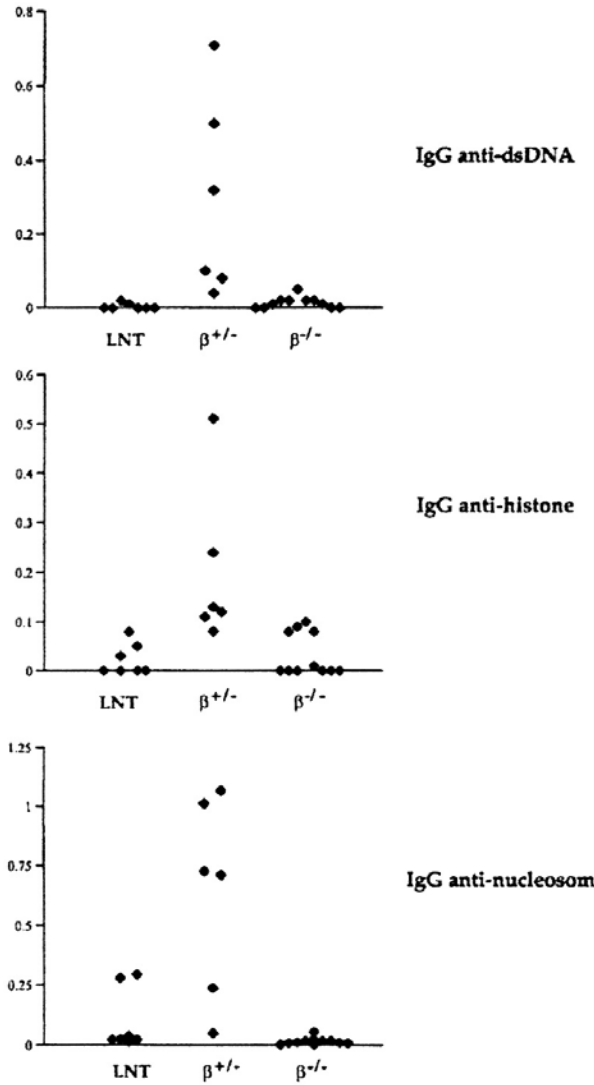

Fig. 4. ELISA assays of anti-dsDNA, anti-histone and anti-nucleosome levels in $a\beta$ T cell-deficient IFN-γ transgenic mouse serum. Sera from $\beta^{+/-}$, $\beta^{-/-}$ IFN-γ transgenic mice and transgene-negative littermates (*LNT*) are shown. (Reproduced from Seery et al. 1999, with permission)

When we examined sections of the skin of IFN-γ transgenics, we noted abnormal clusters of apoptotic cells in the interfollicular epidermis and hair follicles (Seery et al. 1999). IFN-γ transgenics have an elevated number of apoptotic keratinocytes, regardless of the T-cell status of the animals. In addition there are large amounts of TUNEL-positive material in the dermis, some of which appears to have been phagocytosed. Apoptotic cells in epithelia are known to be rapidly phagocytosed by macrophages (Metcalfe and Streuli 1997), and the majority of infiltrating cells in the dermis are of the monocyte/macrophage lineage (Carroll et al. 1997).

These observations led us to propose the following model. IFN-γ induces keratinocyte apoptosis, possibly via facilitation of Fas-Fas ligand interactions (Takahashi et al. 1995). Autoantigens from the epidermis are taken up by Langerhans cells and presented to antigen-specific autoreactive $\alpha\beta$ T cells in the draining lymph nodes, as a result of which antinuclear antibody-producing B cells are stimulated (Seery et al. 1999). The concept that apoptotic nuclei can act as antigens in the generation of antinuclear antibodies does have experimental support (Mevorach et al. 1998; Korb and Ahearn 1997; Burlingame et al. 1993).

In order to test our model, we examined the effects of a pan-caspase inhibitor, ZVAD-fmk, on IFN-γ transgenic mice (Seery et al. 2001). The animals received daily subcutaneous injections for 21 days. None of the animals showed an adverse response to the drug.

ZVAD-fmk has no effect on macroscopic skin disease. After treatment the mice still have persistent skin erythema, hair hypopigmentation, and dermal infiltration. ZVAD-fmk does not prevent an anuclear stratum corneum from forming, in contrast to the reported effects of caspase inhibitors on keratinocytes in culture (Weil et al. 1999). However, the number of TUNEL-positive cells in the interfollicular epidermis and underlying dermis is lower in ZVAD-fmk-treated animals than in saline-treated controls (Seery et al. 2001). One mouse that was treated with the drug for 3 weeks and then sacrificed 4 days after the final treatment had large numbers of apoptotic cells in the skin, demonstrating that the suppressive effect is dependent on continuous application (Seery et al. 2001).

The levels of anti-dsDNA produced by individual IFN-γ-transgenic mice treated with ZVAD-fmk show considerable variability.

Although the average anti-dsDNA levels of the treatment group fell by 24% from starting levels, this did not reach statistical significance ($p<0.1$). Four out of 17 mice showed a rapid and sustained fall in anti-dsDNA levels to values comparable to those of nontransgenic mice; these animals did not produce antihistone antibodies before or after treatment. Five animals showed sustained reciprocal changes in anti-dsDNA and anti-histone levels: in one a fall in antihistone was accompanied by a rise in anti-dsDNA, while in the other four animals the levels of anti-dsDNA fell and anti-histone rose.

In spite of the variable effects of the drug on autoantibody levels, ZVAD-fmk-treated IFN-γ transgenic animals demonstrate a marked reduction in the severity of renal disease (Seery et al. 2001; Fig. 5). The absence of severe kidney damage cannot be explained solely on the basis of changes in autoantibody levels, as some transgenic animals had no evidence of renal disease at the time of sacrifice even though they had high levels of anti-dsDNA. The sample size was too

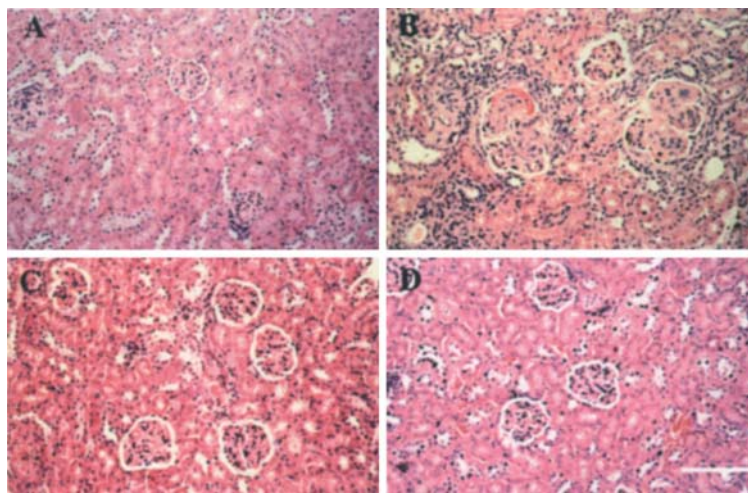

Fig. 5 A–D. Effect of ZVAD-fmk on kidneys of IFN-γ transgenic mice. **A** Wild-type littermate, **B–D** transgenics, **B** untreated, **C, D** treated with ZVAD-fmk for 3 weeks. Scale bar, 260 μm. (Reproduced from Seery et al. 2001, with permission)

small to conclude whether there had been any reduction in IgG deposition in the kidneys, although it was clear that drug treatment did not abolish immune complex deposition.

16.8 Conclusions

Although we originally set out to examine the role of IFN-γ in skin inflammation, we ended up with a murine model of SLE. The studies with ZVAD-fmk and the TCR knockout mice establish that the epidermal hyperproliferation and skin inflammation induced by IFN-γ are independent of autoantibody production and kidney disease (Seery et al. 1997, 1999).

We designed the ZVAD-fmk experiments with the premise that apoptotic keratinocytes provide the source of self-nuclear antigens that drive antinuclear antibody production in IFN-γ transgenics. Although anti-dsDNA levels normalised in some of the treated mice, it is unlikely that removing the source of antigen would switch off a mature T-cell response within a matter of days, and indeed the autoantibody titres, such as the reciprocal relationship between anti-histone and anti-dsDNA levels in some mice, suggest more complex changes. Recent studies suggest that predisposing factors to systemic autoimmunity may be incomplete induction of tolerance to apoptotic antigens, decreased apoptotic cell clearance, or abnormal signalling thresholds on responding lymphocytes (White and Rosen 2003). Our mice provide a valuable model with which to examine these issues.

Treatment with ZVAD-fmk resulted in a significant reduction in the severity of renal disease in IFN-γ transgenics. This is of interest because the severity of renal disease is the primary determinant of prognosis in the human condition (Berden 1997). ZVAD-fmk may act both systemically and locally to reduce renal damage in IFN-γ transgenic mice. By decreasing apoptosis both in the skin and, potentially, in other organs, the drug may reduce the rate of deposition of nucleosomes in the glomeruli, even in the presence of high anti-dsDNA levels and IgG deposits in the kidneys.

Apoptosis is a normal physiological process, and long-term administration of caspase inhibitors could result in significant side effects. However, SLE characteristically runs a relapsing/remitting

course (Berden 1997), and intermittent treatment with apoptosis inhibitors might therefore be a useful form of treatment.

Acknowledgements. The generation of a model of SLE represents a significant departure from the main research expertise of my lab. I am very grateful to the many people, including anonymous referees, who provided extremely helpful input along the way. Joseph Carroll initiated the project and the characterisation of the renal disease was the work of Victoria Cattell. The studies would have been impossible without the initiative and inspired experimentation of John Seery.

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