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Bruce Beutler
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Immunology, Phenotype First

How Mutations Have Established New Principles and Pathways in Immunology

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Preface

This monograph deals with the impact of classical genetics in immunology, providing examples of how large immunological questions were solved, and new fields opened to analysis through the study of phenotypes, either spontaneous or induced. As broad as biology has become, there are those who do not fully understand what the genetic approach is, and how it differs fundamentally from most of the methods available to natural scientists. They may hold the opinion that genetics has run its course since Mendel read his paper on peas in 1865. "Why bother with classical genetics," they may ask. "Won't all genes be knocked out soon anyway?" Or they are intimidated by genetics, with its heavy reliance on model organisms that seem so alien. "What has *C. elegans* to do with me?" the questioning might go. "It doesn't even have lymphocytes." Such skeptics may be unaware that the mouse is fast becoming as tractable a model organism as the fly, and that humans may not be too far behind. So I would like to introduce the topic with a few words about the power of genetics, and why it has contributed so much to immunology, and to biology in general.

Genetics, as the word is used here, is not merely the science of heredity, but much more than that. It is the science of exceptions: the science that takes note of heritable variation and seeks to explain it at the most fundamental level. It is the science that splits phenomena into phenotypes; then assigns them to individual genes and even portions of genes. Through genetics, unambiguous conclusions can be drawn about the function of every protein we have. Although all science seeks to explain phenomena, "phenotype" is available only to biologists. Only in biology is an organism's life-plan written in its genes, and subject to alteration by changing a letter here or a word there.

Geneticists do not shrink from applying the scientific method, but it is not their primary tool. They have something special, something that solves problems that are ineluctable through hypothesis and experimentation. Why is genetics so powerful? Several reasons might be cited.

First, genetic analysis is unbiased, while hypothesis-driven research is not. In principle, hypotheses are merely tools and there is nothing personal about them, and no reason to attach a bias to them. But people like to be right about things, even when being wrong might better serve the advancement of human understanding. Time and again, scientists try to "prove the hypothesis" (and occasionally even

write that they have done so) though they have been taught from their earliest days that the goal is to "test the hypothesis."

Genetic inquiry is different. Either the phenotype exists or it does not; either the phenotype is strong enough to map or it is not; either the mutation is found or it is not. Finding a mutation may be disappointing insofar as it may reside in a gene with well-known functions, in which case little progress may have been made. But there is no question of bending the rules. The geneticist is an explorer. His or her prior conceptions about how a biological system works will help in forming a decision as to whether a particular phenomenon is worth investigating, and may also help in deciding how to construct a screen. But preconceptions will not mislead.

Second, genetics is calculated to produce surprise. In foreswearing hypotheses, there is a certain humility, an admission that biological complexity outstrips our ability to guess at how a given process works. Instead, we surrender to the possibility of surprise, and even trust in surprise. Of course, it may be argued that hypothesis-driven research also produces surprise. One devises experiments to test hypotheses, and the outcome may run contrary to expectation. All the same, the genetic approach does not even ask a question. It merely seeks exceptions to the status quo. And some of those exceptions may be bizarre, or even undreamed of.

Third, genetics asks why things go wrong. It is a deconstructive process, rather than one of invention. It must be granted that looking at the effects of damage is not unique to genetics, but all the same, it is fundamental to genetics, and is a powerful approach whenever it is applied in biology. By studying the effects of strokes, tumors, and traumatic injuries, clinical neurologists and pathologists were able to deduce the function of many parts of the human brain. For example, they inferred that a "homunculus" must exist in the posterior frontal cortex (and close by it, a second homunculus in the anterior parietal cortex), wherein each part of the body is spatially reflected, so that a lesion might affect adjacent areas of the body: the face, neck, arm, and trunk, for example, or the trunk and the legs and feet; but never the face and feet without involvement of intermediate structures. Geneticists follow much the same practice as neurologists, focusing intently on the effects of spontaneous mutations or those induced at random by mutagens, brought to their attention because something has gone wrong. They are able to decipher the function of each part of the genome, which contains its own "homunculus" just as the brain does, but one that is enormously more fractured and complex. The proteins that are required for limb development (or innate immune sensing, or any complex function) each have their physical representation in the genome, and though the corresponding genes may be widely scattered, they can all be found through mutagenesis and careful phenotypic screening.

Fourth, genetic conclusions are comparatively solid. The reliability of genetic conclusions is derived from the reliability of the technology upon which genetic research is based (the unbiased mapping of phenotypes to critical regions, and ultimately, DNA sequencing). This is not to say that geneticists are never wrong, or that there was never a case in which a phenotype was incorrectly attributed to a particular mutation. But such mistakes are rare. When genetic data conflict with biochemical data, or data developed from immunological

assays, or data from cell transfection studies, or any combination thereof, the genetic data are usually correct.

The stories told in this book are some of the most important in immunology. Each begins with a phenotype and comes to a profound conclusion about cause. In some cases autoimmunity was at issue; in others cancer; in others a failure to detect or respond to infection. But in all instances, the biological function of a given protein or protein family was discovered. Finding the mechanism through which that protein functions presents the next challenge, and in all cases, the challenge has yet to be met in full. Ultimately, the geneticist must usually make hypotheses after all. Usually he or she is not alone: the field has been opened to many other workers once the key genetic advance has been made.

Reverse genetic methods are among the most powerful tools to be used in testing these hypotheses. Again, the situation might be compared to that of the neuroscientist, who creates brain lesions in experimental animals in order to test the function of distinct parts of the brain, alone or in conjunction with one another. Reverse geneticists, who deliberately target genes for destruction, attempt to test the function of particular parts of the genome. In both cases, nothing may be found, either because of functional redundancy, or because the investigator simply does not know what to look for. But at times, concrete and specific understanding is gained.

The interpretation of phenotypes is facilitated when there is a strong conceptual framework within which to operate. This is certainly the case in immunology, a relatively sophisticated science that has taught us quite a lot, though enormously less than it has left to teach. We know of innate immunity and adaptive immunity; we know of humoral immunity and cellular immunity. We know of antibody and complement. And we know of T cells, B cells, T-regulatory cells, antigen-presenting cells, natural killer cells, macrophages, and granulocytes. Each has a distinct role to play in protecting us from infection, or conversely, in causing inflammatory disease. Mutations can make things go very wrong where every cell and protein just mentioned is concerned. Yet we still lack a fully coherent understanding of exactly why we reject cells from unrelated individuals yet tolerate the placental allograft. We do not know why some among us develop autoimmunity while the majority does not. We do not understand why all microbes are recognized (for some, recognition receptors have yet to be found), and why some defy the immune response so effectively even when they are detected. This is the perfect playground for a geneticist: a desirable mixture of ignorance and understanding. And it is likely to remain this way for a very long time.

Yes, all genes will soon be knocked out. But many knockout mutations will be embryonic lethal, or will have other effects that mask the essential immunological function of the proteins concerned. Others will present no obvious phenotype, not because the gene in question has no function, but because we simply do not know what to look for. There is no escape from starting with phenotype. Biologists will always return to the phenotype-first approach.

We live at the dawn of a golden age of genetics, in which a phenovariant may be seen in the morning and the causal mutation known by noon. Stories formally similar to the ones presented here may soon be increasingly common, and we must all hope

that they will be. But it should not be forgotten that these particular discoveries—whether in mice or in humans, most of them pursued before the respective genomes were sequenced and some of them at a time when sequencing was performed mostly manually—were heroic in their own time and have laid the foundation for some of the most important concepts in immunology.

La Jolla, USA Bruce Beutler

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Part I
Immunodeficiency

The Forward Genetic Dissection of Afferent Innate Immunity

B. Beutler(✉), E.M.Y. Moresco

Contents

Abstract Recognition of the microbial world is mediated chiefly by a small group of immune receptors that activate a characteristic host inflammatory response, the innate immune response. Known as the Toll-like receptors (TLRs), these molecules are represented among most metazoans. In mammals, forward genetic analysis of the lipopolysaccharide (LPS) response led to the identification of TLR4 as the LPS receptor. Through a combination of forward and reverse genetic studies, a relatively detailed understanding of the functions of mammalian TLRs has now been achieved. As discussed here, mutagenesis has revealed proteins that participate in TLR signaling pathways, and informed our understanding of the subtleties of these molecules' structure and function.

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Introduction

The molecular basis of mammalian innate immune perception remained obscure for many years after microbes were first identified as the causal agents of infectious disease. From the earliest decades of the twentieth century it was widely assumed that specific receptors must detect microbes or molecules that they manufacture. Some of the microbial molecules that served as targets for recognition were established at that time, and their structures elucidated soon thereafter, because they elicited powerful inflammatory effects evocative of an authentic infection. But the host receptors for these molecules proved highly elusive.

In recent times the terms "pattern recognition receptors," "pathogen-associated molecular patterns," and "danger signals" have been introduced into the innate immunity field. But while these were convenient and all-embracing terms for conserved molecules of microbial origin, they brought the field no nearer to finding the receptors. Whatever one called them ("pattern recognition receptors" or "danger receptors"), the primary molecular sensors of infection remained beyond reach until genetic methodologies advanced to the point that they could be found.

The question as to precisely how we sense microbes was an important one for several reasons. First and foremost, it went to the heart of self/non-self discrimination: a topic that is fundamental in immunology. Second, whatever the receptors were, they transduce the very first molecular events that transpire after the inoculation of microbes. These initial events "light the fuse" for all that follows during infection, including the development of the inflammatory response, which limits infection when circumscribed but can prove fatal when generalized. Third, the inflammatory response seen during infection is biochemically similar to the inflammatory response seen during sterile inflammatory diseases. The same gateways to inflammation that are triggered by microbes might be activated to our detriment in rheumatoid arthritis, systemic lupus erythematosus, and other diseases in which the immune system plays a destructive role. Simply put, these receptors orchestrate the most powerful inflammatory events we know of.

A pure genetic approach ultimately led to the identification of the Toll-like receptors (TLRs) as innate immune sensors. This approach is recounted here. It was followed by extensive mutagenic analysis of the TLR signaling pathways, which further informed us of the key proteins used by TLRs to elicit changes in gene expression, leading to the activation of an antimicrobial state. The genetic approach, which depends on identifying mutations that cause phenotypes rather than the formation and testing of hypotheses, has many advantages. Importantly, it leads one to discover the unexpected. In addition, because it is unbiased, it is not subject to the type of manipulation that besets experimentalists who want to "prove the hypothesis correct."

The LPS Receptor, and How We Know It Exists

The most potent and best-studied molecule of microbial origin that triggers innate immune responses is lipopolysaccharide (LPS), a major constituent of the outer membrane of gram-negative bacteria. Famous for its ability to cause fever and

shock, LPS in fact recreates many of the effects of an infection. It does so through an effect on cells of hematopoietic origin (Michalek et al. 1980), and specifically, does so by eliciting cytokine production by these cells. In 1985, tumor necrosis factor (TNF), elaborated in large amounts by LPS-activated macrophages (Beutler et al. 1985a, b) was shown to be a major contributor to the lethal effect of LPS in vivo (Beutler et al. 1985c).

In 1965 it was noticed that mice of the C3H/HeJ substrain were impervious to the lethal effect of LPS (Heppner and Weiss 1965), a finding confirmed and extended by Sultzer, who also observed that C3H/HeJ mice do not mount a normal exudative response to LPS when injected intraperitoneally with the substance (Sultzer 1968). By 1975 the existence of a single locus governing responses to LPS had been established (Watson and Riblet 1975), and by 1978 this locus had been mapped to mouse chromosome 4 using classical phenotypic markers (Watson et al. 1977, 1978). Coutinho and colleagues subsequently identified a second LPS-resistant strain (C57BL/10ScCr), showing that the mutation responsible for resistance in this strain was allelic with the resistance mutation in the C3H/HeJ strain (Coutinho et al. 1977; Coutinho and Meo 1978). Depending on the assay used, the phenotype of LPS resistance was either recessive or co-dominant, and it extended to all aspects of the LPS response, including, for example, the adjuvant effects of LPS (Skidmore et al. 1975), and the ability of mice to make antibodies against LPS itself (Coutinho and Gronowicz 1975). The universal dependence of LPS effects on the *Lps* genotype spoke strongly in favor of a single, nonredundant pathway for LPS perception in mammals, no matter how many protein components that pathway might incorporate. The cytokine response to LPS was ultimately used as an endpoint in detecting LPS responses and in cloning the *Lps* locus. Some investigators used other endpoints [B-cell mitogenesis (Peavy et al. 1970; Andersson et al. 1972; Coutinho and Gronowicz 1975); changes in pulmonary compliance (Peiffer-Schneider et al. 1997)] in the attempt to confine the mutation to a manageable critical region.

Identification of TLR4 as the LPS Receptor

Before positional cloning was feasible in the mouse, many attempts were made to identify the LPS receptor, and some of these efforts made use of LPS nonresponder mice. In two early studies (Forni and Coutinho 1978; Coutinho et al. 1978), antibodies raised in rabbits against C3H/Tif (normal LPS responder) strain B cells and exhaustively absorbed using cells from C3H/HeJ mice were found to be differentially reactive with LPS responder strains and the nonresponder strains C3H/HeJ and C57BL/10ScCr. However, the antiserum was never successfully used to isolate an LPS receptor.

Using an endogenously ^{14}C-labeled LPS preparation, Kabir and Rosenstreich tested C3H/HeJ and C3H/HeN splenocytes for differences in binding, and found no significant difference (Kabir and Rosenstreich 1977). Similarly, Watson and Riblet found that ^{3}H LPS bound equally well to C3H/HeJ and C3HeB/FeJ spleen cells

(Watson and Riblet 1975). They concluded that while a membrane-associated signaling molecule was likely defective in C3H/HeJ mice, the primary interaction between LPS and the lymphocyte was not dependent upon this molecule, and likely occurred through hydrophobic interaction with the membrane.

Affinity chromatography was used to search for LPS binding sites on human erythrocytes (Yokoyama et al. 1978) and mouse lymphocytes (Yokoyama et al. 1979), with the finding that band III protein and PAS (Periodic Acid-Schiff)-1 glycoprotein bound LPS in the former instance, and class I MHC bound LPS in the latter instance. These were two rather early examples of approaches taken by many investigators, who sought to find the LPS receptor through biochemical means (Wright and Jong 1986; Lei and Morrison 1988a, b, 1993; Lei et al. 1990, 1993; Bright et al. 1990; Wright 1991). Some putative binding molecules were never identified; others (like those just mentioned) are now considered to be irrelevant to LPS responses. Of particular note was the theme that CD18, complexed with one or more of the CD11 integrins, was essential to LPS responses (Golenbock et al. 1990; Lynn et al. 1991; Ingalls and Golenbock 1995; Ingalls et al. 1997, 1998a, b, 1999; Flaherty et al. 1997; Bhat et al. 1999).

Highly informative biochemical studies of LPS activity established that a plasma protein called LPS binding protein (LBP) engages LPS (Wright et al. 1989; Schumann et al. 1990) and that LPS signaling subsequently depends upon CD14 (Wright et al. 1990). This conclusion depended upon antibody depletion studies, and was validated later by the phenotype of mice that lack CD14 (Haziot et al. 1996; Jiang et al. 2005). CD14 therefore seemed to be at least a key part of the LPS receptor. Because the protein had no transmembrane domain, however, and was instead tethered to the surface by a glycosylphosphoinositide anchor, it was believed that it must participate in a complex with another protein(s) in order to signal. Moreover, the *Cd14* locus maps to chromosome 18 in the mouse; the *Lps* locus was by that time known to reside on chromosome 4 (Watson et al. 1978).

The early confinement of *Lps* to a position between the major urinary protein (*Mup1*) and polysyndactyly (*Ps*) loci (Watson et al. 1978) encompassed the type I interferon (IFN) genes, and as we now know, covered approximately 35 Mb of genomic DNA within which approximately 205 annotated genes reside. At the time, however, the total number of genes was unknown, and the type I IFN genes were regarded as early candidates. They were ultimately excluded by genetic mapping.

Prior to the year 2000 the mouse genome was largely *terra incognita*, and not only the total number of genes, but their relative locations within the genome, were open to discovery. After the landmark work of Watson and colleagues (1977, 1978), mapping efforts remained in abeyance for nearly 15 years. Although loose confinement of the *Lps* locus was made by recurrent crossing of C3H/HeJ to BALB/c, with the development of a congenic interval approximately 5.5 cM in size (Vogel et al. 1994), efficient mapping through parallel examination of thousands of meioses was not undertaken until the mid-1990s. A series of deletion constructs, spanning a large part of chromosome 4 including the *brown* (*b*) locus (Rinchik et al. 1994), were also used in an attempt to narrow the location of the gene, but without success,

and ultimately ended with the erroneous conclusion that the genotype Lps^{d}/Lps^{θ} supports normal LPS signal transduction (Vogel et al. 1999). It is not entirely clear why this effort failed, as it is now quite clear that the Lps^{d}/Lps^{θ} genotype actually yields a nonresponder phenotype. For whatever cause, the location of the gene was not tightly confined by these approaches, however promising they might have seemed.

As the density of markers in the mouse genome grew, several laboratories attempted to narrow the position of *Lps*. During this later phase of investigation, the construction of contigs (from YAC or BAC clones) was undertaken independently in at least two laboratories. The de novo search for genes was then attempted (in the early days) through exon trapping; later, as expressed sequence tag (EST) libraries grew more complete, the search continued through basic local alignment search tool (BLAST) analysis of shotgun sequences of genomic DNA, while at all times it continued through computer-aided recognition of coding regions (programs such as Genscan and GRAIL).

Malo and colleagues mapped *Lps* on 1,604 meioses to a position between a proximal cluster of genes including *Cd30l*, *Hxb*, and *Ambp*, and the distal markers D4Mit178 and D4Mit7, which at the time had not been resolved from one another (Qureshi et al. 1996). Schwartz and colleagues mapped the locus on the basis of changes in pulmonary compliance occurring following intratracheal administration of LPS (Peiffer-Schneider et al. 1997).

The *Lps* locus was mapped to maximum resolution (2.6 Mb) on 2,093 meioses and positionally cloned by Poltorak and colleagues, who first succeeded in finding the relevant gene in the *Lps* critical region (Poltorak et al. 1998b) and, within it, the mutation responsible for LPS resistance (Poltorak et al. 1998a). In C3H/HeJ mice a single nucleotide substitution altered the cytoplasmic domain of Toll-like receptor 4 (TLR4), while in the C57BL/10ScCr mice and in the C57BL/10ScN strain (from which the former substrain was derived), a small deletion removed the *Tlr4* gene entirely (Poltorak et al. 1998a, b). Later, the exact limits of this deletion were determined (Poltorak et al. 2000), and it was further shown that C57BL/10ScCr (but not C57BL/10ScN) had a point mutation in the interleukin (IL)-12 receptor β2 chain, which caused a different form of immunocompromise superimposed on the LPS sensing defect (Poltorak et al. 2001).

The identity of the *Lps* locus was subsequently confirmed by Malo and her co-workers (Qureshi et al. 1999a, b). Later, the *Tlr4* gene was targeted for deletion by Akira and colleagues, who found an LPS-resistant phenotype (Hoshino et al. 1999). In still later work, the gene was inserted into the genome of C57BL/10ScCr animals by bacterial artificial chromosome (BAC) transgenesis, which restored LPS sensing (Kalis et al. 2003). This latter study also revealed that the *Tlr4* locus is haploinsufficient; moreover, the gene copy number determines LPS signaling intensity over a fairly wide range (Kalis et al. 2003).

Prior to the positional cloning of *Lps*, the function of the *Tlr4* locus was unknown. TLR4 was one of several homologs of the *Drosophila* protein Toll, which had been known since the early 1990s to exist in mammals (Nomura et al. 1994; Taguchi et al. 1996). Toll had initially been known for its developmental role in the

fly, but was shown in 1996 to have an immunological function as well (Lemaitre et al. 1996), as described in detail in the following section. Pursuant to this realization, it was shown that TLR4 could activate nuclear factor (NF)-κB in mammalian cells (Medzhitov et al. 1997), and it was speculated that it might have a role in mammalian immunity (both innate and adaptive), just as Toll was known to be important in *Drosophila* immunity. However, the genetic demonstration that TLR4 served as the membrane-spanning component of the mammalian LPS receptor, required for surviving gram-negative infections, gave the key insight into how mammals sense infection.

Innate Immunity in *Drosophila melanogaster*

Concurrent with the *Lps* locus positional cloning effort, Jules Hoffmann and colleagues worked to understand resistance to infection in insects. Among the key effectors of insect immunity are antimicrobial peptides, seven classes of which were identified by the Hoffmann lab. These included the Drosocin, Diptericin, Drosomycin, Metchnikowin, Cecropin, Attacin, and Defensin classes of peptide. It was observed that the genes encoding these proteins had motifs similar to those known to recognize NF-κB in their promoter regions; subsequently, it was found that the promoters would in fact respond to NF-κB activating stimuli (Reichhart et al. 1992; Georgel et al. 1993; Kappler et al. 1993; Meister et al. 1994). Drosomycin was particularly important for the containment of fungal infections, and was induced by fungal infection, while Diptericin was important for the containment of gram-negative infections, and was induced by gram-negative infection.

Only three NF-κB variants (Dorsal, Dif, and Relish) exist in *Drosophila*. The Toll signaling pathway, found by Nüsslein-Volhard and colleagues to be required for dorsoventral patterning in the fly embryo, was known as a possible source of NF-κB activation, triggering the nuclear translocation of Dorsal. Indeed, Toll was found to be essential for Drosomycin production in adult flies challenged with fungus (Vitaterna et al. 1994). So too was the Toll ligand Spaetzle, which was generated by proteolytic cleavage from a precursor in response to a then-unknown cascade triggered by infection. The NF-κB analog Dif, however, was used in preference to Dorsal (Rutschmann et al. 2000).

On the other hand, the gram-negative response pathway was clarified by positional cloning of a spontaneous mutation called Immune deficiency (Imd), which proved to affect a receptor-interacting protein (RIP)-like cytoplasmic protein linked to a transmembrane peptidoglycan recognition protein (PGRP). The Imd pathway also involved *Drosophila* FADD, an ortholog of the Fas-associated death domain linker protein in mammals, DREDD, a homolog of Caspase 8 in mammals, and Tab2. The pathway ultimately triggered the activation of Relish. It was strongly evocative of the TNF signaling pathway (Georgel et al. 2001).

The identification of Toll as a mediator of immunity in *Drosophila* was completed in 1996, while the identification of Imd as a mediator of immunity was

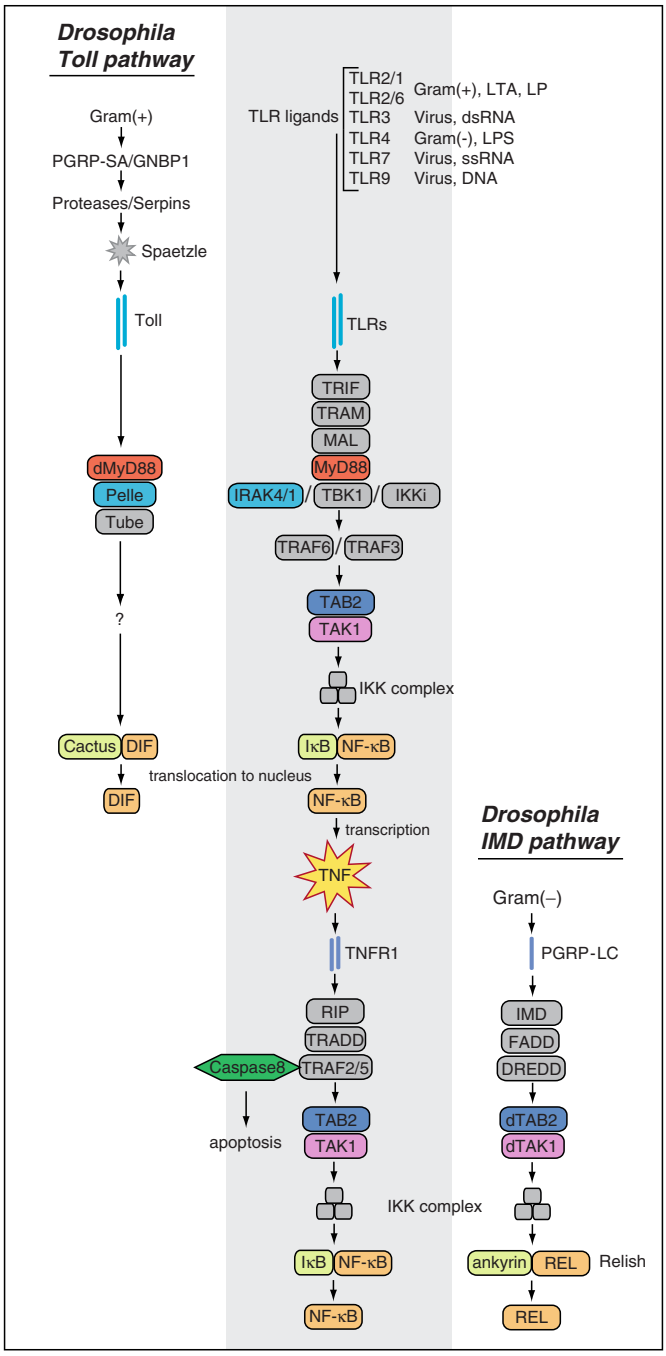
achieved in 2001. The fact that Toll and the mammalian TLRs (as recognized in 1998) have conserved defensive functions was interesting in its own right. But the added fact that the Imd pathway is in many ways similar to the TNF pathway indicated something else. While Imd and Toll pathways operate with complete independence in *Drosophila*, any stimulus that activates a mammalian TLR will also activate TNF production and thence the TNF signaling pathway (Fig. 1). The two pathways are linked to one another in mammals. The special importance of the TNF pathway in defense in mammals, where it clearly does protect against infection by mycobacteria (Kindler et al. 1989), *Listeria monocytogenes* (Havell 1987), and other microbes, is reflected by its existence as a major response pathway with its own sensing mechanism in *Drosophila*. The efficacy of interdiction of TNF in the treatment of inflammatory diseases such as rheumatoid arthritis, Crohn's disease, psoriasis, and other ailments also speaks to its key importance as a linker of pathways that, in some organisms, have each assumed large duties in the inflammatory response.

The General Role of the TLRs in Innate Immune Recognition, and Their Conserved Structure

TLR4 is a large, single-spanning type I membrane protein marked by leucine-rich repeats (LRRs) throughout the length of its ectodomain, except in a centrally placed "hinge" region (Kim et al. 2007). The cytoplasmic domain of the protein is almost entirely devoted to a single TIR (Toll/IL-1R/Resistance) domain—a fold also observed in the IL-1 receptor and IL-18 receptor subunits—and in certain other receptors that have immunoglobulin-type repeats in their ectodomains (SIGIRR, TIGIRR, and ST2 proteins). The TIR domain has been identified in many proteins; both in plants and animals it is associated with defensive function (McHale et al. 2006; Roach et al. 2005). Microbes have also captured the TIR motif, and may use it to thwart immune signaling (Stack et al. 2005).

In humans there are 10 TLR paralogs; in mice, 12; in both species combined, 13. While humans lack TLRs 11, 12, and 13, they express TLR10 (which mice lack) and have an active TLR8 (no known activity is associated with TLR8 in mice). The TLRs are probably all dimeric (or heterodimeric) in structure. All TLR ectodomains probably assume a "curved solenoid" shape characteristic of LRR proteins (Jin et al. 2007; Kim et al. 2007).

When the function of TLR4 was determined, other members of the TLR family were already known in mammals. TLR1 had been the first mammalian homolog of Toll to be identified (Nomura et al. 1994; Taguchi et al. 1996), and by 1998 TLRs 2, 3, and 5 had been found by homology searches. TLRs 6–10 followed soon after as mouse and human genomic sequences approached completion; in the mouse, TLRs 11–13 (not represented in humans) are now known to complete the family (Tabeta et al. 2004). Because each of the TLRs was endowed with a similar cytoplasmic domain, conserved in Toll, IL-1 receptor chains, and IL-18 receptor chains, it appeared that they might each transduce similar signals, but perhaps in response


Drosophila
Toll pathway
Gram(+)
PGRP-SA/GNBP1
Proteases/Serpins
Spaetzle
Toll
dMyD88
Pelle
Tube
?
Cactus
DIF
DIF
translocation to nucleus
TLR ligands
TLR2/1
TLR2/6
TLR3
TLR4
TLR7
TLR9
Gram(+), LTA, LP
Virus, dsRNA
Gram(-), LPS
Virus, ssRNA
Virus, DNA
TLRs
TRIF
TRAM
MAL
MyD88
IRAK4/1
TBK1
IKKi
TRAF6
TRAF3
TAB2
TAK1
IKK complex
IκB
NF-κB
NF-κB
transcription
TNF
TNFR1
RIP
TRADD
Caspase8
TRAF2/5
apoptosis
TAB2
TAK1
IKK complex
IκB
NF-κB
NF-κB
Drosophila
IMD pathway
Gram(–)
PGRP-LC
IMD
FADD
DREDD
dTAB2
dTAK1
ankyrin
REL
Relish
REL

to different ligands. It made sense to hypothesize that the other TLRs each recognize various molecules of microbial origin as well.

When Akira and colleagues deleted TLR2 by gene targeting, they found that it was required for detection of lipopeptides (Takeuchi et al. 1999) and other components of gram-positive bacterial cell walls. TLR2 was found to operate in conjunction with TLRs 1 or 6 (Ozinsky et al. 2000), each heterodimeric complex showing a different ligand specificity (Takeuchi et al. 2001, 2002). TLR5 was eventually found to be a receptor for flagellin (Hayashi et al. 2001) while TLR3 was a receptor for poly I:C (Alexopoulou et al. 2001), TLR9 a receptor for unmethylated DNA (Hemmi et al. 2000), and TLR7 and TLR8 (the latter only active in humans) could detect ssRNA and the inflammatory nucleotide-based imidazoquinoline drugs resiquimod, imiquimod. and loxoribine (Hemmi et al. 2002; Heil et al. 2003).

In some cases (e.g., in the case of the TLR1/2 heterodimer), TLRs directly engage their microbial ligands; in other cases, the ligands probably interact first or exclusively with accessory proteins that then alter the shape of the TLRs and trigger a response. For example, TLR4 was shown to be tightly associated with MD-2 (Shimazu et al. 1999), a small protein later shown quite convincingly to bind LPS (Ohto et al. 2007) and presumably to transmit information about the LPS to the

Fig. 1 Mammalian TLR and *Drosophila* Toll and IMD pathways. The mammalian TLR and TNF pathways (schematized within the *gray bar*) are connected: any TLR-activating stimulus will lead to TNF production and subsequent TNF pathway activation. The Toll signaling pathway in flies (*left*) is homologous to the mammalian TLR pathway; the IMD pathway in flies (*right*) is homologous to the mammalian TNF pathway. TLR activation by various microbial ligands recruits between one and four of the adaptors TRIF, TRAM, MAL, and MyD88 in mammals and leads to the activation of IRAK family protein kinases or the TBK1 or Ikki family kinases, TRAF6 or TRAF3, and ultimately the IKK complex. The IKK complex phosphorylates IκB, which releases NF-κB for transcription of targets, including TNF. Activation of the TNF pathway then follows, stimulating TNF receptors to recruit RIP, TRADD, and TRAF2 and/or -5. These activate the TAB2/TAK1 complex, which leads to NF-κB activation in subsequent steps. Alternatively, TRAF2/5 may activate caspase 8, resulting in apoptosis. In the Toll pathway (*left*), the ligand for the Toll receptor is Spaetzle, which must first be cleaved by proteases or serpins initially activated through binding of gram-positive bacteria to PGRP-SA (peptidoglycan-recognition protein-SA) or GNBP1 (gram-negative binding protein 1). As in the TLR pathway, Toll requires the function of an adaptor, dMyD88, which forms part of the trimeric TISC (Toll-induced signaling complex) with Pelle (an IRAK homolog) and Tube. Through unknown mechanisms, TISC signals to Cactus (IκB homolog) to release DIF (dorsal-related immunity factor, an NF-κB homolog) for transcription activation. The IMD pathway (*right*) functions independently from the Toll pathway, is activated by gram-negative bacteria, and leads to activation of a distinct NF-κB homolog, Relish. PGRP-LC senses gram-negative peptidoglycan and recruits IMD (RIP homolog), FADD (FAS-associated death domain, a TRADD homolog) and DREDD (death-related ced-3/Nedd2-like protein), which in turn activate dTAB2 and dTAK1. As in the mammalian TNF pathway, dTAB2 and dTAK1 lead to Relish (NF-κB homolog) activation. In the case of Relish, the inhibitory C-terminal ankyrin repeats of Relish are cleaved, and remain in the cytoplasm. The active REL moiety translocates to the nucleus and stimulates transcription. *dsRNA*, double-stranded RNA; *LTA*, lipoteichoic acid; *LP*, lipopeptides; *ssRNA*, single-stranded RNA

TLR4 subunit (Kim et al. 2007). In the absence of any of three proteins: MD-2, CD14, or TLR4, responses to glycosylated LPS are abolished (Jiang et al. 2005). As described in "Requirements for TLR2 and TLR4 Complex Signaling", both CD36 and CD14 participate in signaling via TLR2 complexes (Hoebe et al. 2005; Jiang et al. 2005).

The Adaptors That Serve TLR Signaling

Muzio et al. (1997) observed that a cytoplasmic adaptor protein called MyD88 had homology to the IL-1 receptor cytoplasmic domains, and offered evidence that this protein participates in IL-1 signal transduction, an observation confirmed by the phenotype of the MyD88 knockout mouse (Adachi et al. 1998). MyD88 recruits members of the IL-1 receptor-associated kinase (IRAK) family to the activated complex via death domain interactions. MyD88 also supports signaling from most of the TLRs, with the exception of TLR3, which is fully MyD88 independent, and TLR4, which retains some signaling potential in the absence of MyD88. A total of four adaptors, however, are now known to be influential in mammalian TLR signaling: MyD88, MAL, TRIF, and TRAM (Fig. 2).

Two of these adaptors were sufficiently homologous to the TLRs to be identified by BLAST searches of EST databases. These were MyD88 and its closest homolog, MAL (also known as Tirap) (Fitzgerald et al. 2001; Horng et al. 2001). MyD88 is required for all signaling via the TLR2 complexes, TLR5, and TLRs 7, 8, and 9, as well as IL-1R and IL-18R. It is partly required for signaling via TLR4. It is not required for signaling via TLR3. MAL is partly required for signaling via the TLR2 complexes and TLR4, but no other TLRs (Fitzgerald et al. 2001).

Even when both MyD88 and MAL are targeted and a compound homozygous mutant is produced, residual signaling via TLR4 and unimpaired signaling via TLR3 are observed (Yamamoto et al. 2002). In particular, production of type I IFN (IFN-β) is unimpaired, suggesting that in the absence of MyD88 or MAL (or both), IRF-3 (interferon response factor-3), the transcription factor required for IFN-β production, undergoes phosphodimer formation and activates the IFN-β gene. This residual signaling occurs by way of the so-called "MyD88 independent pathway," which studies of the *N*-ethyl-*N*-nitrosourea (ENU)-induced phenotype *Lps2* (See "TRIF is the MyD88-Independent Adaptor") and knockout mice demonstrated relies on the adaptor TRIF (Yamamoto et al. 2003a; Hoebe et al. 2003). IFNβ production is the hallmark of MyD88-independent signaling, and it is now known that TLR3 and TLR4 recruit TRIF and TBK1 (TRAF-family-member-associated NF-κB-activator-binding kinase 1), the critical kinase required for activation of IRF3 (McWhirter et al. 2004).

While the functions of MyD88 and MAL were first resolved by gene knockout work, the functions of the MyD88-independent adaptor proteins were deduced independently by both forward and reverse genetic methods, and in the former case, ENU mutagenesis was used to create the phenotypes of interest.

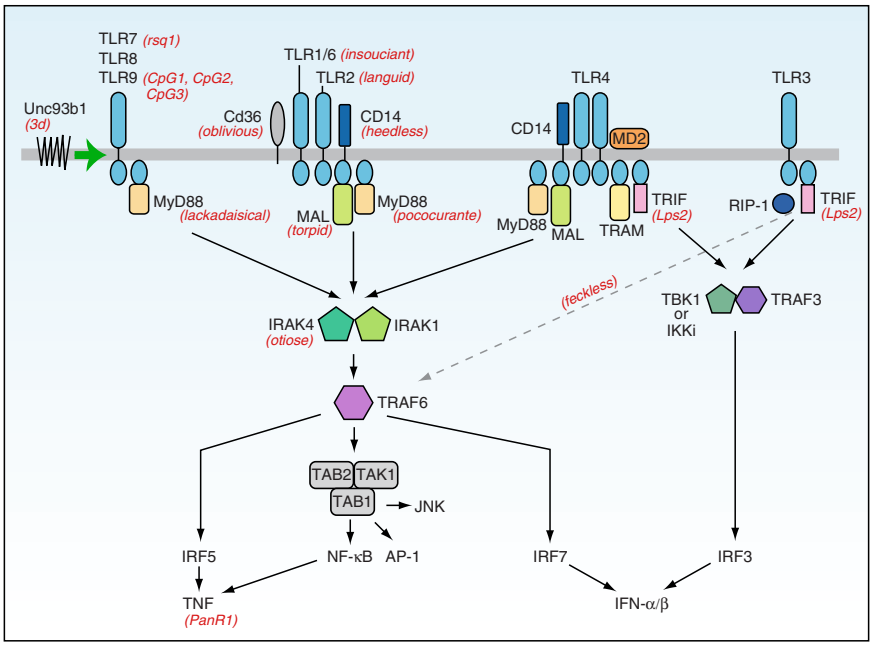


Fig. 2 Overview of TLR signaling pathways. The pathways represent data obtained from both forward and reverse genetic studies. *N*-Ethyl-*N*-nitrosourea (ENU)-induced phenotypes are shown in *red text*. Note that TLRs 3, 7, and 9 are endosomal proteins, while TLRs 1, 2, 6, and 4, as well as TLR5 (not shown), are expressed on the plasma membrane. Unc93b1 is an endoplasmic reticulum protein that influences endosome function. TLR activation recruits TRIF, TRAM, MAL, and/or MyD88 and leads to the activation of IRAK1 and IRAK4, or the TBK1 or Ikki family kinases. TRAF6 signaling to the TAB1/TAB2/TAK1 complex activates NF-κB, AP-1, and/or c-Jun N-terminal kinase (*JNK*). As mentioned in the text, IRF5 is another means by which signaling through MyD88 leads to TNF production. MyD88 and TRAF6 may interact directly with IRF5 in a complex, thereby activating IRF5 and promoting its translocation to the nucleus. Additionally, MyD88, together with TRAF6 and IRAK4, has also been shown to bind IRF7 directly in order to stimulate IFN-α production. This occurs downstream of TLR7, TLR8, and TLR9 in plasmacytoid dendritic cells and requires the phosphorylation of IRF7 by IRAK1 (Uematsu et al. 2005). In the MyD88-independent pathway, TLR3 or TLR4 recruit TRIF and TBK1, the critical kinase required for activation of IRF3. TRAF3 mediates the interaction between TRIF and TBK1. The kinase RIP-1 is required for NF-κB activation downstream of TRIF, and likely requires the direct interaction between TRIF and RIP-1. The point at which RIP-1 signaling impinges on the NF-κB pathway is unknown (*dashed gray arrow*)

ENU Mutagenesis in the Identification of TLR Signaling Proteins

The unbiased forward genetic approach to TLR signaling began in the year 2000 and has continued to the present time, with the production of more than 100,000 germline mutants over a 7-year time frame, most of them third generation (G3)

offspring homozygous for a fraction of the mutations induced by the germline mutagen ENU in G0 mice. More than 30,000 mice have been screened for germline mutations that alter signaling from seven of the TLR complexes, causing impairment of TNF production as measured by a biological assay (L-929 cell cytotoxicity). A total of 19 transmissible mutations have been detected. Of these, 15 have been identified at the nucleotide level. Of the seven TLRs held under surveillance, four have been struck (one of them 3 times and the others once each). Three of the four adaptor proteins known to exist have been struck (one of them twice). Hence, an appreciable level of saturation—though certainly not complete saturation—has been achieved.

As described elsewhere in detail, ENU is the most widely used mutagen for the generation of phenotypical variance in mice, and is applied with the goal of positionally cloning the mutations responsible for phenotypes of interest (Beutler et al. 2007a). It causes mutations at a frequency sufficiently high to produce phenotype variance in abundance, low enough to permit unambiguous assignment of responsibility to a single mutation in the majority of cases, and to do so with only a modest amount of effort invested in mapping. A particular advantage of variant alleles produced by the ENU approach consists in the fact that they reside on a pure, defined genetic background (in our laboratory C57BL/6J). Knockouts, by contrast, generally exist on a rather poorly defined background, and this may sometimes create confusion in assessing phenotypes. ENU has the added virtue of producing viable alleles of genes for which null alleles are lethal in the homozygous state.

TRIF Is the MyD88-Independent Adaptor

An ENU-induced mutation called *Lps2* was the first germline mutation to affect MyD88-independent signaling (Fig. 2). Identified by Hoebe et al. after screening 4,000 mice, roughly half of them G3 and half G1 mutants, the *Lps2* phenotype was so named because it approximated the classical *Lps* phenotype (Hoebe et al. 2003). Mice showed a much diminished TNF response to LPS, and were entirely unable to produce type I IFN in response to LPS. Notably, however, TLR3 signaling was entirely abolished by the *Lps2* mutation (not a characteristic of the *Lps* mutation in Tlr4). It thus appeared that MyD88-independent signaling was selectively ablated. It also seemed clear that TLR4-driven TNF production was dependent partly on MyD88-independent signaling. Finally, it was clear that TLR3 and TLR4 must share a common transducer.

The *Lps2* mutation was mapped on more than 1,600 meioses to a 216-kb region of mouse chromosome 17, containing eight genes, one of which was annotated only as "novel," but on inspection proved to have a TIR domain and to be identical to a newly recognized TIR adaptor protein elsewhere called Ticam1 (Oshiumi et al. 2003a) or TRIF (Yamamoto et al. 2003a). This adaptor had been identified in one laboratory on the basis of two-hybrid system analyses using the TIR domain of TLR3 as bait to search for a novel binding protein (Oshiumi et al. 2003a), and in

another laboratory by advanced homology searches (Yamamoto et al. 2003a). It was believed to support MyD88-independent signaling because, when overexpressed, it would drive type I IFN signaling. It was not at all clear, however, which TLRs depended upon it, with one group suspecting that it was utilized by all TLRs and another suggesting that it was utilized only by TLR3. In *Lps2* mutant mice, the TRIF coding region was disrupted by a single base pair deletion, leading to alteration and premature truncation of the protein. The product was, as a result, unstable and nonfunctional.

One interesting feature of the *Lps2* phenotype was observed on FACS analysis of macrophages from mice with the mutation. A fraction of cells were *Lps2*-independent, in the sense that when stimulated with LPS, they made TNF despite the mutation. No such population was evident when poly I:C was used as a stimulus. This implied that still another adaptor might substitute for TRIF in a fraction of macrophages, signaling downstream of TLR4 (but not TLR3) in the absence of TRIF.

Once TRIF was identified, its close homolog TRAM [initially called Adaptor X in our own laboratory (Hoebe et al. 2003) and also known as Ticam2 (Oshiumi et al. 2003b)] could easily be identified by BLAST searches. TRAM proved to be capable of substituting for TRIF in a fraction of macrophages (Hoebe et al. 2003), and to be primarily responsible for carrying the TLR4 signal initiated by certain ligands, notably glycoprotein G of vesicular stomatitis virus (Georgel et al. 2007).

The *Trif* (Yamamoto et al. 2003a), *Tram* (Yamamoto et al. 2003b), *MyD88* (Adachi et al. 1998), and *Tirap* (Yamamoto et al. 2002) genes have each been inactivated by targeting. Three of the four loci (*Trif*, *MyD88*, and *Tirap*) have been encountered through mutagenesis screening, and the ENU alleles have produced certain surprises, in that some are phenotypically distinguishable from the knockouts. While the *Trif* allele *Lps2* is essentially null, two hypomorphic and receptor-selective alleles of *Myd88* were produced by ENU mutagenesis (*pococurante* and *lackadaisical*; discussed in "The Nature of Receptor: Adaptor Interaction"). An allele of *Tirap* (*torpid*) was identified in screening and found to be rather different from the knockout in its effects: less drastic phenotypes than in the knockout are reported (see http://mutagenetix.scripps.edu for details).

More Distal Elements of the TLR Signaling Pathway

A mutation in *Irak4* (*otiose*) was identified through mutagenesis screening and, to all appearances so far, produces a phenotype identical to that caused by *Myd88* mutations (see http://mutagenetix.scripps.edu for details). A mutation in *Tnf* (used as the endpoint of screening) known as *PanR1* ("pan-resistance") was detected as a dominant phenovariant in G1 mice (Rutschmann et al. 2006). The *PanR1* allele does not affect the development of lymphoid organs as severely as knockout mutations of the *Tnf* locus do. On the one hand, minimal residual activity of the TNF protein may account for this. On the other hand, the knockout allele may affect

neighboring genes such as the lymphotoxin-encoding locus, and may exert an effect on lymphoid development indirectly.

Requirements for TLR2 and TLR4 Complex Signaling

Further screening disclosed *oblivious*, a mutation that prevented signaling by some, but not all, TLR2/6 ligands (Hoebe et al. 2005). In particular, MALP-2 (a diacyl lipopeptide), lipoteichoic acid (a component of gram-positive bacteria that may well be contaminated with diacyl lipopeptides when obtained from commercial sources), and gram-positive peptidoglycan (another component of bacteria that generally is contaminated with lipopeptides) stimulated less than the normal amount of TNF production when added to *oblivious* macrophage cultures. Zymosan, the tri-acyl lipopeptide PAM_3CSK_4 (which signals entirely via TLR2/TLR1 complexes) and the diacyl lipopeptide PAM_2CSK_4 (which signals partly via TLR2/1 complexes) stimulated normal TNF production. *Oblivious* mice were also hypersusceptible to infection with *Staphylococcus aureus*, and developed spontaneous infections of the surface of the eye, which became colonized with *Staphylococcus lentus* (Hoebe et al. 2005). The mutation proved to be a nonsense allele of *Cd36*, which encodes a class B scavenger receptor. CD36 thus acts as part of the TLR2/TLR6 receptor complex, although its physical relationship to the other components of the complex remains unclear. *Oblivious* mice also show a defect of CD4 priming (Janssen et al. 2006), although the mechanism of this effect is unknown.

Heedless, a phenotype traced to a mutation of the CD14 protein (Jiang et al. 2005; Huber et al. 2006), had a dramatic effect on TLR4 signaling and a striking though partial influence on signaling from the TLR2/6 complex as well, although it did not affect TLR2/1 complex signaling. Remarkably, *heedless* influenced sensing of rough and smooth LPS variants differently. Rough LPS (minimally glycosylated) could activate MyD88-dependent signaling in *heedless* macrophages. Smooth LPS (luxuriantly glycosylated) could not. Neither LPS chemotype could activate MyD88-independent signaling in *heedless* macrophages.

The differential effects of both *Cd36* and *Cd14* mutations on the detection of specific ligands suggests that the encoded proteins may coordinate the tertiary structure of the TLR subunits, permitting them to respond to some ligands and not others, and influencing signaling in a qualitative manner as well. Possibly TLR2 and TLR4 can adopt multiple active conformations. Further to this hypothesis, the *pococurante* allele of MyD88 restricts signaling from some (but not all) TLR2 ligands (as discussed below in Sect. 6.5).

Insouciant (Jiang et al. 2006) and *languid* (http://mutagenetix.scripps.edu) were mutations of TLR6 and TLR2, respectively. Each produced a phenotype indistinguishable from that of the knockout mutations, produced in the Akira laboratory. To date, no functionally abnormal alleles of TLR1 or TLR4 have been identified in ENU mutagenesis screens.

The Nucleic Acid Sensing TLRs

TLRs 3, 7, 8, and 9, and very likely TLR13 (in the mouse), exist predominantly within the endosomes and endoplasmic reticulum rather than on the cell surface. TLRs 3, 7, 8, and 9 signal from acidified compartments (late endosomes or early lysosomes). A mutation termed *3d* ("triple defect") was found to suppress all signaling from these TLRs (Tabeta et al. 2006). *3d* mutant mice also failed to cross-present antigen, and had marked impairment (though not complete ablation) of class II restricted antigen presentation as well (Tabeta et al. 2006). Individual mutations affecting the TLR signaling apparatus (in TLR3, TLR9, TRIF, or MyD88) cause enhanced susceptibility to mouse cytomegalovirus (MCMV) (Tabeta et al. 2004). Almost certainly because of their inability to signal via TLRs 3 and 9, *3d* mutants were highly susceptible to MCMV infection. In addition, augmented susceptibility to *Listeria monocytogenes* and *Staphylococcus aureus* infection was observed (Tabeta et al. 2006).

The *3d* mutation was positionally cloned, and found to specify a missense error in *Unc93b1*, a gene encoding a 12-spanning protein restricted to the endoplasmic reticulum (ER) (Tabeta et al. 2006). Subsequent work revealed that UNC-93B is probably required to escort TLRs 3, 7, 9, and 13 to the endosomal compartment (Brinkmann et al. 2007). Hence, signaling does not occur in *3d* mutant mice because the TLRs do not encounter exogenously presented nucleic acids. In humans mutations of the orthologous gene were found to cause enhanced susceptibility to herpes simplex encephalitis, as well as the characteristic defect of nucleic acid sensing (Casrouge et al. 2006). The phenotype of humans with UNC-93B mutations is very similar to that of mice.

UNC-93B is one of three proteins of a small family that also includes UNC-93A and UNC-93C. The function of these remaining paralogs is unknown, but is being examined by gene targeting. In *Caenorhabditis elegans*, the UNC-93 protein fulfills a neurological function, as mutants are "uncoordinated." Based on suppression screens, it is possible (though uncertain) that the protein is a regulatory subunit of a two-pore potassium channel (de la Cruz et al. 2003). In mammals, the 12-spanning structure and the phenotype of the *3d* mutant, in which exogenous antigens are taken up by phagocytosis but not permitted access to the cell surface in conjunction with class I MHC proteins, suggests that a channel function may also exist. However, speculation holds that UNC-93B may comprise a channel for polypeptides rather than for ions.

The Nature of Receptor: Adaptor Interaction

There has been much speculation concerning the detailed interaction between TIR adaptor proteins and their receptors in vivo. Most commonly offered is a model of "bridging" in which the adaptors MAL and TRAM directly interact with TLR4 and

permit MyD88 and TRIF to dock with them in turn. Some workers, however, have reported a direct interaction between MyD88 and TLR4, and it is not at all clear precisely how the four adaptors contact one another and the receptors themselves. This is despite the fact that the TIR domain structures of TLR1 and TLR2 have been solved (Xu et al. 2000; Jin et al. 2007), and other TIR domains may be modeled at low resolution based on these structures. All TIR domains consist of six α-helices (αA, αB, αC, αC′, αD, and αE) and five β-strands (βA, βB, βC, βD, and βE) that are connected by seven loops (named for the α-helix and β-strand they connect; e.g., AA connects βA with αA). The crystal structures of the TLR1 and TLR2 TIR domains reveal that they fold into a structure with a central five-stranded parallel β-sheet surrounded by five helices.

Molecular modeling studies, interpreted in the light of the *pococurante* ("*Poc*") phenotype caused by a point mutation within the TIR domain of MyD88, suggest that docking between MyD88 and TLR4 may indeed occur. The Poc site is located near to the site of the classical "Lps" mutation (corresponding to P712H in the TLR4 sequence), which resides within the BB loop. Poc and Lps mutations have identical effects, wherever they are engrafted. When introduced into MyD88 itself, the mutations prevent signaling from all of the MyD88-dependent TIR domain receptors except the TLR2/TLR6 complex. Similarly, when engrafted into TLR2, either mutation will permit residual signaling from the TLR2/TLR6 complex. But if both mutations are engrafted either into TLR2 or into MyD88, all signaling is abolished. It is clear that the Poc and Lps mutations prevent receptor:adaptor interaction because they prevent physical recruitment of MyD88 to those receptors for which signaling function is destroyed. On the other hand, mutations in the αE helices of receptors or adaptors (antipodal to the Poc and Lps sites) have different effects. In the case of the receptor, recruitment is abolished. In the case of MyD88, recruitment is observed, but signaling is abolished. This has been interpreted to mean that the αE helix is involved in receptor and adaptor oligomerization, while the Poc and Lps sites are involved in receptor:adaptor interaction (Jiang et al. 2006).

The *lackadaisical* phenotype is also caused by a receptor-selective MyD88 mutation, this time one that prevents signaling via TLRs 7 and 9 in part, but allows signaling via all other TLRs that require MyD88. The mutation, in this case, lies between the death domains of MyD88 and the TIR domain, a region for which there is no structural information. We can surmise that this part of the molecule is somehow important in choosing receptors, and may have direct contact with the receptors (Jiang et al. 2006).

How Far from Saturation, and How Far to Go?

A reasonably cohesive picture of the biochemical pathways required for TLR signaling has begun to emerge (Fig. 2). Not only ENU-induced mutations, but gene targeting as well, has helped us to assemble this picture. For example, gene targeting experiments have demonstrated that IRF5 (Takaoka et al. 2005) and IRF7 (Kawai et al. 2004; Honda et al. 2004) have specific roles to play in MyD88-dependent

signaling; RIP-1 connects TLR3 signaling to the activation of NF-κB (Meylan et al. 2004); and TRAF3 signals in conjunction with TBK1 to allow MyD88-independent activation of IFN-β (Hacker et al. 2006). But surely the picture is incomplete, and some phenotypes induced with ENU have yet to be solved. *Feckless*, a mutation that prevents poly I:C-mediated activation of NF-κB but permits activation of IFN-β synthesis, has proved unusually stubborn (Z. Jiang, M. Berger, B. Beutler, unpublished data). Beyond this, there is the larger question of how far one should go with random germline mutagenesis. Ultimately, any screen is going to be exhausted, and there is less and less to be "mined" from the phenotype under analysis.

It is estimated, as a rule of thumb, that about 10% saturation results from the analysis of 10,000 G3 mice, produced in such a manner as to capture G1 mutations with 50% efficiency. Hence, about 30% of the targets that can cause phenotypical variations may now have been captured in the TLR signaling system. This assumes, however, that all aspects of the TLR signaling phenomenon have been maintained under surveillance, and this is almost certainly not true. Not all cytokine effectors have been monitored, nor all surface molecules induced. Only the core of the pathway has been studied with real attention. Therefore, there may be many molecules yet to find.

It is also true that some "secondary" phenotypes have much to tell and are not covered by the TLR signaling screen. For example, while we know that TLR signaling is important for the containment of MCMV, we do not know what role it may play in other physiologically important processes. It is here that the choice of screen (and the far-sightedness of the investigator) may determine the importance of what is ultimately discovered. Do TLRs play a role in inflammatory disease? Very likely so, and indeed, MyD88 mutations are known to suppress lupus-like disease in some mouse models (Christensen et al. 2006). But surely there is far more to be learned. It is also in combining mutations that much can be learned. In the absence of both MyD88 and TRIF, no TIR domain signaling can occur, and mice are severely immunocompromised. Yet to the surprise of many, they retain excellent adaptive immune competence (Gavin et al. 2006; Nemazee et al. 2006).

Concluding Thoughts on Immune Sensing and the Forward Genetic Approach

Forward genetics is a powerful approach in all aspects of biology, but it is particularly powerful in immunology, where a strong conceptual framework exists for the interpretation of new phenotypes. Yes, we knew that there was an LPS receptor, but it was seemingly impossible to isolate without genetics. Yes, we knew that there were endosomal and surface TLRs. But how did they reach their destinations? Many of the surprises that arise from forward genetics are easy to interpret, though this never diminishes their impact. Quite to the contrary, it enhances it.

Where the sensing pathways of the innate immune system are concerned, their beauty lies partly in their simplicity. Simple considerations suggested that there must be a limited number of germline-encoded receptors for innate immune recognition.

But there were other possibilities. Could alternative splicing be a means of generating diversity? Are there 100 receptors rather than only 10? Yet here is the situation as we see it: our most powerful inflammatory responses originate from an exceedingly small collection of molecules.

Not discussed in this chapter is that the retinoic-acid-inducible gene I (RIG-I)-like helicases and the NOD (nucleotide-binding oligomerization domain)/NALP family of proteins also serve sensing functions (Beutler et al. 2007b; Tschopp et al. 2003). The sequential dissection of all of these pathways is now a realistic prospect, as is the search for nonredundant pathways in adaptive immune activation.

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Genetic Analysis of Resistance to Infections in Mice: A/J meets C57BL/6J

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Contents

Abstract Susceptibility to infectious diseases has long been known to have a genetic component in human populations. This genetic effect is often complex and difficult to study as it is further modified by environmental factors including the disease-causing pathogen itself. The laboratory mouse has proved a useful alternative to implement a genetic approach to study host defenses against infections. Our laboratory has used genetic analysis and positional cloning to characterize single and multi-gene effects regulating inter-strain differences in the susceptibility of A/J and C57BL/6J mice to infection with several bacterial and parasitic pathogens. This has led to the identification of several proteins including Nramp1 (Slc11a1), Birc1e, Icsbp, C5a, and others that play critical roles in the antimicrobial defenses of macrophages against intracellular pathogens. The use of AcB/BcA recombinant congenic strains has further facilitated the characterization of single gene effects in complex traits such as susceptibility to malaria. The genetic identification of erythrocyte pyruvate kinase (Pklr) and myeloid pantetheinase enzymes (Vnn1/3) as

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key regulators of blood-stage parasitemia has suggested that cellular redox potential may be a key biochemical determinant of *Plasmodium* parasite replication. Expanding these types of studies to additional inbred strains and to emerging stocks of mutagenized mice will undoubtedly continue to unravel the molecular basis of host defense against infections.

Introduction

Infectious diseases continue to be a major cause of morbidity and mortality worldwide. In developing countries, infectious diseases are responsible for about half the burden of premature death and disability. Although antimicrobial drugs and vaccination programs have made headway in combating some pathogens, the absence of effective vaccines and the widespread emergence of microbial drug resistance continue to hamper the prevention and treatment of major foes such as malaria, tuberculosis, and human immunodeficiency virus (HIV). Additional socio-economic factors such as climate change, deforestation, floods, armed conflicts, population migration, and increased travel have contributed to the emergence and dissemination of novel viral and bacterial pathogens that constitute novel global health threats (reviewed in Khasnis and Nettleman 2005). The rapid microbial adaptation to antibiotics, together with the reduced interest of the pharmaceutical industry in the field of antimicrobial drug discovery (Norrby et al. 2005), are sure to compound the problem of infectious diseases in the years to come. On the other hand, most individuals coming in contact with an infectious agent do not develop disease, suggesting that the natural defenses of the body are generally well equipped to resist assault by microbial pathogens. A better understanding of such host defense mechanisms, therefore, may provide insight in understanding not only pathogenesis, but it may also suggest some novel host-based pathways and targets for pharmacological prevention and the treatment of the corresponding disease. Such defense mechanisms may manifest themselves as genetic variations in innate susceptibility to infections in humans and in animal models of disease.

In humans, an apparent heritability of infectious disease susceptibility has long been recognized. More recent population and epidemiological studies relating to geographic distributions, effect of race, first contact epidemics, and studies in twins have confirmed a genetic component to infection susceptibility in humans (Clementi and Di Gianantonio 2006; Cooke and Hill 2001; Frodsham and Hill 2004; Hill 2001, 2006). In the case of malaria, there is evidence of co-evolution of the pathogen and host genomes, with otherwise deleterious allelic variants in human erythrocyte proteins being retained in the population as they confer a protective advantage against the *Plasmodium* parasite (Min-Oo and Gros 2005). Except for some rare exceptions (Casanova and Abel 2007), the genetic component to susceptibility to infections is believed to be complex and multigenic, most probably because of the expected plurality in cell types and biochemical pathways ultimately involved in host response to a pathogen. In addition, the host:pathogen interaction

is dynamic in nature with adaptive mechanisms at play on both sides of this interaction. Thus, the study of this genetic component in humans is complicated by many environmental factors, none of which is more important than pathogen-associated virulence determinants (strain, dose, co-infection, etc.). Such a complex gene–environment interaction results in apparent genetic heterogeneity, incomplete penetrance, and variable expressivity, all reducing the power of standard genetic association or linkage studies. Furthermore, ascertainment of disease status is sometimes difficult in field studies further complicating these types of studies. Nevertheless, numerous association studies with candidate genes identified in animal models have been published (Marquet and Schurr 2001). Obvious limitations of such studies are that many genes are left out of the analysis, and that only a single gene is analyzed at one time. More recently, genetic studies by whole genome scans have also been published for major diseases such as tuberculosis, leprosy, and malaria (Casanova and Abel 2007). Although major gene effects have been identified in a few studies (Baghdadi et al. 2006; Picard et al. 2006), the norm has been that the genetic control is complex and multigenic with an often modest contribution of individual loci.

The laboratory mouse has been extremely useful for genetic analysis in infectious diseases. First, there exist several excellent experimental mouse models of infection that mimic different aspects of the corresponding human infection. Second, pathogen-associated parameters such as strains, virulence, dose, and route of infection can be controlled. Third, there are many inbred, recombinant and naturally occurring or experimentally induced mutant mouse strains that are commercially available, which can be used to identify major gene effects influencing onset, progression, host response, and ultimate outcome of infection with a specific pathogen. Fourth, genetic analysis can be conducted in fairly easy-to-produce informative crosses that can be analyzed for the presence of major gene effects or quantitative trait loci (QTLs), using readily available single-nucleotide polymorphisms (SNP) or di-nucleotide markers. Additional mapping stocks such as recombinant inbred and recombinant congenic stocks can be used in large multistrain intercrosses to break down multigenic effects into monogenic traits. Fifth, the mouse genome has been sequenced and annotated, and tissue expression data are available for most of the genes, facilitating the prioritization of positional candidates contained within a genetic interval of interest. Importantly, germ-line modification in transgenic mice can be used to generate gain- or loss-of-function mutants, and thereby validate the role of individual genes in host defenses against infection with a specific pathogen (Yap and Sher 2002). Finally, genes, proteins, and biochemical pathways identified in mouse studies provide candidate genes for validation in parallel genetic studies in human populations from areas of endemic disease (De Jong et al. 1998; Dupuis et al. 2003; Greenwood et al. 2000; Jouanguy et al. 1999).

Our laboratory has worked for the past 20 years on the mapping and characterization of host genes that affect susceptibility to various infectious diseases. Our approach has been to sample the genetic diversity of a small number inbred mouse strains to identify and clone major gene effects. In a few instances we have also

succeeded in using AcB/BcA recombinant congenic mouse strains to identify single gene effects in complex traits. Our findings will be herein reviewed.

Simple Traits

Our definition of a simple trait corresponds to an infection-associated phenotype(s) that shows clear variation among inbred strains, with no overlap in quantitative evaluation of the phenotype or phenotypes between individuals of different such strains. Furthermore, when allowed to segregate in informative backcross or F_2 progeny, these traits show a classical Mendelian segregation pattern indicative of a single gene effect that can be identified by positional cloning. We have generally achieved further validation of positional candidates through direct sequence analysis, transfection studies in vitro, or through the creation of transgenic animals in vivo.

Mycobacterium *Species*

Nramp1

Susceptibility to infection by *Mycobacterium bovis* (bacillus Calmette-Guerin, BCG), as measured by early in vivo replication in the liver and spleen, is under simple genetic control in inbred strains, with resistance dominant over susceptibility. The locus responsible was detected some 30 years ago, and was mapped to the proximal part of chromosome 1 using recombinant inbred strains and classical crosses, and given the designation *Bcg*. It was immediately obvious that *Bcg* was either identical or tightly linked to two other previously mapped loci, *Ity* and *Lsh*, that independently control susceptibility to infection with *Salmonella typhimurium* and *Leishmania donovani* (Skamene et al. 1998). Experiments ex vivo demonstrated that macrophages are responsible for the phenotypic expression of infection resistance/susceptibility in mice (Gros et al. 1983). The *Bcg/Ity/Lsh* locus was identified by a laborious early positional cloning approach based on high-resolution linkage mapping, physical mapping by pulse-field gel electrophoresis, and the creation of a transcript map of the region using CpG clustering and exon trapping (Marquis et al. 2007; Poon and Schurr 2004). A positional candidate, designated as *Nramp1* (natural resistance associated macrophage protein, currently annotated as *Slc11a1*), was identified based on its exclusive expression in macrophages and macrophage-rich tissues. Sequence analysis showed that *Nramp1* encoded an integral membrane protein of 60 kDa composed of 12 transmembrane (TM) domains, and further revealed that susceptible inbred strains carried a single nonconservative Gly-to-Asp substitution at position 169 in (predicted) TM4 of the protein. This mutation was subsequently shown to impair protein folding, processing, and targeting, resulting in the absence of

mature protein being expressed in the membrane compartment of susceptible macrophages (Vidal et al. 1993, 1996). Validation of the causal relationship between *Nramp1* and *Bcg/Ity/Lsh* came in the form of the construction and characterization of a null allele of *Nramp1* by gene targeting, which abrogated resistance to infection with all three infectious agents. Conversely, introduction of a wildtype, resistance-associated $Nramp1^{G169}$ allele on the otherwise susceptible background of $Nramp1^{D169}$ restored resistance to infection both in transfected RAW macrophages and in transgenic mice (reviewed in Lam-Yuk-Tseung and Gros 2003; Poon and Schurr 2004).

Nramp1 protein is not present at the plasma membrane but is exclusively expressed in the Lamp1-positive lysosomal compartment of macrophages and the gelatinase-positive granules of neutrophils (Canonne-Hergaux et al. 2002; Gruenheid et al. 1997). Upon phagocytosis of inert particles or live microorganisms including *Salmonella*, *Leishmania*, *Mycobacterium*, and *Yersinia* (Cuellar-Mata et al. 2002; Govoni et al. 1999; Searle et al. 1998), Nramp1 is quickly recruited to the membrane of the maturing phagosomes (Gruenheid et al. 1997). Subsequent studies by microfluorescence imaging using solid particles coupled to a metal sensitive fluorophore showed that Nramp1 in macrophages functions as a manganese transporter at the phagosomal membrane (Jabado et al. 2000). Nramp1 was shown to function as an efflux pump, moving Mn^{2+} ions down a concanamycin-sensitive proton gradient created by the vacuolar H^+/ATPase (Jabado et al. 2000). Subsequent studies in transfected cells expressing a mutant Nramp1 variant at the plasma membrane showed that Nramp1 can act both on Mn^{2+} and Fe^{2+}, although it appears to show a preference for the former (Forbes and Gros 2003). Finally, Nramp1 is part of a large family of metal transporters that has been highly conserved in evolution from bacteria to humans (Cellier et al. 1995). Of note is the Nramp2/Slc11a2 protein, which functions as the general Fe^{2+} acquisition system in humans for both nutritional iron at the duodenal brush border and more generally for transferrin iron at the membrane of recycling endosomes (Canonne-Hergaux et al. 1999). Mutations in human and mouse Nramp2/Slc11a2 cause a severe form of microcytic anemia (Lam-Yuk-Tseung et al. 2006).

Mycobacteria survive within macrophages by inhibiting the maturation of phagosomes into fully bactericidal phagolysosomes, as demonstrated by reduced recruitment of lysosomal enzymes and vacuolar H^+/ATPase and reduced acidification (Clemens and Horwitz 1995; Clemens et al. 2000a, b; Russell et al. 1996; Schaible et al. 1998; Sturgill-Koszycki et al. 1994, 1996). Early experiments by Hackam and colleagues (1998) using macrophages from $Nramp1^{-/-}$ mice showed that recruitment of Nramp1 to the membrane of *M. bovis*-containing phagosomes caused a significant increase in acidification. Subsequent electron microscopy studies showed that recruitment of Nramp1 to the membrane of *M. avium*-containing phagosomes causes bacteriostasis, increased bacterial damage, increased acidification and increased fusion to lysosomes when compared to $Nramp1^{-/-}$ phagosomes (Frehel et al. 2002; Hackam et al. 1998). A simple explanation of these results is that inhibition of phagosome maturation by mycobacteria requires a metal-dependent, active process that can be antagonized by Nramp1-mediated metal efflux

from the phagosomal lumen. Globally, Nramp1-induced depletion of phagosomal iron could impair the ability of mycobacteria to modulate phagolysosomal fusion. Similar conclusions have been reached in the study of *Salmonella* and *Leishmania*-containing phagosomes (see the following section).

The human *NRAMP1* gene maps to chromosomal region 2q35, in close proximity to the interleukin 8 receptor gene (Cellier et al. 1994). The gene is composed of 15 exons including one that is alternatively spliced. Comparison of the human and mouse predicted NRAMP protein sequences revealed a remarkable degree of conservation between the two polypeptides, with 88% identical residues and 93% overall sequence similarity (Cellier et al. 1994). In humans, *NRAMP1* mRNA is expressed in spleen, lung, and at high levels in peripheral blood leukocytes, macrophages, and neutrophils (Canonne-Hergaux et al. 2002; Cellier et al. 1997). A possible role of *NRAMP1* in susceptibility to infectious diseases and to autoimmune disorders in humans has been intensely investigated (for complete reviews, please see Marquis et al. 2007; Poon and Schurr 2004). It suffices to note that *NRAMP1* has been consistently associated with pulmonary tuberculosis susceptibility in African and Asian populations but not in populations of European descent (Li et al. 2006), with direct genetic linkage data obtained during a tuberculosis outbreak in a Canadian aboriginal family (Greenwood et al. 2000) and in pediatric tuberculosis among children (Malik et al. 2005). Additional studies have shown that *NRAMP1* is involved in susceptibility to two other common mycobacterial diseases, leprosy (Abel et al. 1998; Alcais et al. 2000), and Buruli ulcer (Stienstra et al. 2006).

Together, these studies of Nramp1/Slc11a1 provide a clear example of how a gene discovered using genetic analysis in mice can subsequently be shown to contribute to a complex disease trait in humans.

Icsbp1

In inbred mouse strains, there is a strict correlation between allelic combination at *Nramp1* alleles and susceptibility to infection with *M. bovis* (BCG) (Malo et al. 1994), with the notable exception of the recombinant inbred strain BXH-2 (Skamene et al. 1982). BXH-2 is a recombinant inbred mouse strain derived from C3H/HeJ and C57BL/6J (Taylor 1978) that is known to develop a myelogenous leukemia by a two-step mutagenic process including an inherited mutation that causes a myeloproliferative syndrome, with a second retroviral-mediated insertion mutation resulting in clonal expansion of leukemic cells (Bedigian et al. 1981, 1984, 1993; Jenkins et al. 1982). Using splenomegaly as a phenotypic marker of myeloproliferation in F_2 crosses derived from BXH-2, we showed that this latter trait is determined by a single recessive locus in BXH-2 that we designated *Myls* (Turcotte et al. 2004). Positional cloning showed that the gene was located on the distal portion of chromosome 8 near marker *D8Mit13* [logarithm of differences (LOD)>44; map position 125 Mb (Turcotte et al. 2004)]. The *Myls* interval contains several positional candidates, including *Icsbp1* [interferon consensus sequence-binding protein 1, also known as interferon regulatory factor 8 (*IRF8*)]. Icsbp1 is a transcriptional regulator

that plays an important role in transcriptional activation of interferon γ-responsive genes that bear an interferon-stimulated response element (ISRE) sequence element in their regulatory regions. BXH-2 mice carry an R294C mutation within the predicted interferon regulatory factor (IRF)-association domain of the protein. The R294C allele is associated with a complete failure of BXH-2 splenocytes to produce interleukin-12 and interferon-γ in response to activating stimuli.

Despite a C3H-derived resistance *Nramp1*^{G169} allele, BXH-2 mice are susceptible to infection with *M. bovis* (BCG). Susceptibility appears somewhat variable, however, when tested at 3 weeks post-infection, with spleen colony-forming units (CFU) counts 5- and 100-fold (Skamene et al. 1982) superior to those seen in parental C3H controls, depending on the experiment. The effect of the R294C mutation in *Icsbp1* on susceptibility to *M. bovis* (BCG) was analyzed using a number of F_2 crosses between BXH-2 (*IRF-8*^{C294}, *Nramp1*^{G169}) and other inbred strains of known *Nramp1* genotype. These studies showed that the *Icsbp1*^{R294C} mutation increased susceptibility to *M. bovis* (BCG), and this effect was most visible in segregating F_2 mice fixed for homozygosity or heterozygosity for resistance *Nramp1*^{G169} alleles (Turcotte et al. 2005). Subsequent studies showed that BXH-2 mice cannot control *M. bovis* (BCG) replication during the late stages of infection, and display continuous growth in the spleen associated with complete absence of granuloma formation. Additional preliminary data also indicate that BXH-2 mice present a severe susceptibility phenotype to pulmonary tuberculosis following an intravenous challenge with highly virulent *M. tuberculosis* H37Rv (J.F. Marquis, R. LaCourse, L. Ryan, R.J. North, and P. Gros, unpublished data). In addition, the effect of the *Icsbp1*^{R294C} mutation appears pleiotropic as BXH-2 mice also show susceptibility to infection with the unrelated pathogens *Salmonella typhimurium* and *Plasmodium chabaudi* (Turcotte et al. 2007). In the case of *P. chabaudi*, although BXH-2 can clear the initial burst of parasitemia, they fail to mount a long-term protective immune response since the animals develop multiple waves of recurring parasitemia late in the infection. These findings together suggest that *Icsbp1* plays a critical role in both innate and acquired immune responses to intracellular pathogens.

Genetic *Nramp1* Modifier Detected in *Mus spretus* Wild Mice

The presence of genetic modifiers of *Nramp1*-dependent susceptibility to *M. bovis* (BCG) infection was investigated in the wild-derived mouse strain *Mus spretus*. *Mus spretus* is a wild-derived inbred strain that is phylogenetically distant from *Mus musculus* from which common laboratory inbred strains are derived. The evolutionary distance and associated genetic diversity separating wild-derived mice, such as *M. spretus* and *M. musculus*, are advantageous for the identification of novel gene effects in crosses derived from the two strains. Despite the presence of a fixed *Nramp1*^{G169} resistance allele, *M. spretus* (SPRET/EiJ) is quite susceptible to infection with a low dose of *M. bovis* (BCG). The presence of possible modifiers of the protective effect of *Nramp1*^{G169} alleles in

SPRET/EiJ was investigated by whole-genome scans using 159 informative markers distributed along 19 autosomes and the X chromosome of 175 (SPRET/EiJ×B6) F_1 × B6 backcross mice, using splenic *M. bovis* (BCG) bacterial load as a quantitative phenotype. As expected, *Nramp1* had a major effect (*D1Mcg4*) on splenic bacterial loads. Several additional weaker gene effects were noted, however, on chromosomes 4 (*D4Mit150*) and X (*DXMit249*) in male mice, and on chromosome 9 (*D9Mit77*) and 17 (*D17Mit81*) in female mice. The chromosome 17 QTL showed a strong interaction with *Nramp1* in female mice. It overlaps the major histocompatibility (MHC) locus, a region that contains many genes regulating early (innate) and late phase (acquired immunity) of host response to infection with mycobacteria including *M. bovis* (BCG) and *M. tuberculosis* (Lavebratt et al. 1999; Sanchez et al. 2003). The effect of chromosome 17 on host response to *M. tuberculosis* has recently been attributed to a functional polymorphism in the tumor necrosis factor (TNF)-α gene (Kahler et al. 2005), suggesting a possible modifying effect of this pleiotropic proinflammatory cytokine on *Nramp1*-mediated resistance.

QTLs detected as modifiers of Nramp1 action in mice may represent novel and valuable entry points for the parallel search for mycobacterial susceptibility loci in humans.

Salmonella typhimurium

Salmonella are facultative intracellular gram-negative bacteria of major global health importance. Almost all *Salmonella* serotypes belong to the same species designated *Salmonella enterica*. Over 2,500 serovars of *S. enterica* have been identified that are differentiated by their flagellar, carbohydrate, and lipopolysaccharide (LPS) structures (Fierer and Guiney 2001; Ochman and Groisman 1994). *S. enterica* species are typically orally acquired pathogens that cause one of four major syndromes: enteric fever (typhoid), enterocolitis/diarrhea, bacteremia, and chronic asymptomatic carriage (Fierer and Guiney 2001). In humans, serovars Typhi, Paratyphi, and Sendai cause enteric fever, while most serovars cause enterocolitis. While serovar Typhi is largely restricted to humans, serovar Typhimurium causes disease in both humans and other animals. *Salmonella typhimurium* infection in mice recapitulates the pathophysiology of the acute human infection with *Salmonella* Typhi or Paratyphi. Following infection by the oral or parenteral route, *S. typhimurium* localizes to the spleen and the liver where it replicates rapidly, causing death of the susceptible mice within a week of infection.

Tlr4 (Lps)

Bacterial LPS is a major constituent of the outer membrane of gram-negative bacteria and is essential for virulence of *Salmonella* in vivo (Rietschel et al. 1994).

In addition, bacterial LPS is a major mediator of pathogenesis in vivo, being a potent inducer of inflammatory responses in macrophages and mitogenic activity in B lymphocytes (Rosenberger et al. 2000; Royle et al. 2003). Inbred strains of mice vary dramatically in their degree of susceptibility and resistance to infection with *S. typhimurium* as determined by the extent of microbial replication in spleen and liver, and overall survival to the acute infection. In addition, a robust response to LPS is required in mice for survival to acute infection with *S. typhimurium*. Inbred mouse strains such as C3H/HeJ and C57BL/10ScCr do not respond to LPS in vitro and are susceptible to *S. typhimurium* infection (MacVittie et al. 1982; O'Brien et al. 1980, 1985; Vazquez-Torres et al. 2004; Weinstein et al. 1986). The acute susceptibility of C3H/HeJ mice was studied by linkage analysis and was found to be inherited in a recessive manner, which segregated as a monogenic trait (Watson and Riblet 1974; Watson et al. 1977). The locus was named *Lps*, and two alleles were defined: Lps^n and Lps^d for normal and defective response to LPS, respectively (Watson et al. 1978). High-resolution genetic, physical, and transcriptional maps of the area were used to identify the gene responsible for the *Lps* effect (Poltorak et al. 1998; Qureshi et al. 1996). These studies led to the identification of the gene encoding Toll-like receptor 4 (*Tlr4*) as the gene mutated at *Lps* (Poltorak et al. 1998; Qureshi et al. 1999). Tlr4 functions as a pattern recognition receptor that recognizes LPS of gram-negative bacteria such as *Salmonella*. Confirmation of the role of *Tlr4* in LPS hyporesponsiveness was obtained through examination of mice that had been rendered deficient for *Tlr4* (Hoshino et al. 1999). These studies were the first to show that the Tlr family plays a major role in innate defense mechanisms. In particular, they are critical for the recognition of microbial products based on a set of molecular determinants (leucine-rich repeats) unrelated to the immunoglobulin super-family.

Nramp1 (Ity)

Early studies in recombinant inbred strains together with direct progeny testing experiments strongly suggested that the *M. bovis* susceptibility locus *Bcg* was identical to two other host resistance loci, *Ity* and *Lsh*, independently described as affecting susceptibility to infection with *S. typhimurium* and *L. donovani*, respectively (Skamene et al. 1998). Subsequent gene targeting and transfection experiments formally demonstrated that *Nramp1*, *Bcg*, *Ity*, and *Lsh* were indeed the same locus controlling susceptibility to infection with unrelated intracellular pathogens (Govoni et al. 1996; Vidal et al. 1995).

The effect of Nramp1 on the biochemical composition and physiological properties of *Salmonella*-containing phagosomes formed in macrophages has been well studied and has proved useful in understanding the mechanistic basis of the protein's effect on intracellular pathogens. As with mycobacteria, *Salmonella* survive within macrophages by interfering with normal phagosome maturation (toward phagolysosome), and reside in specialized *Salmonella*-containing vacuoles (SCV), also known as "spacious" phagosomes (Knodler and Steele-Mortimer 2003). As opposed

to phagosomes containing mycobacteria, SCVs formed in both *Nramp1*$^{+/+}$ and *Nramp1*$^{-/-}$ macrophages acidify fully and recruit the lysosomal marker Lamp-1. In *Salmonella*-permissive *Nramp1*$^{-/-}$ macrophages, however, SCV exhibit reduced incorporation of the late endosomal marker mannose-6-phosphate receptor (M6PR), remain negative for endosomal markers (EEA1), and are inaccessible to endosomal vesicles loaded with fluid-phase tracers after invasion. By contrast, SCVs formed in nonpermissive *Nramp1*$^{+/+}$ macrophages recruit M6PR and EEA1 (Cuellar-Mata et al. 2002) and show increased microbicidal activity (Govoni et al. 1999). Additional studies in vitro in explanted macrophages have shown that the addition of membrane-permeant iron chelators can recapitulate the Nramp1 effect, and stimulate recruitment of M6PR and EEA1 to SCVs formed in otherwise *Nramp1*$^{-/-}$ cells (Jabado et al. 2003). These studies show that Nramp1-mediated metal depletion at the phagosomal membrane antagonizes the ability of *Salmonella* to become secluded from the degradative pathways of macrophages (Cuellar-Mata et al. 2002). *Salmonella* included in SCVs have been found to respond to the presence of Nramp1 at the membrane by transcriptional induction of a number of virulence genes, including *ssrA* and *sseJ*, that map within *Salmonella* pathogenicity island 2 (*SPI2*) (Zaharik et al. 2002). Thus, Nramp1-mediated metal depletion at the membrane of SCV is associated with major changes in biochemical and fusogenic properties of these vesicles, resulting in increased bacteriostatic activity of macrophages. Indeed, adequate supplies of iron had been known to be essential for *Salmonella* virulence in vivo, and for intracellular replication in macrophages in vitro (Kehres and Maguire 2003; Ratledge 2004). *Salmonella* possess several high- or low-affinity, ATP-dependent or proton-coupled (*tonB*-dependent) iron transporters such as *fepBCDG*, *sitA-D*, *FeoABC*, *CorAD*, and the *Nramp* homolog *MntH* (Hantke 1997; Kammler et al. 1993; Kehres et al. 2002; Tsolis et al. 1996; Zhou et al. 1999). Many of these transporters have been shown to be essential for *Salmonella* virulence in vivo (Bearden and Perry 1999; Boyer et al. 2002; Janakiraman and Slauch 2000; Tsolis et al. 1996). Single mutations at *feoB* or *sitA-D* reduced virulence, while double mutations at *MntH*, *sitA-D*, or *feoB* completely abrogated *Salmonella* virulence in *Nramp1*$^{-/-}$ mutant 129 Sv mice in vivo. Together, these studies indicate that iron plays a critical role at the interface of host:pathogen interaction. Macrophage metal transporters such as Nramp1 and ferroportin (Nairz et al. 2007) thus represent major defenses acting to restrict intracellular access to this essential nutrient.

Finally, this hypothesis is in good agreement with results obtained with *L. donovani* (Huynh et al. 2006). Huynh and colleagues (2006) recently identified *LIT1* as a major Fe^{2+} transporter of *Leishmania*. LIT1 protein is expressed at the plasma membrane and is present only in the amastigote intracellular form of the parasite but is absent from the extracellular promastigote form. LIT1 was further shown to be a critical virulence determinant as its inactivation resulted in (1) reduced viability and impaired replication of the parasite in bone marrow macrophages in vitro and (2) reduced virulence in vivo in permissive BALB/c mice (*Nramp1*^{D169}). By comparing the timing and extent of expression of LIT1 protein following phagocytosis of *L. amazonesis* amastigotes by normal and Nramp1-defective macrophages, the authors observed accelerated LIT1 protein expression under iron-poor conditions of Nramp1-positive phagolysosomes

(Huynh et al. 2006). Therefore, LIT1 is an intracellular iron acquisition system essential for survival of *Leishmania* parasites in macrophages. LIT1 is subject to iron-specific regulation sensitive to the presence of Nramp1, confirming the critical role of this protein in regulating intra-phagosomal iron pools (Marquis and Gros 2007).

Modifiers of *Nramp1* Effect in *Salmonella* Infection

Genetic modifiers of *Nramp1*-mediated resistance to *Salmonella* were studied using the wild-derived mouse strains *M. musculus molossinus* (MOLF/Ei). MOLF/Ei mice are extremely susceptible to infection with *S. typhimurium* despite the presence of resistance alleles at *Nramp1* and *Tlr4*, with survival times comparable to that of C57BL/6J (*Nramp1*^{s}) controls. Linkage analysis using 252 (C57BL/6J×MOLF/Ei) F_2 animals identified two QTLs that significantly affect survival time following lethal infection with *S. typhimurium*, one on chromosome 11 (*Ity2*; LOD=7.0; 10% of phenotypic the variance), and one on chromosome 1 (*Ity3*; LOD=4.8; 7% of phenotypic variance) (Sebastiani et al. 1998, 2000). Several candidate genes were detected in the *Ity2* region, including granulocyte/macrophage colony-stimulating factor (*Csfgm*), interleukin 3 (*Il3*), myeloperoxidase (*Mpo*), and inducible nitric oxide synthase (*Nos2*). MOLF/Ei mice showed a decreased capacity to induce *Nos2* mRNA and to produce NO (Sebastiani et al. 2002) following *Salmonella* infection. The observations that *Nos2*-null mutant mice are unable to suppress bacterial growth in the late phase of the infection and eventually die from infection (Mastroeni et al. 2000) strengthen the candidacy of *Nos2* as the gene responsible for the *Ity2* effect. The *Ity3* region contains a number of positional candidates (*C4bp*, *Cfh*, *Ptgs2*, and *Daf1*), including the *Tlr5* gene (Sebastiani et al. 2000). mRNA expression studies during infection with *S. typhimurium* show that *Tlr5* mRNA levels in liver are consistently lower in MOLF/Ei (~50% reduction) compared to other inbred mouse strains including C57BL/6J, 129S6/SvEvTac, C3H/HeJ, and C57BL/10J. Finally, sequence analysis defined a unique *Tlr5* haplotype in MOLF/Ei mice (distinct from 47 other strains tested) associated with a lower level of *Tlr5* mRNA expression (Sebastiani et al. 2000). The subsequent demonstration that Tlr5 acts as a cellular receptor for *S. typhimurium* flagellin suggests a mechanism by which Tlr5 could underlie the effect of *Ity3* in response to infection in vivo (Gewirtz et al. 2001; Hayashi et al. 2001).

In an effort to identify additional modifiers of the *Nramp1* effect, 36 strains from the AcB/BcA set of recombinant congenics derived from A/J (*Salmonella*-resistant; *Nramp1*^{G169}) and C57BL/6J (*Salmonella*-susceptible; *Nramp1*^{D169}) were infected with 10^3 *S. typhimurium* intravenously, and bacterial replication in organs (spleen and liver) and overall survival to infection were used as measures of susceptibility (Roy et al. 2006). Several strains showed a phenotype that was deviant from that expected from their *Nramp1* genotype. Scoring for survival time, AcB61 were found to be susceptible despite an *Nramp1*^{G169} resistance allele, while AcB64 (*Nramp1*^{G169}) were significantly more resistant than the corresponding A/J parental control. Infected were 247 (AcB61×129S6) and 249 (AcB64×DBA/2J) informative F_2 mice (where *Nramp1*^{G169} alleles were fixed), and a whole genome scan was conducted using survival time as a

measure of susceptibility. In the AcB64 cross, five novel *Salmonella* susceptibility QTLs mapping to chromosomes 3 (*Ity4*), 2 (*Ity5*), 14 (*Ity6*), 7 (*Ity7*), and 15 (*Ity8*) were detected. The genes underlying the effects of these QTLs remain unknown. In the AcB61 cross, a major QTL was detected (*Ity4*) on chromosome 3 that accounts for 42.1% of the phenotypic variance. The *Ity4* region contains an obvious candidate previously shown to affect susceptibility to malaria: the liver- and red blood cell-specific pyruvate kinase gene (*Pklr*), in which mutations cause a very severe anemia that protects mice against the lethal effects of *P. chabaudi* infection (Min-Oo et al. 2003).

Together, these studies demonstrate that susceptibility to acute infection with *S. typhimurium* is under complex genetic control in A/J and C57BL/6J strains, with a major role played by *Nramp1/Slc11a1*. They also provide an example of the usefulness of crosses with wild-derived mice and recombinant congenic lines in localizing some of these modifier loci.

Legionella pneumophila

Legionella pneumophila is a gram-negative bacterium that causes Legionnaire's disease, a severe form of pneumonia in humans. The intracellular survival of *L. pneumophila* in human macrophages depends on its ability to segregate into an endoplasmic reticulum (ER)-derived vacuole that does not mature, that does not acquire lysosomal markers, and that becomes studded with ribosomes. Macrophages from most inbred mouse strains are nonpermissive to intracellular replication of *L. pneumophila* ex vivo, with the exception of the A/J strain, which is uniquely permissive and which has been used as a model-system to better understand *L. pneumophila* pathogenesis in human cells. Genetic analyses in recombinant inbred mouse strains as well as in informative backcrosses derived from the A/J strain established that permissiveness to *L. pneumophila* replication in macrophages infected ex vivo was controlled by a single gene designated *Lgn1*, with nonpermissiveness completely dominant over permissiveness. High-resolution linkage mapping has located *Lgn1* to a 0.32-cM interval on mouse chromosome 13. This is a region of high genomic complexity that includes a large duplication that contains multiple intact and re-arranged copies of the *Birc1* (baculoviral inhibitor of apoptosis protein repeat-containing 1, formerly *Naip*) gene (reviewed by Fortier et al. 2005). The minimal physical 140-kb interval of *Lgn1* contains two such proteins, *Birc1b* (*Naip2*) and *Birc1e* (*Naip5*). Birc1 proteins were found expressed in macrophages and their levels of expression were shown to be upregulated following phagocytosis of infectious agents or inert particles. In addition, A/J macrophages (*Lgn1*^{s}) were found to express a lower level of Birc1 proteins compared to C57BL/6J macrophages (*Lgn1*^{r}). In vivo genetic complementation studies in A/J transgenic mice carrying genomic bacterial artificial chromosome (BAC) clones from the region were used to pin down the identity of *Lgn1*. Testing for complementation of the A/J-derived macrophage susceptibility phenotype indicated that the two BAC clones that rescued the phenotype both had the full-length *Birc1e* transcript in

common (Diez et al. 2003). These results indicate that *Birc1e* is likely allelic with *Lgn1*. The possibility that transgenic rescue is caused by a gain-of-function on a haploid-insufficient background (overexpression of *Birc1e* by multiple BAC copies) remains to be determined. Parallel studies by W. Dietrich's group showed that morpholino-based antisense inhibition of *Birc1e* can partly reverse the nonpermissiveness of macrophages from mice containing a transgenic copy of *Birc1e* (Wright et al. 2003).

The term *Naip* (neuronal apoptosis inhibitory protein) was initially coined to reflect the candidacy of *Naip* for the human spinal atrophy locus. The Naip protein also has three BIR domains (baculovirus IAP repeats) that are present in other IAP family members, and that have been shown to interact with cellular caspases, thus implicating Naip in regulating cellular apoptosis (Maier et al. 2002; Martinon and Tschopp 2007). A role for Naip protein in survival of neurons following mechanical damage has been reported (Perrelet et al. 2000). On the other hand, Naip/Birc proteins have been recently re-classified as NBS-LRR proteins (now NLR) on the basis of their nucleotide-binding domain, and the presence of long leucine-rich repeats that are known to act as pattern recognition motifs in Toll-like receptor proteins. In fact, the NLR protein family includes nucleotide-binding oligomerization domains (NODs), Ipaf, and others, and has been shown to act as intracellular sensors for the presence of bacterial products (Fritz et al. 2006).

Recent studies have shed light on the ligand and signaling mechanism of Birc1e/Naip5 in macrophages (Molofsky et al. 2006; Ren et al. 2006; Zamboni et al. 2006). Zamboni and colleagues showed that infection with *L. pneumophila* induces caspase-1-dependent cell death in cells expressing the B6 (nonpermissive) but not the A/J (permissive) copy of *Birc1e/Naip5*. Studies of different *L. pneumophila* mutants indicated that activation of caspase-1 requires type IV secretion system-mediated transfer of products into the cytosol, but not intracellular replication or residency in the ER. Finally, macrophages from either caspase-1-deficient (*Casp1*$^{-/-}$) or Ipaf-deficient (*Card12*$^{-/-}$) mice were found to be more permissive to *L. pneumophila* replication than wildtype B6 mice, and co-immunoprecipitation studies demonstrated a direct interaction between Birc1e/Naip5 and Ipaf. A model was proposed in which recognition of *L. pneumophila* products by the LRR domain of Birc1e/Naip5 would cause caspase-1/Ipaf-mediated activation of the inflammasome, resulting in cell death and restriction of *L. pneumophila* replication in macrophages in nonpermissive strains. Recent genetic screens for *L. pneumophila* mutants capable of growing in nonpermissive B6 macrophages identified several mutations in the structural gene for flagellin (*flaA*) (Molofsky et al. 2006; Ren et al. 2006). The authors showed that flagellin is required to induce cell death in B6 macrophages, and A/J macrophages are resistant to flagellin-induced death. Therefore, the role of Birc1e/Naip5 in *Legionella* susceptibility may involve (1) regulation of Ipaf-dependent caspase-1 activation or (2) caspase-1 activation following a direct recognition of flagellin or another bacterial compound by Birc1e/Naip5, or (3) a caspase-1-independent mechanism. On the other hand, recent studies from our group using macrophages from A/J mice and from A/J transgenic mice harboring a functional *Birc1e* copy have suggested additional complexity and diversity in

Birc1e protein function. Indeed, we found that *Legionella* phagosomes formed in the two types of macrophages differ markedly, and that presence of Birc1e is associated with reduced acquisition of endoplasmic reticulum markers (calnexin) and enhanced acquisition of lysosomal markers (cathepsin D, Lamp1) (Fortier et al. 2007). The Birc1e effect on phagosome maturation was very rapid, occurring within the first hour of infection, suggesting that Birc1e-mediated activation of caspase-1 may affect early protein targets distinct from late targets associated with cell death or processing of IL-1. The nature of such targets is of great interest but still needs to be discovered.

Candida albicans

In humans, *Candida albicans* exists as a commensal in the gastrointestinal and genitourinary tracts but can also cause opportunistic infections in the immunocompromised host. It is a common cause of fungal infection in humans (Verduyn Lunel et al. 1999). Superficial candidiasis include thrush, chronic atrophic stomatitis, chronic mucocutaneous candidiasis, and vulvovaginitis (Eggimann et al. 2003). These infections tend to be self-limited in immunocompetent hosts (Eggimann et al. 2003). Invasive candidiasis refers to *Candida* infections that occur at sites other than the skin or mucous membranes, with most cases caused by bloodstream dissemination; this condition is almost exclusively limited to immunocompromised hosts (Kullberg and Filler 2002). The incidence of nosocomial infection with *Candida* has been on the rise, a problem compounded by the appearance of antifungal drug resistance (Verduyn Lunel et al. 1999). In disseminated severe forms of *Candida* infection, the major target organs are the digestive tract, lungs, kidney, heart, and brain (Odds 1988).

The pathogenesis of systemic and acute infection with *C. albicans* has been extensively studied in animal models (rats, rabbits, guinea pigs), with the mouse being the most widely used. Systemic infections in these animals resemble human candidiasis, with the kidney being the major target of infection (Ashman et al. 1996). Although *C. albicans* can cause systemic infection when introduced by the intra-peritoneal or gastrointestinal route, the intravenous route has been favored to induce acute infection (De Repentigny 2004) with major colonization of heart, kidney, liver, and brain. Additional models of cutaneous *C. albicans* infection (vaginitis) have also been used (Fidel and Sobel 1999). The median lethal dose (LD_{50}) for most *C. albicans* isolates introduced intravenously in immunocompetent mice is between 10^4 and 10^6 blastospores, depending on the strain of *C. albicans*, the growth conditions used to prepare the inoculum, and the genetic make-up of the murine host (Odds 1988; Odds et al. 2000). Besides time of survival, fungal burden and tissue damage in infected organs have been used as phenotypic markers of susceptibility in mouse (Ashman 1998). A correlation between kidney fungal load and mortality has been reported in some mouse strains, but not in others (Marquis et al. 1988; Mencacci et al. 1998; Salvin and Neta 1983). In other mouse strains, however,

deleterious host response in the face of low to moderate fungal load has been associated with early death from acute *C. albicans* infection (Tuite et al. 2004).

A genetic approach in mice has been used to identify major determinants of susceptibility to acute infection with *C. albicans*. A reverse genetic approach, in which the effect of individual genes are assessed in gene knockout studies, and a forward genetic approach investigating natural differences in susceptibility to *C. albicans* infection in vivo have been undertaken (for a complete review, see Tuite et al. 2004). Early strain surveys showed that inbred strains such as C57BL/6J, BALB/cJ, CBA/J, and DBA/1 are resistant while A/J, DBA/2J, NZB/J, and AKR/J are susceptible. These studies further identified a partial correlation between the complement competence status (C5a) and susceptibility to infection (Wetsel et al. 1990). Recent studies using intravenous infection with *C. albicans* showed that A/J is highly susceptible while C57BL/6J is highly resistant to infection. Susceptibility was associated with increased fungal loads in kidney and heart, and very early death of A/J mice compared to B6. Histopathological analysis revealed that A/J did not mount a proper inflammatory response following infection and died within 48 h. By contrast, B6 mice developed much higher fungal loads over a 3-week period and ultimately died of renal failure (which was not seen in moribund A/J). Linkage analysis in 128 (A/J×B6) F_2 progeny using fungal load in kidney or heart and overall levels of TNF-α produced at 24 h as a readout showed that susceptibility behaved as an autosomal recessive monogenic trait which was mapped by whole genome scanning to the proximal part of chromosome 2, with the highest LOD score (LOD = 22.7) for a marker tightly linked to the structural gene for the C5 component of complement (Tuite et al. 2005). Up to 40% of the commonly used inbred mouse strains (Cinader et al. 1964) have a 2-bp deletion in an exon near the 5′-end of the mRNA, introducing a premature stop codon 4-bp downstream of the deletion (Wetsel et al. 1990). This leads to the production of a truncated 216-amino acid translation product compared to the wildtype 1,680-amino acid protein. This truncated protein is not secreted (Wetsel et al. 1990). Thus, C5 deficiency in mice is associated with severe susceptibility to acute *C. albicans* infection.

The functional consequences of a C5 deficiency on response to systemic candidiasis have been studied (Ashman et al. 2003; Lyon et al. 1986; Morelli and Rosenberg 1971). In our laboratory, we have used the recombinant congenic strain BcA70, which has the C5 mutant allele of A/J fixed on the genetic background of resistant C57BL/6J (B6) (Fortin et al. 2001b). BcA70 is as susceptible to infection as A/J. C5 is proteolytically processed to C5a, -b, and -c, which react with opsonized microbes to form a membrane attack complex (MAC) that creates pores in the membrane of invading microbes following binding of antibodies. In addition, C5a acts as a major chemoattractant to recruit neutrophils and macrophages at the site of infection, a response that is impaired in C5-deficient mice (Gerard and Gerard 1994; Mullick et al. 2006). Analysis of the profile of cytokines produced during infection of A/J and BcA70 shows a pattern of extreme inflammatory and allergic response, suggesting unregulated production of proinflammatory molecules including TNF-α, IL-6, monocyte chemoattractant protein-1 (MCP1), macrophage inflammatory protein (MIP2), tissue inhibitor of metalloproteinase (TIMP1), and keratinocyte-derived

cytokine (KC) (Mullick et al. 2006). This results in cardiomyopathy (elevated creatine kinase and cardiac troponin I), hypoglycemia, and rapid death.

Finally, a systematic screening of AcB/BcA recombinant congenic lines for modifiers of the C5-deficiency effect on susceptibility to *C. albicans* infection identified two strains with discordant phenotypes (kidney fungal loads): BcA67 shows an intermediate level of susceptibility despite presence of wildtype C5-sufficient alleles, while BcA72 females are as resistant as B6 controls despite being genetically C5-deficient. These observations suggest additional complexity in the genetic control of susceptibility to *C. albicans* in A/J and B6 mice.

Complex Traits

Mycobacterium tuberculosis

Tuberculosis is caused by aerosol infection with the bacterial pathogen *Mycobacterium tuberculosis*. Although *M. tuberculosis* can infect and replicate in several organs, tuberculosis is almost exclusively a pulmonary disease. Tuberculosis still remains a global health problem of enormous proportions, with 32% of the world's population believed to be or certainly infected (WHO 2000), an estimated 8 million new cases of active disease per year (WHO 2002) and 1–1.5 million deaths annually. Globally, migration of populations from countries with endemic disease, HIV infection, poverty, unemployment, homelessness, overcrowding, and population aging have contributed to the persistence of tuberculosis in developed countries (Parry and Davies 1996). Moreover, the emergence of multidrug resistance also represents an increasing threat to tuberculosis control (Young and Duncan 1995).

Although the majority of people infected with *M. tuberculosis* remain asymptomatic, 5%–10% of them have a lifetime risk of developing active disease. Genetic factors have long been thought to play a role in onset, progression, and ultimate outcome of infection with *M. tuberculosis* (Levin and Newport 2000). This includes epidemiological data pointing to sex (Hinman et al. 1976; Rieder et al. 1991) and racial differences in susceptibility (Stead et al. 1990), as well as geographical distribution and familial aggregation of disease (Casanova and Abel 2002). In addition, population studies in endemic areas of disease and during first contact epidemics (Motulsky 1960; Sousa et al. 1997), together with studies in twins (Comstock 1978), have clearly established a genetic component of susceptibility to tuberculosis in humans. Moreover, case control studies in areas of endemic disease have pointed to several gene variants contributing to tuberculosis risk, including those encoding human leukocyte antigen (HLA) (Delgado et al. 2006; Goldfeld et al. 1998), the natural resistance-associated macrophage protein (*Nramp*) 1 (Bellamy et al. 1998; Cervino et al. 2000; Gao et al. 2000; Greenwood et al. 2000; Li et al. 2006; Malik et al. 2005; Ryu et al. 2000), the vitamin D receptor (Bellamy

et al. 1999; Wilkinson et al. 2000), the mannose-binding protein (Selvaraj et al. 1999), the IL12/23-IFN pathway (Lio et al. 2002; Lopez-Maderuelo et al. 2003; Rossouw et al. 2003; Tso et al. 2005), and the genes encoding DC-SIGN (*CD209*) (Barreiro et al. 2006), chemokine monocyte chemoattractant protein-1 (MCP-1) (Flores-Villanueva et al. 2005), and *SP110* variants (Thye et al. 2006; Tosh et al. 2006). Whole-genome scanning experiments have also identified suggestive linkages on chromosomes 15 and X, identified in African families from The Gambia and South Africa (Bellamy et al. 2000), and on two regions of chromosomes 11 and 20 previously detected in Brazilian families (Miller et al. 2004). Of particular interest is the recent identification in a Moroccan population of a highly significant major locus on chromosome 8q12-q13 that confers predisposition to pulmonary tuberculosis in adults (Baghdadi et al. 2006).

The complex genetic component of susceptibility to tuberculosis is inherently difficult to study in humans. Mouse models of infection can provide a valuable alternative in which major gene effects and positional candidates can be detected, validated in vivo in gene transfer experiments, and ultimately tested for a parallel effect in human field studies. The mouse constitutes an excellent model to study human tuberculosis. Many key parameters of the host response to *M. tuberculosis* in the mouse closely parallel those observed in the human disease (see North and Jung 2004 for a recent review). The efficacy of the host response to pulmonary tuberculosis is under complex genetic control in the mouse, with a broad spectrum of disease severities observed among different strains. Genetic analyses have located a number of tuberculosis susceptibility loci (Kramnik et al. 2000; Lavebratt et al. 1999; Mitsos et al. 2000, 2003; Sanchez et al. 2003; Sapoval et al. 2002; Yan et al. 2006), but so far only a single such locus (*Ipr1*) has been identified (Pan et al. 2005). Please see Fortin et al. (2007) for a complete review of the genetic control of susceptibility to infection with mycobacteria in mice and humans.

Inbred strains have been classified as highly susceptible (CBA, C3H, DBA/2, 129SvJ) or highly resistant (C57BL/6J, BALB/c) to intravenous or aerosol infection with *M. tuberculosis* (Medina and North 1996, 1998). Replication of *M. tuberculosis* in the lungs of innately resistant (C57BL/6J) and susceptible (DBA/2) inbred strains follows a biphasic course. Following infection, *M. tuberculosis* initially (1–3 weeks) replicates rapidly in the lungs of both C57BL/6J and DBA/2 mice. The infection is subsequently (4 weeks to 5 months) held stationary in C57BL/6J while there is continuing microbial replication in the lungs of DBA/2 (Mitsos et al. 2003), which is accompanied by strong inflammatory response and premature death, with a mean survival time (MST) of 110 days. Resistant C57BL/6J mice can control the infection, although they ultimately succumb with a MST of 245 days (Medina and North 1998). Similar patterns of resistance and susceptibility were obtained following intravenous infection (1×10^5 CFU) (Mitsos et al. 2000).

The genetic basis for differential susceptibility of B6 and D2 strains was investigated in our laboratory by whole genome scanning in informative (C57BL/6J×DBA/2) F_2 mice infected by different routes and with different doses of *M. tuberculosis* H37Rv (Mitsos et al. 2000, 2003). An initial genome scan was

conducted using survival time following intravenous infection with 10^5 CFU *M. tuberculosis* H37Rv as a phenotypic marker of susceptibility (Mitsos et al. 2000). Two significant linkages were identified: *Trl-1* on distal chromosome 1 (LOD = 4.80) and *Trl-3* (LOD=4.66) on proximal chromosome 7. A third suggestive linkage, *Trl-2* (LOD=3.93), was localized to proximal chromosome 3. For each of these loci, resistance was associated with homozygosity for the C57BL/6J alleles. The second genome scan used bacterial load in the lungs at 90 days post-infection following aerosol infection with 2×10^2 CFU as a measure of susceptibility (Mitsos et al. 2003). This scan confirmed the *Trl-3* locus (LOD = 3.1) and also identified an additional locus, *Trl-4* (LOD = 5.6), that mapped to the distal portion of chromosome 19, with the C57BL/6J allele at this locus conferring resistance in a partially dominant manner. A strong genetic interaction was detected between *Trl-3* and *Trl-4*, with two-locus linkage analysis yielding a LOD=10.09 and explaining 38% of the variation in raw CFUs. Remarkably, F_2 mice homozygous for C57BL/6J alleles at both *Trl-3* and *Trl-4* were as resistant as C57BL/6J parents, whereas mice homozygous for DBA/2 alleles were as susceptible as DBA/2 parents (Mitsos et al. 2003). At present, the *Trl-3* and *Trl-4* QTLs affecting susceptibility to pulmonary tuberculosis have been retained and validated for the following reasons: (1) *Trl-3* appears to affect both the rate of pulmonary *Mtb* replication and survival to infection, (2) *Trl-4* is the strongest QTL detected to date in two genome scans, (3) the combined effect of both loci explains approx. 50% of the phenotypic variance in the (C57BL/6J×DBA/2) F_2 cross used, with (4) strong interaction between the loci.

Although QTL analysis has been successful in identifying loci involved in the control of susceptibility to *M. tuberculosis*, cloning the gene of interest remains an enormous challenge, not only because of the large size of the chromosomal regions and corresponding transcript maps but also because each locus often accounts only for a small fraction of the total phenotypic variance. To evaluate the individual contribution of *Trl-3* and *Trl-4* to the overall tuberculosis susceptibility phenotype, we generated individual mouse lines congenic for these loci by using a speed-congenic protocol (Bennett and Johnson 1998). In this protocol, successive F_1 backcross males are partially genotyped to identify those with the most biased parental genotype content for selection for further backcrossing. Once at the N4 generation, heterozygotes are intercrossed to generate the homozygote congenic lines and also to produce the double *Trl-3/Trl-4* congenic line, in order to test the separate and combined effects of C57BL/6J resistance alleles on the DBA/2 background. In these mice, the chromosome(s) carrying the QTL(s) of interest (chromosome 7 or 19) from C57BL/6J strain is transferred by breeding to the genetic background of the DBA/2 strain. For *Trl-3*, we are backcrossing a segment of the proximal half of chromosome 7 derived from C57BL/6J, and donated by the BXD19 strain, onto DBA/2 background. For *Trl-4*, we are backcrossing chromosome 19 from C57BL/6J, donated by the BXD9 strain, onto DBA/2 background. Recently, the breeding of both single congenic lines (BXD19 and BXD9) has been completed. Preliminary results have revealed that both congenic strains were about 50% less susceptible to aerosol infection than the DBA/2 parental strain (lung CFUs) (J.F. Marquis, R. LaCourse, L. Ryan, R.J. North, and P. Gros, unpublished data).

Plasmodium chabaudi

Malaria is caused by infection with members of the protozoan parasite family *Plasmodium*. *Plasmodium falciparum* and *Plasmodium vivax* are responsible for a large proportion of the human disease (Marsh and Snow 1997). Between 300 and 500 million cases of malaria are believed to occur each year, with a reported 1 million fatalities, mostly in young children from impoverished countries. Severe anemia and cerebral malaria are major disease manifestations of blood-stage malaria, especially in Africa, where transmission rates are high. There is no effective vaccine against malaria, and this global health problem has been exacerbated by the development of malarial drug resistance in the *Plasmodium* parasite and by insecticide resistance in the *Anopheles* insect vector (Marsh and Snow 1997). The malarial parasite has a complex life cycle in its mammalian host that involves sequential replication in the erythrocyte and sequestration in different tissues such as the liver and brain microvasculature, where it causes disease. Protective immunity against *Plasmodium* species is poorly understood but involves different cell types and physiological and biochemical pathways at each stage of the infection. An effective antimalarial vaccine, especially against the asexual, erythrocytic parasite that causes the symptoms associated with malaria, is still not available despite an enormous effort worldwide (Good 2005). Thus, a better understanding of the innate and adaptive immune mechanisms of host defense against the blood-stage *Plasmodium* parasite, which may manifest themselves as genetic determinants of susceptibility in endemic areas and during epidemics, may provide new targets for therapeutic intervention in this disease.

Malaria is one of the clearest manifestations of genetic control of disease. Population studies in areas of endemic disease indicate important three-way interactions among host genes, the environment, and the malaria parasite. It has also been recognized that normal or disease-associated alterations in certain erythrocyte proteins affect susceptibility to malaria in humans, with positive selection of these variants by the parasite in endemic areas (co-evolution). For a more comprehensive description of the genetic component of susceptibility to malaria in humans, the reader is referred to recent comprehensive reviews on this subject (Kwiatkowski 2000; Min-Oo and Gros 2005). The complex genetic control of susceptibility to malaria has been studied in mice where models for the blood-stage infection and for the cerebral disease have been developed using the murine parasites *Plasmodium chabaudi AS* and *Plasmodium berghei*, respectively. Mouse models of malaria, using *P. chabaudi*-parasitized erythrocytes, mimics several pathophysiological aspects of the blood-stage infection in humans, including host response, genetic control of parasitemia, and ultimate outcome of infection. This model has been effective in localizing several major QTLs, with the genes underlying two such QTLs recently identified by positional cloning. Infection in susceptible mouse strains such as A/J is characterized by heightened parasitemia at the peak of infection, muted inflammatory and erythropoietic responses, and a decreased survival time, compared to resistant strains such as C57BL/6J. Whole-genome scans have

been conducted in backcross and F_2 mice bred from resistant and susceptible parents, and using peak parasitemia and mortality as quantitative phenotypes (Burt et al. 1999; Foote et al. 1997; Fortin et al. 1997). These early studies led to the mapping of three major QTLs on distal chromosome 9 (*Char1*), central chromosome 8 (*Char2*), and chromosome 17 (*Char3*, H-2 locus). Large genetic intervals defined by these QTLs, coupled with the relatively small phenotypic variance explained by each locus have so far impeded cloning of the corresponding genes.

A parallel approach was used to help characterize the complex genetic factors determining the A/J (susceptible) vs B6 (resistant) inter-strain difference in susceptibility to infection with *P. chabaudi*. We phenotyped the AcB/BcA set of reciprocal recombinant congenic strains (derived from A/J and B6 by systematic inbreeding of a second backcross) (Fortin et al. 2001b) for susceptibility to malaria. The usefulness of recombinant congenic strains (RCS) for the study of complex traits has been discussed but can be summarized as follows. By virtue of the breeding scheme used in their derivation, individual AcB/BcA strains carry a small portion of one parental genome fixed as a set of congenic segments on the genetic background of the other strains. Individual genetic effects contributing to a complex trait may have segregated in individual RCS and can be studied in isolation, both for identifying the gene involved but also to elucidate unigenic contributions to the overall phenotype. The relatively small size of the congenic segments fixed in individual RCS facilitates the search and testing of candidate genes. In addition, secondary genetic effects can be detected in strains fixed for certain alleles at major mapped loci, but yet showing deviations from expected phenotypes. Furthermore, reassortment of parental haplotypes or appearance of novel mutations during the breeding of individual strains may generate "hyper-phenotypes" that segregate as simple traits and can be quickly cloned. With the advent of whole genome mRNA transcript profiling, AcB/BcA strains can be used to map *cis*-acting gene expression polymorphisms (eQTLs) and associated regulatory regions that genetically differ between A/J and B6.

A subset of 18 AcB/BcA strains was tested for susceptibility to malaria, using level of parasitemia at the peak of infection and overall survival as phenotypic traits. In general, there was a good correlation between resistance/susceptibility and haplotypes at *Char1* and *Char2* (Fortin et al. 2002). Strains AcB55 and AcB61, however, showed a discordant phenotype and were very resistant to *P. chabaudi* infection despite A/J-derived susceptibility alleles at *Char1* and *Char2* (Fortin et al. 2001a). Additional linkage studies to locate a possible B6-derived segment conferring resistance to AcB55 were carried out in 200 informative (AcB55×A/J) F_2 mice, leading to the identification of a locus (*Char4*) on chromosome 3 (LOD=6.57) that regulates peak parasitemia following infection. Phenotypic characterization of AcB55 and AcB61 strains identified splenomegaly in these mice, together with elevated reticulocyte numbers in peripheral blood and elevated numbers of $Ter119^+$ cells in the bone marrow. Additional transcript profiling using spleen RNA indicated that enhanced erythropoietic activity is a common phenotypic feature of both strains (Min-Oo et al. 2003). Reticulocytosis was found to be inherited as a monogenic trait in the aforementioned (AcB55×A/J) F_2 cross, co-segregating with *Char4* alleles and associated with resistance to malaria. Recombination between the

reticulocytosis trait and markers from the B6 congenic segment on chromosome 3 linked to *Char4*, however, suggested that the locus may map outside this B6 congenic segment. Further analysis in a fully informative (AcB55×DBA/2) F_2 cross showed that this was indeed the case. The transcript map of the chromosomal region contained a strong positional candidate liver- and red-cell specific pyruvate kinase (*Pklr*) based both on its essential role for ATP production in erythrocytes and the fact that mutations in *PKLR* cause hemolytic anemia in humans. Sequencing revealed the presence of an isoleucine-to-asparagine substitution at residue 90 of the Pklr protein in AcB55 and AcB61, a mutation that has also been described in a human case of pyruvate kinase deficiency (Min-Oo et al. 2004). Recently, a second mutant allele at the *Pklr* locus (G338D) was identified in a CBA/N mouse genetic background (CBA/N-Pk^{slc}). As for the I90N allele, this new allele was shown to cause severe hemolytic anemia, and also conferred dramatic protection against *P. chabaudi* infection (Min-Oo et al. 2007b). These findings indicate that loss of function at *Pklr* in mice protects against malaria.

Finally, we have obtained evidence indicating that the protective effect of pyruvate kinase deficiency may be further modulated by other host genetic factors. In addition to *Char4*, linkage analysis in (AcB55×A/J) F_2 mice identified a second suggestive QTL on chromosome 10 (*D10Mit189*) that maps to a 14-Mb C57BL/6J-derived congenic segment fixed in AcB55. C57BL/6J alleles at this locus are protective (reduced peak parasitemia), inherited in a co-dominant fashion, and show an additive effect with *Char4* (Fortin et al. 2001a). This locus was given the temporary designation *Char9* (Min-Oo et al. 2007a). The B6-derived 14-Mb congenic segment on chromosome 10 of AcB55 defining *Char9* is predicted to contain 77 genes that were characterized with respect to (1) tissue-specific expression, (2) the presence of strain-specific alterations in the level of gene expression, and (3) strain-specific polymorphic variants in coding and regulatory regions of positional candidates. *Vnn1/Vnn3* were identified as the likely candidates responsible for *Char9*. *Vnn1/Vnn3* map within a conserved haplotype block and show expression levels that are strictly *cis*-regulated by this haplotype. The absence of *Vnn* messenger RNA expression and lack of pantetheinase protein activity in tissues are associated with susceptibility to malaria and are linked to a complex rearrangement in the *Vnn3* promoter region. The A/J strain also carries a unique nonsense mutation that leads to a truncated protein. *Vanin* genes code for a pantetheinase involved in the production of cysteamine, a key regulator of host responses to inflammatory stimuli. Administration of cystamine in vivo partially corrects susceptibility to malaria in A/J mice, as measured by reduced blood parasitemia and decreased mortality. These studies suggest that pantetheinase is critical for the host response to malaria (Min-Oo et al. 2007a). They also raise the possibility that cysteamine may be a valid, host-based molecule for therapeutic intervention in malaria, alone or in combination with current "parasite-based" antimalarial drugs such as mefloquine. This example clearly illustrates the power of the AcB/BcA set to isolate a gene effect contributing to a complex phenotype in a single mouse strain. The small size of the syntenic fragment can in this case be a major advantage in restricting the size of the QTL. The positional cloning of the gene responsible can then be undertaken by a combination of haplotype mapping, transcript profiling, and nucleotide sequencing.

Conclusions and Future Perspectives

Genetic analyses in mice have proved extremely useful for identifying genes, proteins, and pathways playing a critical role in host defense against infections. In our laboratory we have focused our studies on two inbred mouse strains, A/J and C57BL/6J, and this has led to the identification of several monogenic traits and corresponding proteins, including *Nramp1*, *Birc1e*, *C5*, *Icsbp*, and others that are important determinants of innate immune responses to infection with several intracellular pathogens. We have also used recombinant congenic strains derived from these two parent strains to start studying more complex genetic traits. Although the genetic diversity represented by A/J and B6 is fairly modest, there exist a large number of commercially available and phylogenetically distant strains that may contain a large pool of hypomorphic or mutant alleles and that could similarly be used to identify additional genes and pathways participating in host response to infections. A limitation of this approach is that many of these gene effects may be partial and behave as QTLs for which the underlying gene or genes may be difficult to identify. The parallel production of large numbers of *N*-ethyl-*N*-nitrosourea (ENU)-mutagenized mice may alleviate this problem by providing mutants in which the effect on host response to infection can be readily studied and for which the corresponding mutant gene can be identified by direct sequencing. Genes discovered in the mouse can provide novel entry points to parallel studies in humans using populations at risk or focusing on areas of endemic disease. Finally, validated genes and metabolic pathways may also suggest novel strategies for therapeutic intervention in the corresponding infections.

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Host Defenses Against Human Papillomaviruses: Lessons from Epidermodysplasia Verruciformis

G. Orth

Contents

Abstract Epidermodysplasia verruciformis (EV) is a rare, autosomal recessive genodermatosis associated with a high risk of skin carcinoma (MIM 226400). EV is characterized by the abnormal susceptibility of otherwise healthy patients to infection by specific, weakly virulent human papillomaviruses (HPVs), including the potentially oncogenic HPV-5. Inactivating mutations in either of the related *EVER1/TMC6* and *EVER2/TMC8* genes cause most EV cases. New insights in EV pathogenesis have been gained from the following recent observations:

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(1) EV-specific HPVs (betapapillomaviruses) are defective for an important growth-promoting function encoded by an E5/E8 gene present in other HPVs, and inactivation of EVER proteins may compensate for the missing viral function; (2) the transmembrane viral E5/E8 and cellular EVER proteins interact both with the zinc transporter ZnT1, and are likely to modulate zinc homeostasis. EV may thus represent a primary deficiency in intrinsic, constitutive immunity to betapapillomaviruses, or constitute a primary deficiency in innate immunity (or both). Keratinocytes, the home cells of HPVs, are likely to play a central role in both cases. An important issue is to establish which cellular genes involved in intrinsic and innate antiviral responses play a part in the outcome of infections with other HPV types, such as genital oncogenic HPVs.

Abbreviations EV: Epidermodysplasia verruciformis; HPV: Human papillomavirus; PID: Primary immunodeficiency disease; NMSC: Nonmelanoma skin cancer; ORF: Open reading frame; TGF-β: Tumor growth factor beta; CMI: Cell-mediated immunity; NK cell: Natural killer cell; TNF-α: Tumor necrosis factor alpha; SCID: Severe combined immune deficiency; γc: Common γc cytokine receptor subunit; JAK-3: Janus kinase-3; WHIM syndrome: Warts, hypogammaglobulinemia, infections, and myelokathexis syndrome; SCC: Squamous cell carcinoma; EGFR: Epidermal growth factor receptor; CRPV: Cottontail rabbit papillomavirus; ERK1/2: Extracellular signal-regulated kinase 1/2; AP-1: Activating protein-1; PCDH1: Protocadherin 1; MTF1: Metal-responsive element-binding transcription factor 1; cM: Centimorgan; TMC: Transmembrane channel-like; TLR: Toll-like receptor; PAMP: Pathogen-associated molecular pattern

Introduction

Epidermodysplasia verruciformis (EV) was clinically described in 1922 by Lewandowsky and Lutz, and first considered a congenital epidermal anomaly. The first patient, a 29-year-old woman born from a consanguineous marriage, presented with reddish scaly papules covering her whole skin (Fig. 1), which were allegedly present at birth. The patient developed an invasive carcinoma on her forehead at the age of 25 (Lewandowsky and Lutz 1922). The parental consanguinity and familial aggregation observed for a proportion of reported cases later led to the postulate, in 1933, that EV was probably transmitted by a recessive gene (Cockayne 1933). For a long time, EV was considered either as an inborn anomaly of epidermal differentiation, involving a predisposition to the development of skin cancers, or as a particular form of generalized verrucosis (reviewed in Orth 2006).

The viral etiology of the disease was established by autoinoculation and heteroinoculation experiments (Lutz 1946; Jablonska and Formas 1959) and the regular observation of wart virus particles in benign lesions (reviewed in Jablonska et al. 1972; Lutzner 1978; Majewski and Jablonska 1995; Orth 1987, 2006). This was further substantiated by the demonstration that EV was associated with specific, related human papillomavirus (HPV) types (Pass et al. 1977; Orth et al. 1978, 1979), and by the detection of the

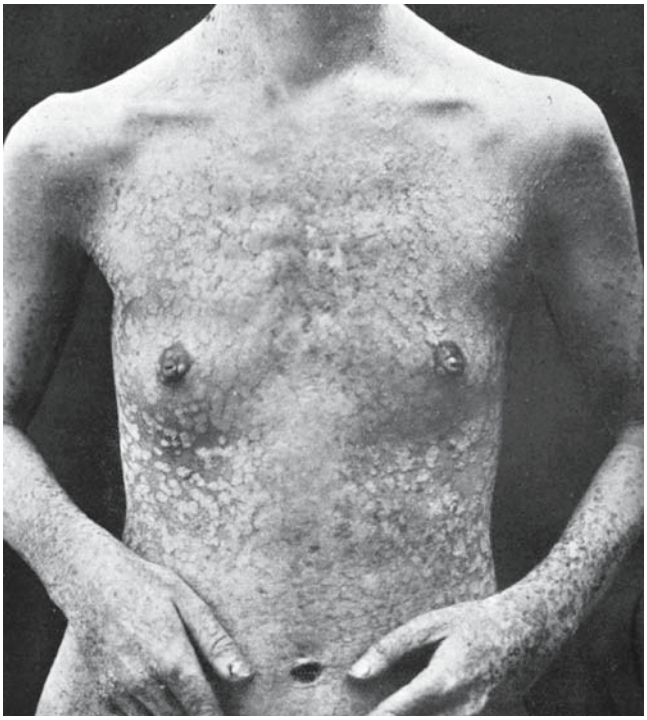

Fig. 1 First EV case described (Lewandowsky and Lutz 1922)

genome of HPV type 5 (HPV-5) in EV carcinomas (Orth et al. 1980; Ostrow et al. 1982; Pfister et al. 1983). Two susceptibility loci for EV were mapped to chromosomal regions 17q25 (*EV1*) and 2p21-p24 (*EV2*) (Ramoz et al. 1999, 2000), and this led to the identification of inactivating mutations that cause EV in either of two novel genes, *EVER1* and *EVER2*, located in the *EV1* locus (Ramoz et al. 2002).

EV can now be defined as a rare, lifelong, skin disease associated with a high risk of nonmelanoma skin cancer (NMSC), which results from a genetically determined, recessively transmitted, abnormal susceptibility to a specific group of HPV genotypes and their carcinogenic potential, mainly that of HPV-5 (Orth et al. 2001). More than 100 HPV genotypes have been fully characterized to date. According to a recently adopted phylogenetic nomenclature, HPV types are grouped into five genera comprising a variable number (1 to 15) of species (de Villiers et al. 2004). The HPV types associated with EV (EV HPVs) are usually considered innocuous for the general population, and belong to the genus *Betapapillomavirus*. The genus *Alphapapillomavirus* comprises types differing by their genital, oral, or cutaneous tropism and by their pathogenicity. It includes the causative agents of genital warts (HPV types 6 and 11) and viruses etiologically related to the development of carcinomas of the uterine cervix and other anogenital cancers (mostly the potentially carcinogenic HPV types 16 and 18) (Bosch et al. 2002; Schiffman and Kjaer 2003).

The genera *Gammapapillomavirus*, *Mupapillomavirus*, and *Nupapillomavirus* comprise types associated with cutaneous warts (de Villiers et al. 2004). Infections caused by cutaneous and mucosal HPV types are widespread and often asymptomatic. HPV-associated diseases and asymptomatic infections usually clear spontaneously. Only persistent lesions associated with potentially carcinogenic types may evolve into invasive carcinomas. The host, viral, and environmental factors that determine the outcome of HPV infections remain poorly understood (Orth 2008).

EV is likely to represent the first identified primary immunodeficiency disease (PID), since this Mendelian condition was clinically described 30 years before the recognition of X-linked agammaglobulinemia (Bruton 1952; Casanova and Abel 2007). Recent advances in our understanding of the pathogenesis of EV provide some insights into mechanisms conditioning the outcome of HPV infections.

The EV Phenotype

EV HPVs can only express the full spectrum of their biological properties at the expense of individuals endowed with a genetically determined vulnerability (Orth et al. 2001). The corresponding phenotype is defined by a number of clinical, viral, and immunological features that characterize EV patients (EV phenotype).

Clinical Features

EV usually begins during infancy or early childhood. About 10% of the patients are born of consanguineous marriages. The disease is characterized by the presence of skin lesions presenting as flat warts or as scaly, reddish, brownish, or achromic plaques. Typical common warts are only occasionally observed, and anogenital warts only exceptionally. EV lesions are refractory to conventional wart treatments. In their second, third, or fourth decade, over half of EV patients start developing precancerous lesions of the skin (actinic keratoses, Bowen's carcinoma in situ) and invasive NMSCs (mostly squamous cell carcinomas), most frequently localized in sun-exposed areas of the skin. Cancers develop slowly, and are mainly locally destructive (reviewed in Lutzner et al. 1984; Jablonska and Orth 1985; Majewski and Jablonska 1995). In general, EV patients are otherwise healthy. It should be mentioned, however, that EV was found associated with mental retardation or other congenital abnormalities in a minority of cases (Lutzner 1978).

Virology

The genus *Betapapillomavirus* comprises 25 fully characterized HPV types, which are distributed into five species (de Villiers et al. 2004). Most of these viruses have been detected in benign EV lesions by molecular hybridization techniques:

HPV types 5, 8, 12, 14, 19, 20, 21, 24, 25, 36, 47 (species 1), 9, 15, 17, 22, 23, 37, 38 (species 2), and 49 (species 3). EV HPVs have a worldwide distribution, and HPV-5 is the most frequently found genotype. Patients are usually infected with multiple EV HPV types and, often, with HPV-3, an alphapapillomavirus that causes flat warts in the general population. A very high virus content characterizes the polymorphic benign EV lesions, and the viral genomes extracted from these lesions can even be identified by direct visualization of their restriction patterns in ethidium bromide-stained gels. This high level of intranuclear viral replication is associated with a specific cytopathic effect, namely the presence of large "dysplastic" keratinocytes with a pale-stained cytoplasm within the spinous and granular layers of the epidermis. This histological feature is pathognomonic of EV (Orth et al. 1980; Orth 1987, 2006).

Only a subset of betapapillomaviruses is associated with the malignant conversion of EV lesions, usually HPV-5 and occasionally, HPV types 8, 14, 17, 20, or 47. Viral genomes are maintained as high copy number episomes, and high levels of transcripts of the viral E6 and E7 open reading frames (ORFs) are detected in premalignant lesions and invasive carcinomas. This further indicates that this subset of betapapillomaviruses is endowed with an oncogenic potential. Recent in vitro studies have shown that the HPV-5 E6 protein, but not the E6 protein encoded by HPV-9, a nononcogenic EV HPV, represses the tumor growth factor beta (TGF-β) signaling pathway by binding to SMAD3, suggesting that a downregulation of this pathway could be involved in HPV-5-associated skin carcinogenesis (Mendoza et al. 2006). Most (but not all) EV carcinomas arise on sun-exposed areas of the skin. Sunlight represents the major risk factor for NMSC in the general population (Brash 1997; Ullrich 2005). The early development of EV carcinomas is likely to involve a synergistic carcinogenic role of HPV-5 and sunlight (reviewed in Orth 1987, 2006).

Immunology

The patients suffering from EV are not abnormally prone to bacterial, fungal, or viral infections. This includes infections caused by HPV types that induce genital diseases and skin warts in the general population, except HPV-3 and HPV-3-related types (Jablonska and Orth, 1985; Majewski et al. 1997). The risk for cancers other than NMSCs does not seem to be abnormally high in EV patients (Lutzner 1978). Humoral immunity appears to be preserved (Jablonska et al. 1979). Antibodies to the major L1 capsid protein of HPV-5 have been detected in all patients studied so far (Favre et al. 1998). Antibodies to the HPV-5 E6 or E7 (or both) oncoproteins were found in about 70% of EV patients with carcinomas (M. Favre, E. Mahé, S. Majewski, S. Jablonska, and G. Orth, unpublished results).

The first evidence for an impaired cell-mediated immunity (CMI) in EV patients was obtained 30 years ago (Glinski et al. 1976; Prawer et al. 1977). Most, but not all, EV patients studied since then were reported to have an impaired CMI on the basis of in vitro and in vivo tests. Decreased T lymphocyte counts and $CD4^+/CD8^+$ T cell ratios, as well as a reduced T cell responsiveness to mitogens, were reported for some patients. An anergy to common skin antigens was observed in most

patients. The most common feature has been an anergy to sensitization to dinitrochlorobenzene (Glinski et al. 1981; Majewski et al. 1986, 1997; Majewski and Jablonska 1992; Oliveira et al. 2003). Patients with EV were found to display normal or increased natural killer (NK) cell activity, using the standard K-562 cells as targets (Majewski et al. 1986). It should be mentioned that the normal number of Langerhans cells, the epidermal antigen presenting cells, appears to be preserved in EV patients (Majewski and Jablonska 1992).

HPV-associated lesions usually regress spontaneously, as a consequence of specific cell-mediated immune responses. Cytotoxic T lymphocyte responses or delayed-type hypersensitivity reactions (or the two together) directed against viral early proteins are thought to play a major role in this outcome (Tagami et al. 1985; Coleman et al. 1994; Stanley 2006). Potentially oncogenic genital HPV types, especially HPV-16, have evolved mechanisms to evade innate and adaptive immunity. This may allow the persistence of intraepithelial lesions, which is a prerequisite to the development of anogenital cancers (Frazer et al. 1999; Woodworth 2002; Stanley 2006). The complete regression of EV lesions has never been observed and innate or acquired cell-mediated immune responses toward keratinocytes infected with EV HPVs are still poorly understood. An increased expression of the tumor necrosis factor alpha (TNF-α) and TGF-β1 genes has been detected in keratinocytes of typical EV lesions, and this has been postulated to play a part in the impaired local immunosurveillance resulting in the persistence of the lesions (Majewski et al. 1991). Available data point to an unresponsiveness of T lymphocytes to autologous HPV-infected keratinocytes (Cooper et al. 1990), and to a reduced NK cell-mediated cytotoxicity against HPV-5-infected keratinocytes in patients with EV (Majewski et al. 1990). Further studies are needed to confirm these early data.

Little information is available on the immunological status of EV patients before the onset of their disease. A still unsolved issue is whether the immunological abnormalities observed to varying extents in most (but not all) patients are secondary to the chronic, massive HPV infection, or result from a primary immune deficiency involved in the pathogenesis of EV.

EV-Specific HPVs in Non-EV Individuals

Betapapillomaviruses Among Other HPVs

Betapapillomaviruses share many properties with the other HPV genotypes, including the general organization of their genome (a circular double-stranded DNA molecule of 7,500–8,000 base pairs), their strict tropism for keratinocytes, and the close link between their life cycle and the biology of their host cells (Howley and Lowy 2007). Generally speaking, cutaneous HPVs most likely target the slow-cycling, self-renewing epithelial stem cells located in the basal layer of the epidermis and in the bulge of hair follicles (Alonso and Fuchs 2003). HPV infections are often

asymptomatic (latent) or may result in the development of a wart. The challenge shared by all papillomaviruses is to infect slow-cycling cells and to replicate in nondividing terminally differentiating keratinocytes. Their strategy relies on the interaction between the viral early proteins E5, E6, and E7 to activate the host cellular DNA machinery and to prevent apoptosis in response to unscheduled DNA synthesis. This leads to the development of a productively infected lesion. The maintenance of the viral DNA as autonomous episomes in latently infected cells would only require the expression of the viral early proteins E1 and E2, which are involved in the replication and segregation of the viral genome (Longworth and Laimins 2004; Münger et al. 2004; Howley and Lowy 2007; Orth 2008).

EV HPVs cause widespread asymptomatic infections in the general population. Our hypothesis is that betapapillomaviruses are defective for an essential growth-promoting function (Nonnenmacher et al. 2006; Orth 2006).

Betapapillomaviruses as Commensals of the Skin

The use of sensitive PCR approaches, designed to detect a broad range of known EV HPVs or putative novel EV HPV-related genotypes, has brought a wealth of information about the epidemiology and biology of betapapillomaviruses. An impressive diversity of putative novel betapapillomaviruses has been disclosed, in addition to the 25 genotypes already characterized (Antonsson et al. 2000). Infections of the skin with EV HPVs or related putative genotypes are highly prevalent in healthy adults (Boxman et al. 1997; Astori et al. 1998; Antonsson et al. 2000), and these infections are acquired very early in infancy (Antonsson et al. 2003). However, the prevalence of antibodies to the L1 major capsid protein of HPV-5 and other EV HPV genotypes is low (lesser than 10%) in the general population (Favre et al. 1998; Stark et al. 1998; Feltkamp et al. 2003; Karagas et al. 2006). Considering that betapapillomaviruses are ubiquitous and cause widespread asymptomatic cutaneous infections, it has been proposed that these viruses are commensals of the human skin (Antonsson et al. 2000).

Rare Phenocopies of EV upon Immunosuppression

Immunosuppression per se is not sufficient to allow EV HPVs to express their pathogenic and oncogenic potentials. This is illustrated by the scarcity of an EV-like syndrome (persistent cutaneous lesions with specific histologic features and a high level of EV HPV DNA replication) among patients with genetic, acquired, or induced depression or suppression of CMI. An increased incidence of warts and skin cancers is observed among organ allograft recipients, who have been intentionally immunosuppressed to prevent rejection of the transplants (Koranda et al. 1974; Euvrard et al. 2003). Only rare cases of HPV-5- or HPV-8-associated EV-like

syndrome have been reported among renal transplant recipients (Lutzner et al. 1980, 1983; Barr et al. 1989). There are few reports of the development of lesions typical of EV in patients infected with human immunodeficiency virus (reviewed in Carré et al. 2003).

That the vulnerability to betapapillomaviruses may require a specific immune dysfunction has been supported by a long-term follow-up study of 41 patients with severe combined immune deficiency (SCID) who had undergone hematopoietic stem cell transplantation early in life. Late-onset, severe cutaneous disease was observed only in patients with SCID associated with either common γc cytokine receptor subunit (γc) or Janus kinase-3 (JAK-3) deficiency. Four of nine such patients had lesions typical of EV associated with HPV types 5 and 14, indicating that γc/JAK-3 signaling in keratinocytes may play a role in immunity against EV HPVs (Laffort et al. 2004). Patients suffering from WHIM syndrome (warts, hypogammaglobulinemia, infections, and myelokathexis), which is associated with mutations in the gene encoding the CXCR4 chemokine receptor, are prone to chronic cutaneous and genital HPV disease, usually associated with alphapapillomaviruses (Gorlin et al. 2000; Hernandez et al. 2003). EV-like lesions have not been described in such patients.

Innocuousness of Betapapillomaviruses?

Whether EV HPVs and related genotypes are harmless in the general population is still a matter of debate (Kawashima et al. 1990; Harwood and Proby 2002; Pfister 2003; Orth 2004, 2005). HPV DNA sequences (most frequently betapapillomaviruses) were detected in low amounts (usually much less than one copy of viral genome per cell) in 25%–65% of basal cell carcinomas and squamous cell carcinomas (SCCs) in immunocompetent individuals and in up to 90% of SCCs in organ transplant recipients. Similar detection rates were found for premalignant skin lesions (reviewed in Harwood and Proby 2002; Pfister 2003). These data, as well as seroepidemiological studies (Feltkamp et al. 2003; Karagas et al. 2006) and functional studies involving ectopic expression of E6 and E7 proteins of cutaneous HPV genotypes (reviewed in Storey 2002), brought some support to the possible role of betapapillomaviruses in the development of NMSCs in non-EV patients. However, mutagenic and immunosuppressive ultraviolet radiations of the sun are considered the major risk factors for skin cancer (Brash 1997; Ullrich 2005), and such a role for HPV remains elusive (Harwood and Proby 2002; Pfister 2003; Orth 2005).

High detection rates of DNA sequences of betapapillomaviruses (especially HPV-5) in the lesional skin have been reported in patients suffering from psoriasis (Favre et al. 1998; Weissenborn et al. 1999). The prevalence of antibodies to HPV-5 L1 protein in these patients (25%) and in patients with extensive, second degree burns or in patients with cutaneous autoimmune bullous diseases (15%–25%) was found to be significantly higher than that in individuals with no known history of

HPV disease (5%) (Favre et al. 1998, 2000). This suggests that the extensive proliferation of keratinocytes involved in epidermal repair processes could favor the expression of betapapillomaviruses (Favre et al. 2000). Whether this could have beneficial (wound healing) or detrimental (psoriasis) consequences remains to be determined (Favre et al. 1998, 2000; Majewski et al. 1999).

Betapapillomaviruses as Defective HPVs

The genome of betapapillomaviruses is characterized by a shorter size, a specific organization of its noncoding regulatory region, and, most importantly, by the lack of an E5 or E8 ORF (Fig. 2) (Fuchs and Pfister 1996; Garcia-Vallvé et al. 2005; Nonnenmacher et al. 2006). The E5 ORF is located between the early (E) and late (L) regions of the genome of alphapapillomaviruses, such as the genital HPV types 6, 16, and 18 and the cutaneous HPV types 2 and 3. It encodes short hydrophobic proteins associated with intracellular membranes (DiMaio and Mattoon 2001; Garcia-Vallvé et al. 2005). The well-characterized HPV-16 E5 protein exhibits a weak transforming activity in vitro, mainly through its ability to upregulate the epidermal growth factor receptor (EGFR) signaling pathway in a ligand-dependent manner. Expression of HPV-16 E5 in the epidermal basal layer of transgenic mice induces epidermal hyperplasia (requiring EGFR signaling) and spontaneous skin papillomas (Genther Williams et al. 2005). The E5 protein is thus assumed to contribute

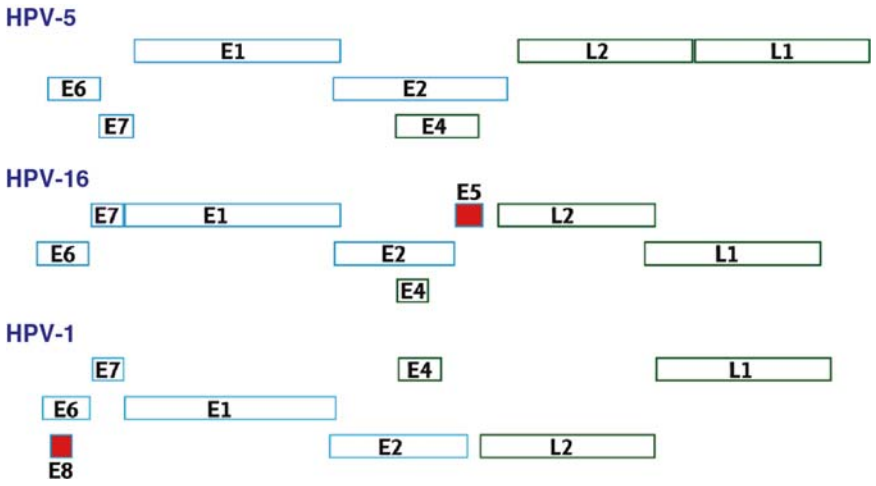

Fig. 2 Linear maps of the open reading frames of HPV-5 (*Betapapillomavirus* genus), HPV-16 (*Alphapapillomavirus* genus), and HPV-1 (*Mupapillomavirus* genus). ORFs are distributed downstream from a long regulatory noncoding region, within the early (*E*) and late (*L*) regions (Howley and Lowy 2007). Note the presence of an E5 ORF in the HPV-16 genome and an E8 ORF in the HPV-1 genome (*red boxes*), and the lack of either ORF in the HPV-5 genome

to the development of a lesion by stimulating cell division, together with the E6 and E7 proteins (DiMaio and Mattoon 2001; Maufort et al. 2007). Cutaneous HPV genotypes belonging to the genera *Gammapapillomavirus* (such as HPV-4) and *Mupapillomavirus* (HPV-1) induce skin warts, but lack an E5 ORF (de Villiers et al. 2004; Garcia-Vallvé et al. 2005; Nonnenmacher et al. 2006). The E6 region of these viruses, however, harbors an E8 ORF with a coding potential for a small hydrophobic protein, structurally related to the E5 proteins (Garcia-Vallvé et al. 2005; Nonnenmacher et al. 2006). The HPV E8 proteins have not been studied yet, but recent in vivo and in vitro studies on the cottontail rabbit papillomavirus (CRPV) indicate that E8 plays a crucial role in viral pathogenesis (Nonnenmacher et al. 2006)

CRPV E8 is a 50-amino acid, membrane-associated protein that shows little transforming activity in vitro (Harry and Wettstein 1996). However, E8-knockout CRPV genomes were found to display a dramatically reduced ability to induce warts upon biolistic inoculation into the skin of immunocompetent and immunosuppressed rabbits. The scarce induced warts showed very slow growth despite sustained expression of E6 and E7 oncogenes. Intriguingly, E8 was found dispensable for wart induction (but not for growth) when the skin was first pretreated with turpentine, which promotes an acute inflammatory response, and then scarified prior to inoculation of E8-knockout genomes (Hu et al. 2002). This experimental procedure induces epidermal hyperplasia and wound healing and increases the susceptibility to CRPV infection (Friedewald 1944). Both epidermal hyperplasia and wound healing involve the release of the epidermal stem cells from their microenvironmental control (Fuchs et al. 2004; Blanpain et al. 2007), and this may allow wart induction by E8 mutants by substituting for E8 function. When expressed in cultured cells, CRPV E8, like HPV-16 E5, was found to increase the EGF-dependent extracellular signal-regulated kinase 1/2 (ERK1/2) phosphorylation, and both the EGF-dependent and the EGF-independent activity of activating protein-1 (AP-1) (Nonnenmacher et al. 2006).

E8 proteins are thus likely to be major players in the development of HPV-associated lesions, by disturbing epidermal homeostasis and promoting the proliferation of quiescent epidermal stem cells (Nonnenmacher et al. 2006). EV HPVs are usually harmless in the general population, and it can be proposed that they behave as defective viruses for an essential (E5/E8-like) growth-promoting function (Orth 2006).

The Missing Viral Function and Zinc Homeostasis

The CRPV E8 protein was found to bind to two cellular transmembrane proteins, the zinc transporter ZnT1 and protocadherin 1 (PCDH1) (Nonnenmacher et al. 2006). ZnT1 is a transporter involved in zinc efflux (Palmiter and Findley 1995) and PCDH1 is a poorly characterized protein potentially involved in cell–cell adhesion and signal transduction (Frank and Kemler 2002). This was an unexpected

finding since neither protein had been linked previously to viral pathogenesis and cell transformation. Most interestingly, the HPV-16 E5 protein was also found to interact with ZnT1 and PCDH1, which supports the hypothesis that E5 and E8 proteins exert similar biological functions (Nonnenmacher et al. 2006). Both E8 and ZnT1 were found to colocalize with EGFR signaling complexes in endosomes, and CRPV E8–ZnT1 interaction was shown to be required for E8-induced AP-1 activation. Moreover, CRPV E8 was found to disrupt a complex formed by ZnT1 and PCDH1 (Nonnenmacher et al. 2006). These findings support the notion that the interaction between E8 and endogenous ZnT1 is required for the upregulation of EGFR signaling. Since zinc is a structural component of a great number of proteins, including signaling proteins and transcription factors, and is essential for their biological activities (Cousins et al. 2006), it may be expected that E8-ZnT1 interaction affects several unrelated cellular functions.

Zinc homeostasis relies on the balanced expression of the ZnT proteins, mediating zinc efflux from the cell or into intracellular organelles, and the Zip proteins, mediating zinc uptake, and on the activity of the cysteine-rich, zinc-storing metallothioneins. The regulation of the expression of ZnT1 and a number of zinc transporters and that of metallothioneins depends on a zinc-sensing protein, the metal-responsive element-binding transcription factor 1 (MTF1) (Cousins et al. 2006). Zinc homeostasis is modulated by various environmental signals, including microbial products, cytokines, antigens, oxidants, nitric oxide, and heavy metals (Spahl et al. 2003; Zhou et al. 2004; Liuzzi et al. 2005; Cousins et al. 2006; Kitamura et al. 2006; Kröncke 2007; Yamasaki et al. 2007). It has recently been proposed that zinc ions (Zn^{++}) act as an intracellular second messenger, with the potential to influence various aspects of cellular signaling (Cousins et al. 2006; Yamasaki et al. 2007).

The specific properties of EV HPVs may be explained, at least in part, by their inability to interact with mechanisms that modulate zinc homeostasis, which are likely to play a crucial role in the life cycle of these viruses. The EV phenotype would thus result from the inactivation of a cellular protein(s) or signaling pathway(s) that would compensate for the missing viral E5/E8 function(s).

The Genetic Etiology of EV: *EVER* Genes

EVER *Genes and EV*

About 10% of EV patients reported in the literature were born from consanguineous parents. About 10% of EV families have more than one affected sibling. The proportion of EV siblings approaches 25%, and the sex ratio for EV is close to one. These observations support the theory that EV is an autosomal recessive disease (MIM 226400) (Lutzner 1978). X-linked recessive inheritance was proposed for a well-documented EV family in which only males were affected (MIM 305350), pointing to a possible genetic heterogeneity of the disease (Androphy et al. 1985).

A genome-wide linkage study performed on consanguineous EV families (first-cousin marriages), using the homozygosity mapping approach, led to the mapping of a first susceptibility locus for EV (*EV1*) on chromosome 17q25, in a 1-cM region (Ramoz et al. 1999, 2000), which was narrowed to a region of about 180 kb (Ramoz et al. 2002). A second locus (*EV2*) was mapped on chromosomal region 2p21-p24, in an 8-cM interval, providing further evidence for nonallelic heterogeneity in the disease (Ramoz et al. 2000).

Mutation analysis of the genes and putative novel genes contained in the *EV1* region allowed identification of truncating mutations segregating with the disease in either of two novel related genes, which were named *EVER1* and *EVER2* (Ramoz et al. 2002). *EVER1* and *EVER*2 are separated by 4.7 kb in an opposite orientation. Forty-one EV patients (either familial or sporadic cases) have now been analyzed in the course of a collaborative study between our group (N. Ramoz, C.J. Kim, P. Cassonnet, M. Favre) and B. Bouadjar (Algiers, Algeria), L.A. Rueda and L.S. Montoya (Bogota, Colombia), K. Fukai (Osaka, Japan), and S. Jablonska and S. Majewski (Warsaw, Poland). Homozygous truncating mutations in either gene were found in 31 (75.6%) cases, and four *EVER1* and six *EVER2* mutant alleles were identified in patients from Algeria, Colombia, Poland, and Japan (Ramoz et al. 2002; Orth 2006; B. Bouadjar, P. Cassonnet, M. Favre, K. Fukai, S. Jablonska, C.J. Kim, S. Majewski, L.S. Montoya, G. Orth, N Ramoz, L.A. Rueda, data to be published). The segregation of mutations in the families revealed a complete penetrance. No correlation between the genotype and the phenotype has been observed so far. Variable nucleotide positions leading to four amino acid substitutions in both EVER proteins were identified, but none of them was found to be associated with the disease. Inactivating mutations in either *EVER1* or *EVER2* have also been identified recently in one compound heterozygous Japanese patient (Tate et al. 2004) and four homozygous patients of Chinese (Sun et al. 2005; Zuo et al. 2006), Pakistani (Gober et al. 2007), and Hispanic (Berthelot et al. 2007) origin. This further stresses the worldwide association of *EVER* mutations with EV. No *EVER* mutations were detected in 25% of the EV patients of our series (including familial cases) and in a Turkish patient (Akgül et al. 2007). This brings further evidence for the genetic heterogeneity of the disease.

EVER *Genes and* TMC *Gene Family*

The *EVER* genes were found to belong to a novel gene family, the transmembrane channel-like (*TMC*) gene family, which comprises eight genes (*TMC1* to *-8*) (Kurima et al. 2003; Keresztes et al. 2003). *EVER1* and *EVER2* are identical to the *TMC6* and *TMC8* genes, respectively. All *TMC* genes are predicted to encode transmembrane proteins with six to ten membrane-spanning domains. The human *TMC* genes and their murine orthologs are highly conserved (75%–95% amino acid sequence identity, pairwise). Homologs of *TMC* genes have been identified in nonmammalian vertebrates and invertebrates (Kurima et al. 2003; Keresztes et al.

2003). All these genes encode a conserved 120-amino acid sequence, the TMC domain (Kurima et al. 2003). Dominant and recessive mutations of *TMC1* and its murine ortholog *tmc1* cause hearing loss (Kurima et al. 2002; Vreugde et al. 2002). *TMC1* and *tmc1* are expressed in cochlear hair cells of the inner ear, and *tmc1* was found to be required for the normal maturation and function of these cells (Kurima et al. 2002; Vreugde et al. 2002; Marcotti et al. 2006). The highly evolutionarily conserved *TMC* genes most likely encode proteins with important cellular roles. It has been speculated that TMC proteins could constitute a novel group of ion transporters or channels, or modifiers of such activities, and could be involved in signal transduction (Kurima et al. 2003; Keresztes et al. 2003).

EVER Proteins and Zinc Homeostasis

Transcripts spanning the full length of the ORFs of *EVER1/TMC6* (20 exons) and *EVER2/TMC8* (16 exons) encode putative proteins of 805 amino acids and 726 amino acids, respectively. Alternative splice events generate smaller *EVER1/TMC6* and *EVER2/TMC8* transcripts, which would encode protein isoforms of 454 amino acids and 503 amino acids, respectively (Ramoz et al. 2002). The EVER1 and EVER2 proteins share 28.4% of their amino acids, the less conserved regions being their amino- and carboxyl-termini. Both proteins are predicted to be integral membrane proteins with ten (EVER1) or eight (EVER2) putative transmembrane domains, two (EVER1) or three (EVER2) putative leucine-zipper motifs and two putative glycosylation sites. The terminal regions are predicted to be lumenal for EVER1 and cytoplasmic for EVER2 (Q7Z403 and Q8IU68, respectively; UniProtKB/Swiss-Prot database). Both proteins were found located in the endoplasmic reticulum when transiently expressed in human keratinocytes (HaCat cells) (Ramoz et al. 2002). All mutations identified so far eliminate the conserved TMC domain located immediately upstream of the ninth (EVER1/TMC6) or seventh (EVER2/TMC8) putative transmembrane domain (Fig. 3).

The functions of the related, membrane-spanning EVER1/TMC6 and EVER2/TMC8 proteins had remained unknown until recent work demonstrating that EVER proteins regulate cellular zinc balance (Lazarczyk et al. 2008). EVER1 and EVER2 proteins were shown to form a complex and to interact with the zinc transporter ZnT1. Neither EVER nor ZnT1proteins interacted with the E6 and E7 oncoproteins of HPV-5 and HPV-16, but they were found to bind the HPV-16 E5 protein, as already reported for ZnT1 (Nonnenmacher et al. 2006). When expressed in human keratinocytes (HaCat cells), EVER and Znt1 were found to downregulate transcription factors stimulated by zinc (MTF-1) or by cytokines (c-Jun and Elk), and this negative regulation was blocked by HPV-16 E5 (Lazarczyk et al. 2008).

The demonstration that the proteins encoded by the viral E5/E8 genes (missing in EV HPVs) and the cellular *EVER* genes share the same cellular partner, ZnT1, and are likely to modulate zinc homeostasis, provides exciting new insights into the pathogenesis of EV.

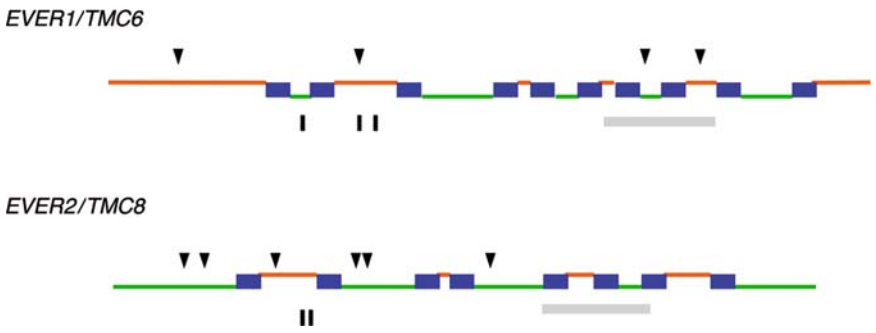

Fig. 3 Schematic representation of *EVER1* and *EVER2* genes showing location of mutations in EV patients. The transmembrane domains are represented by *blue boxes*, the putative cytoplasmic and lumenal regions by *red* and *green lines*, respectively, and the conserved TMC domain by a *gray box*. *Arrowheads* represent mutations identified in our study, and *vertical bars* indicate mutations reported in the literature for *EVER1* (Tate et al. 2004; Zuo et al. 2006; Gober et al. 2007) and *EVER2* (Sun et al. 2005; Berthelot et al. 2007)

EV as a Primary Immunodeficiency Disease

Two Nonmutually Exclusive Hypotheses for EV Pathogenesis

EV is one of the Mendelian conditions manifesting as a narrow susceptibility to infections in otherwise healthy patients (with no overt immunological phenotype), which constitute a newly recognized group of "nonconventional" human PIDs (Casanova et al. 2005). These Mendelian traits include both hematopoietic and nonhematopoietic PIDs, as host defenses not only involve immunological cells of hematopoietic origin but also other cells playing an important role in local immune responses, such as endothelial cells, enterocytes, and keratinocytes (Casanova and Abel 2007). Such diseases have proved to be valuable model systems to dissect the molecular mechanisms underlying immunity to infection in humans (Casanova and Abel 2004, 2007; Fischer 2004).

The strictly epitheliotropic, cutaneous human papillomaviruses most likely target the epidermal stem cells, and their life cycle is tightly linked to the biology of their host cells (Longworth and Laimins 2004; Orth 2008). Keratinocytes are not only responsible for the renewal, cohesion, and barrier function of the skin but also contribute to the skin immune system by secreting, and responding to, various cytokines, chemokines, and growth factors, allowing a cross-talk between keratinocytes and immunocytes (Kupper and Fuhlbrigge 2004; Bos 2005; Nickoloff 2007). Furthermore, keratinocytes express Toll-like receptors (TLRs), and immune responses are initiated after the activation of these receptors by their respective microbial ligands (Lebre et al. 2007; Miller and Modlin 2007). It has been shown that *EVER* genes are transcribed in the normal human skin (Ramoz et al. 2002),

highly transcribed in $CD4^+$ and $CD8^+$ T lymphocytes, B lymphocytes, and NK cells, and, for *EVER1* at least, significantly transcribed in endothelial cells, bone marrow $CD33^+$ myeloid cells, and dendritic cells (Su et al. 2004; http://symatlas.gnf.org).

When discussing the role of *EVER* genes in the pathogenesis of EV, two hypotheses can thus be proposed that are not mutually exclusive: (1) EVER proteins act as restriction factors that hinder the complete expression of the EV HPV genomes in keratinocytes and the development of EV lesions (the EV phenotype), (2) EVER proteins play a specific role in the innate immune responses and the shaping of adaptive immunity against EV HPV-infected keratinocytes (Orth 2006).

A Primary Deficiency in Intrinsic Immunity

Intrinsic immunity is the front line of host defense against viral infections and does not require any virus-triggered signaling or intercellular communication (Bieniasz 2004). It can be assumed that, by modulating zinc homeostasis, EVER proteins contribute to a regulatory pathway controlling the division of epidermal stem cells. It is worth stressing recent studies indicating that the *Caenorhabditis elegans* homolog of ZnT1, the cation diffusion facilitator1, is a positive modulator of EGFR signaling in vulval development (Bruinsma et al. 2002), and that the zinc transporter LIV1/Zip6 controls epithelial–mesenchymal transition during gastrulation in zebrafish (Yamashita et al. 2004). Our hypothesis implies that EVER proteins act as dominant restriction factors for HPVs, and that the function of E5/E8 proteins is, at least in part, to overcome this restriction and to trigger the viral life cycle. It is thus tempting to speculate that the inactivation of *EVER1/TMC6* or *EVER2/TMC8* compensates for the missing EV HPV E5/E8 protein in the induction of EV lesions (Nonnenmacher et al. 2006; Orth 2006). According to this hypothesis, host restriction of betapapillomaviruses in patients harboring wildtype *EVER* genes should be overcome by co-infection of EV HPV-infected keratinocytes with a genotype expressing a functional E5/E8 protein. Notably, high levels of EV HPV DNA (easily detected by Southern blot hybridization) have been detected in warts induced by HPV-3 or related genotypes (alphapapillomaviruses) in some immunosuppressed patients (Obalek et al. 1992).

A Primary Defect in Innate Immunity

If one considers that a primary defect in intrinsic immunity may be responsible for the growth of EV lesions in genetically predisposed individuals, it remains to be understood why these lesions never regress. This is in contrast with cutaneous warts caused by alpha-, gamma-, or mupapillomaviruses, which usually retrogress in the general population under the influence of cell-mediated immune responses (Tagami et al. 1985; Stanley 2006). Typical HPV-5-associated scaly macules were reported to have regressed in a renal allograft recipient (phenocopy of EV) after withdrawal

of azathioprine, an immunosuppressive drug (Lutzner et al. 1983). EV patients may thus present a specific immune defect, as suggested by early studies (Cooper et al. 1990; Majewski et al. 1990).

A Central Role for Keratinocytes?

Since *EVER* genes are expressed in the skin (Ramoz et al. 2002) and various hematopoietic immune cell types (Su et al. 2004; http://symatlas.gnf.org), *EVER* mutations might affect various arms of the immune response to EV HPVs. The phenotype resulting from inactivation of either *EVER* gene in the mouse is, unfortunately, still unknown. As the home cells of HPVs, and as important elements of the skin immune system (Kupper and Fuhlbrigge 2004; Bos 2005; Nickoloff 2007), keratinocytes are thought to play a central role in the innate and adaptive immune responses to papillomaviruses. The resident skin immature antigen-presenting cells, epidermal Langerhans cells and dermal dendritic cells, may also encounter viral particles during primary infection, following a microtrauma of the skin (Orth 2008). Generally speaking, antiviral responses are initiated after receptors expressed in different cell compartments, such as TLRs, recognize pathogen-associated molecular patterns (PAMPs). Pattern recognition results in (1) the production of type I interferons and proinflammatory cytokines and chemokines, (2) the activation of dendritic cells, and (3) the development of adaptive antiviral immune responses (Beutler et al. 2006; Kawai and Akira 2006). The only PAMP identified to date for HPVs is the viral capsid, a highly ordered structure. Papillomavirus-like particles (very similar to the capsids of native virions) have been shown to effectively activate human dendritic cells, but not Langerhans cells, and to directly activate B cells to induce $CD4^+$ T cell-independent humoral responses, both cell activations occurring via TLR4-MyD88-dependent signaling pathways (Fausch et al. 2002; Yang et al. 2004, 2005). Whether functional EVER proteins are required remains to be established. TLRs expressed by keratinocytes have been found to functionally respond to ligands mimicking viral PAMPs, synthetic double-stranded RNA (poly I:C), and CpG-oligodeoxynucleotides (Lebre et al. 2007). No data are yet available on the innate responses of keratinocytes to HPV capsids.

Zinc, EVER Proteins, and Immunity to HPVs

Since the EVER proteins have recently been shown to regulate cellular zinc balance by interacting with ZnT1 (Lazarczyk et al. 2008), it is conceivable that a modulation of the concentration or localization of intracellular free zinc ions is required for signaling pathways triggered by EV HPVs in keratinocytes or other immune cells. It is well known that zinc deficiency results in defects in innate and acquired immunity and in an increased susceptibility to infections (Wellinghausen et al. 1997; Fischer and Black 2004; Rink and Haase 2007). The link between zinc homeostasis

and immunity is well illustrated by acrodermatitis enteropathica (MIM 201100), a rare, autosomal, recessively inherited disease caused by mutations in the gene encoding the zinc transporter Zip4 and a defect in zinc uptake by intestinal cells. This defect results in, among other symptoms, dermatitis and diarrhea, reflecting a dysfunction of the immune system (Wang et al. 2002; Rink and Haase 2007). Mutations in ZnT1 are unlikely to cause any disease, since this gene was found to be essential for early embryonic development in the mouse (Andrews et al. 2004). Interestingly, topical zinc oxide and oral zinc sulfate were reported to represent an efficacious therapeutic option for recalcitrant skin warts (Al-Gurairi et al. 2002; Khattar et al. 2007).

Recent studies have shown how variations in intracellular concentrations of free zinc influence signaling pathways mediating the cellular responses to various stimulations (Spahl et al. 2003; Zhou et al. 2004; Liuzzi et al. 2005; Cousins et al. 2006; Kitamura et al. 2006; Kröncke 2007; Yamasaki et al. 2007). For instance, variations in the concentration or localization of intracellular free Zn^{++} ions are involved in the maturation of murine dendritic cells induced by the TLR4 ligand lipopolysaccharide (LPS) (Kitamura et al. 2006), the activation of mast cells after cross-linking of the high affinity receptor FcεRI (Yamasaki et al. 2007), the response of endothelial cells to inflammatory cytokines (Spahl et al. 2003), and the production of inflammatory cytokines by monocytes (von Bülow et al. 2005). Coexpression of EVER and ZnT1 proteins in human keratinocytes has been shown to downregulate transcription factors stimulated by cytokines (Lazarczyk et al. 2008). How the EVER proteins could participate in the cross-talk between keratinocytes and immunocytes remains to be understood.

If modulations of zinc homeostasis are involved in innate immune defenses and the shaping of acquired immunity to HPVs, the signaling pathways mediated by the EVER proteins should be crucial for protection against betapapillomaviruses, but be redundant for immunity to other HPV genotypes. The other Mendelian traits characterized by a narrow vulnerability to microorganisms have taught us already that major pathways (such as the IL-12–IFN-γ, TIR–NF-κB, or TLR3–IFN-α/β pathways) may be critical for protective immunity against specific viruses or bacteria but redundant for immunity to most other pathogens (Casanova and Abel 2007).

Viral Proteins, Immune Evasion, and EV Persistence

The genital oncogenic alphapapillomaviruses (such as HPV-16 and HPV-18) have evolved strategies to evade host immunity. The multifunctional E6 and E7 oncoproteins are endowed with the capacity to interfere with the expression of, or the response to, type I interferons and inflammatory cytokines or chemokines, and this may contribute to the suppression of antiviral responses and to the persistence of infection (Frazer et al. 1999; Koromilas et al. 2001; Guess and McCance 2005; Stanley 2006). The whole spectrum of viral proteins is expressed in EV lesions, and similar viral mechanisms could contribute to the lifelong persistence of the disease. The overexpression of TNF-α and TGF-β may represent such a mechanism

(Majewski et al. 1991). A defect in local CMI revealed by an anergy to contact sensitizers, and other abnormalities in nonspecific CMI and have been observed in most, but not all, EV patients (Majewski and Jablonska 1995; Oliveira et al. 2003). Whether these abnormalities are linked to *EVER* mutations or are secondary to a longstanding massive infection remains to be understood.

Concluding Remarks

Little is known about the genetic factors controlling HPV infections, from latency to invasive carcinoma. As a Mendelian trait, EV represents an outstanding model for studying the part played by viral and host genetic factors in the outcome of these infections. New insights in the pathogenesis of EV have recently been gained from three major advances: (1) EV-specific HPVs are likely to be defective for an important growth-promoting function encoded by the E5/E8 gene present in other HPV genotypes (Nonnenmacher et al. 2006; Orth 2006); (2) most EV cases are caused by inactivating mutations within the related *EVER1* and *EVER2* genes (Ramoz et al. 2002; Orth 2006); (3) the transmembrane viral E5/E8 and cellular EVER proteins share the same cellular partner, the zinc transporter ZnT1, and both are likely to modulate zinc homeostasis (Nonnenmacher et al. 2006; Lazarczyk et al. 2008). Since zinc is an essential factor required for the structure and function of a great number of proteins, variations in zinc homeostasis may influence many aspects of cellular signaling in immune or nonimmune cells (Cousins et al. 2006; Rink and Haase 2007; Yamasaki et al. 2007). According to the two nonmutually exclusive hypotheses that can be proposed, EV may represent a primary deficiency in intrinsic (constitutive) immunity to betapapillomaviruses, a primary deficiency in innate immunity against these viruses, or both. The mechanisms for the control of EV HPV infections by EVER proteins in keratinocytes, which are likely to play a central role, and immune cells remain to be established. It must be stressed that only 75% of the patients studied so far harbor an *EVER* mutation. A second EV susceptibility locus (2p21-p24) with an autosomal recessive mode of transmission has been identified (Ramoz et al. 2000), and evidence for an X-linked recessive inheritance has been reported (Androphy et al. 1985). Identification of additional genes associated with EV should provide further clues for understanding host defenses against papillomaviruses.

It thus appears likely that, in the general population, susceptibility or resistance to HPV infections and HPV-associated diseases depends both on the diversity of HPV genotypes and on mutations in, or allelic polymorphisms of, host genes involved in intrinsic, innate, or adaptive immunity. We are just beginning to discover the nature of these genes. If EVER proteins play a part in host defenses against all HPVs, as suggested by the interaction between HPV-16 E5 and both ZnT1 and EVER proteins (Nonnenmacher et al. 2006; Lazarczyk et al. 2008), the pathways involved should be redundant for genotypes other than EV HPVs. SCID patients and patients suffering from WHIM syndrome have taught us that γc/JAK-3

and CXCR4 signaling pathways are crucial for immunity against HPVs (Hernandez et al. 2003; Laffort et al. 2004). Focal epithelial hyperplasia of the oral mucosa, or Heck's disease, is another HPV-associated Mendelian trait (MIM 229045) that could allow further advances. Heck's disease is specifically associated with two oral alphapapillomaviruses, HPV types 13 and 32, and is observed predominantly among Eskimos and American Indians (Archard et al. 1965; Beaudenon et al. 1987). No susceptibility locus has been identified so far for this disease.

Unraveling the genetic bases of the susceptibility to HPV infections and diseases represents a major issue. The challenge is to understand why and how genital alphapapillomaviruses, including potentially carcinogenic genotypes, often cause asymptomatic, transient infections, and why only a minority of the infected women will develop invasive anogenital cancers (Bosch et al. 2002; Schiffman and Kjaer 2003).

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Innate Resistance to Flavivirus Infections and the Functions of 2´-5´ Oligoadenylate Synthetases

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Contents

Abstract Mouse susceptibility to experimental infections with flaviviruses is significantly influenced by a cluster of genes on chromosome 5 encoding a family of proteins with enzymatic properties, the 2´-5´ oligoadenylate synthetases (OAS). Positional cloning of the locus in question has revealed that susceptibility of laboratory inbred strains to this class of virus is associated with a nonsense mutation in the gene encoding the OAS1B isoform. Analysis of the molecular structure of the cluster in different mammalian species including human indicates that the cluster is extremely polymorphic with a highly variable number of genes and pseudogenes whose functions are not yet completely established. Although still preliminary, a few recent observations also substantiate a possible role for OAS1 in human susceptibility to viral infections (West Nile virus, SARS, etc.) and its possible involvement in some other diseases such as type 1 diabetes and multiple sclerosis. Finally, convergent observations indicate that the molecules encoded by the *2´-5´OAS* cluster might be involved in other fundamental cellular functions such as cell growth and differentiation, gene regulation, and apoptosis.

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Introduction

The severity of the clinical manifestations of most infectious diseases is greatly influenced by environmental factors and by the physiological status and the immune competence of the infected organism. It is also strongly influenced by genetic factors controlling the virulence of the pathogen and the susceptibility of the host. For this reason, co-evolution of infectious organisms with their hosts has been often compared to the "battle of two genomes" leading in general to infections with less deleterious consequences, which is after all the best way (if not the only way?) to ensure the survival of both organisms in the long term (Lengeling et al. 2001).

Investigations made with the aim of better understanding the genetic mechanisms that operate during the initial steps of infection are of major interest because they can provide information that may, in turn, help in the development of better strategies for fighting infectious diseases. Experiments of that kind, however, are not easy to perform because many parameters interfere with the experimental protocols, often making difficult the unambiguous delineation of "resistant" and "susceptible" phenotypes in a population of experimentally infected animals. In most cases, resistance or susceptibility to a pathogen depends on the complex interactions of multiple genes that control the host response. In a few cases, however, the situation is greatly simplified by the observation of clear-cut phenotypic differences between various inbred strains of laboratory mice after experimental infection. In this review we describe the historical case of genetic resistance to flaviviruses, the experiments that led to its elucidation at the molecular level, and the consequences of these discoveries.

The Pathogenicity of Flaviviruses

Flaviviruses are positive-sense, single-stranded, RNA viruses that are generally transmitted to warm-blooded animals through mosquito or tick bites. Many individuals exhibit flavivirus-specific antibodies, suggesting that infections by these viruses are mild or even unapparent, and revealing that some degree of adaptation has occurred between the virus and its host. In some other cases, however, flaviviruses can cause epidemic outbreaks in humans, and infected patients may exhibit a wide range of symptoms ranging from transient febrile illness to life-threatening hemorrhagic fevers (dengue and yellow fever) and meningo-encephalitis syndromes [Japanese encephalitis and West Nile (WN) fever]. The reasons why some flaviviruses cause severe clinical manifestations only in a small percentage of infected individuals are probably numerous and accordingly they have not yet been completely elucidated, but recurrent epidemiological observations and recent scientific data indicate that host-dependent genetic factors might be important.

Variations in innate flavivirus susceptibility in mice were reported for the first time in the early 1930s, and investigations performed during the following decades have fully confirmed these differences. By and large, one can consider that all laboratory

inbred strains of mice that have been tested so far, with the exception of strain PL/J, are susceptible to experimental infections while most wild mice or mice from inbred strains recently derived from wild progenitors are resistant. To mention just one example of this difference in susceptibility, we reported that a single intra-peritoneal inoculation, equivalent to 100 times the median lethal dose (LD_{50}) of the WN virus (strain IS-98-ST1), administered to adult mice of the classical laboratory inbred strains BALB/c or C57BL/6 was lethal for all the animals 9.5±1.5 days after inoculation, while mice from unrelated inbred strains recently derived from wild ancestors of either the *Mus musculus domesticus* (WMP/Pas), *Mus μ. musculus* (MAI/Pas, MBT/Pas, PWK/Pas), or *Mus spretus* (SEG/Pas, STF/Pas) species were totally resistant to the same treatment (Mashimo et al. 2002). During this experiment, infectious particles of WN virus could be detected in the brain of all infected mice after 5 days of infection, and the amounts of virus peaked at 10^9 focus forming units (FFU)/g of brain tissue by day 7 (Mashimo et al. 2002). High levels of anti-WN antibody could also be detected in surviving animals, indicating that the virus replicated in resistant strains. This experiment was just one of many experiments of the same kind performed over the past 40 years with a variety of flaviviruses, using several routes of inoculation and several doses and strains of virus. All these experiments yielded similar results, confirming that the phenotype of resistance/susceptibility is not WN-specific but, on the contrary, extends to other types of flaviviruses as well.

The resistance to flaviviruses was also demonstrated to be controlled by a major locus on chromosome (Chr) 5, designated flavivirus resistance (symbol *Flv*), with basically two alleles: Flv^r, which is dominant and induces resistance, and Flv^s, which is recessive and correlates with susceptibility. Most classical laboratory inbred strains are homozygous for the Flv^s allele. A third allele leading to "minor resistance" (Flv^{mr}) has also been found segregating in wild mice of the *M. m. molossinus* subspecies [for historical details on the discovery and genetics of the *Flv* locus, readers may refer to Brinton and Perelygin (2003)]. Congenic "resistant" strains have been produced by back-crossing for several generations the successive resistant offspring of an initial cross between resistant wild mice (any species) with a "susceptible" laboratory inbred strain. This classical breeding strategy allowed, at the same time, refinement of the genetic localization of the *Flv* locus on mouse Chr 5 and allowed the production of unlimited populations of "resistant" and "susceptible" mice with an otherwise similar genetic constitution, a very helpful material for experimentation.

Investigating the Molecular Basis of Susceptibility to West Nile Infection

Considering the relatively simple (monofactorial) genetic basis of WN resistance in the mouse and the "genomic" tools that became available after the genome sequencing effort in this species, we decided to embark on the positional cloning of the *Flv* locus. Readers who may be interested in reading our publication about the positional

cloning of *Flv* (Mashimo et al. 2002), must know that, since we had no evidence that the gene we were cloning was identical to *Flv* itself, we provisionally gave it another name (*Wnv* for WN virus—with two alleles Wnv^r and Wnv^s) even though we had little doubt that the two genes were presumably one-and-the-same entity. We now have molecular proof of this identity.. A first difficulty in this project arose when we found that, among the offspring of an intersubspecific backcross of the type (BALB/c×MBT/Pas)F1×BALB/c, which we expected to be a mixed population with 50% of the individuals being Flv^r/Flv^s and the other 50% being Flv^s/Flv^s, all mice heterozygous for Flv^r survived while not all mice with a Flv^s/Flv^s genetic constitution died as we would have expected. This is a good illustration of a major pitfall in this kind of experiment, where it is always risky or even impossible to trust in a "dead-or-alive" phenotype after an experimental infection, even if the latter is performed in the same highly standardized conditions. To bypass this difficulty and be able to achieve a high-resolution genetic mapping of the *Flv* locus, an absolutely necessary step in the positional cloning process, we derived a set of subcongenic mice by selecting, with the help of microsatellite markers, those offspring where a crossover event occurred that reduced the critical genetic interval containing the *Flv* locus. Offspring from these mice (all of the same genetic constitution) were challenged with a standardized dose of virus and finally classified as "resistant" or "susceptible." This rather tedious procedure allowed us to localize, with a very high degree of confidence, the *Flv* locus within an interval flanked by markers *D5Mit408* and *D5Mit242*, which is roughly equivalent to 300 kb of DNA (Fig. 1).

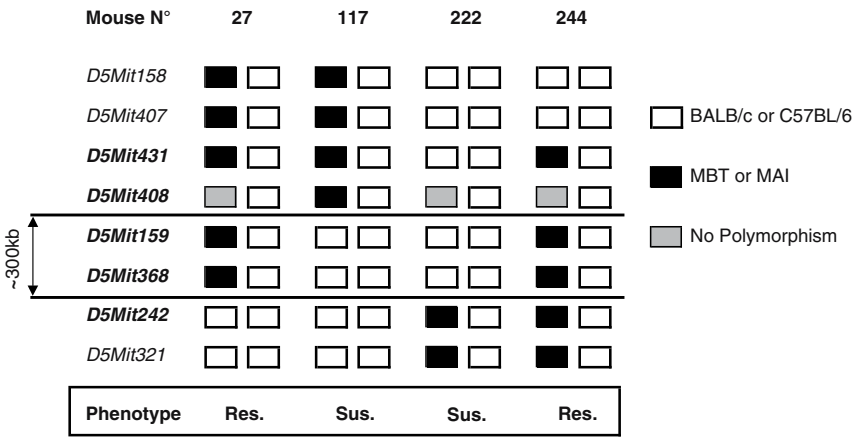


Fig. 1 Over 350 offspring from two intersubspecific backcrosses [(BALB/c×MBT)F1×BALB/c or (C57BL/6×MAI)F1×C57BL/6] were raised and genotyped for the region of Chr 5 flanking the locus for flavivirus resistance and four mice were found with a recombinant haplotype in the critical region (Nos. 27, 117, 222, and 244). These recombinant mice were then mated with either BALB/c or C57BL/6 susceptible partners, and around 30/50 progenies of these crosses were challenged with West Nile virus and classified as "susceptible" or "resistant." This protocol, because it involves a rather large sample of animals with exactly the same genotype, is highly reliable for the purpose phenotyping based on a dead-or-alive phenotype

This genetic interval contains about 30 genes whose sequence and expression pattern are well known (Fig. 2). Among these genes, the cluster encoding the interferon inducible oligoadenylate synthetases (*Oas1*, *Oas2, Oas3* etc.) appeared top-ranked in the list of candidates for reasons that will be explained later. We then decided to have a more careful look at the sequence of these genes in both "resistant" and "susceptible" mice. We observed several single nucleotide polymorphisms (SNPs) among the different strains or species studied, a finding that was not surprising considering the polyphyletic origins of the laboratory inbred strains of mice (Wade et al. 2002). We found it remarkable, however, that in one of the elements of the cluster, namely the *Oas1b* gene, all susceptible mice had a T→C transition in the fourth exon of this gene, replacing an arginine residue with a premature stop codon. The perfect and absolute correlation between susceptibility to viral infection and the occurrence of a stop codon was observed independently in two laboratories (Mashimo et al. 2002; Perelygin et al. 2002) and supported the hypothesis that a truncated, and presumably inactive form of 2´-5´OAS is indeed causative of the innate susceptibility to flavivirus infection. The presence of a stop codon is also compatible with susceptibility behaving as a fully recessive trait and fits perfectly with one of the known functions of the interferon inducible enzyme 2´-5´OAS. In addition to this absolute phenotype/genotype correlation, it has also been reported that a flavivirus-resistant phenotype could be restored in a susceptible mouse strain by replacing the 3´ portion of the susceptible *Oas1b* sequence in 129/SvJ/RW4 ES cells, by homologous recombination with a 129/SvJ/RW4 DNA sequence containing four substitutions characteristic of the *Oas1b* resistance allele, in particular a reversion from TGA to CGA (Scherbik et al. 2007).

The Molecular Organization and Evolution of the *OAS* Gene Family in Mammals

2´-5´OAS are a relatively homogeneous family of enzymatic proteins with a remote evolutionary origin, since molecules with a similar structure have been identified in a wide range of species including most mammals and birds, and even the marine sponge *Geodia cydonium* (Cayley et al. 1982; Wiens et al. 1999; Yamamoto et al. 1998). In the species where the molecular organization has been studied in detail, the genes encoding 2´-5´OAS have been found to be clustered, with variations in gene copy numbers (orthologs and paralogs) among the different species, indicating that rapid evolutionary changes occurred in these regions. In this section, we will summarize the most recent findings with reference to the corresponding publications.

The *human* cluster is the simplest with only three genes *OAS1*, *OAS2*, and *OAS3* within a 130-kb stretch of Chr 12 (12q24.13) (Hovnanian et al. 1998). These three genes share the same order of transcription and are arranged on the chromosome in the following order: centromere 5´–*OAS1*–*OAS3*–*OAS2*–3´ (Hovanessian and Justesen 2007). The size of these genes is relatively short (~12 kb for *OAS1*; ~36 kb for both *OAS2* and *OAS3*) and analysis of their sequence reveals the presence of a conserved domain of five exons (the first 346-amino acid residues of *OAS1*), designated

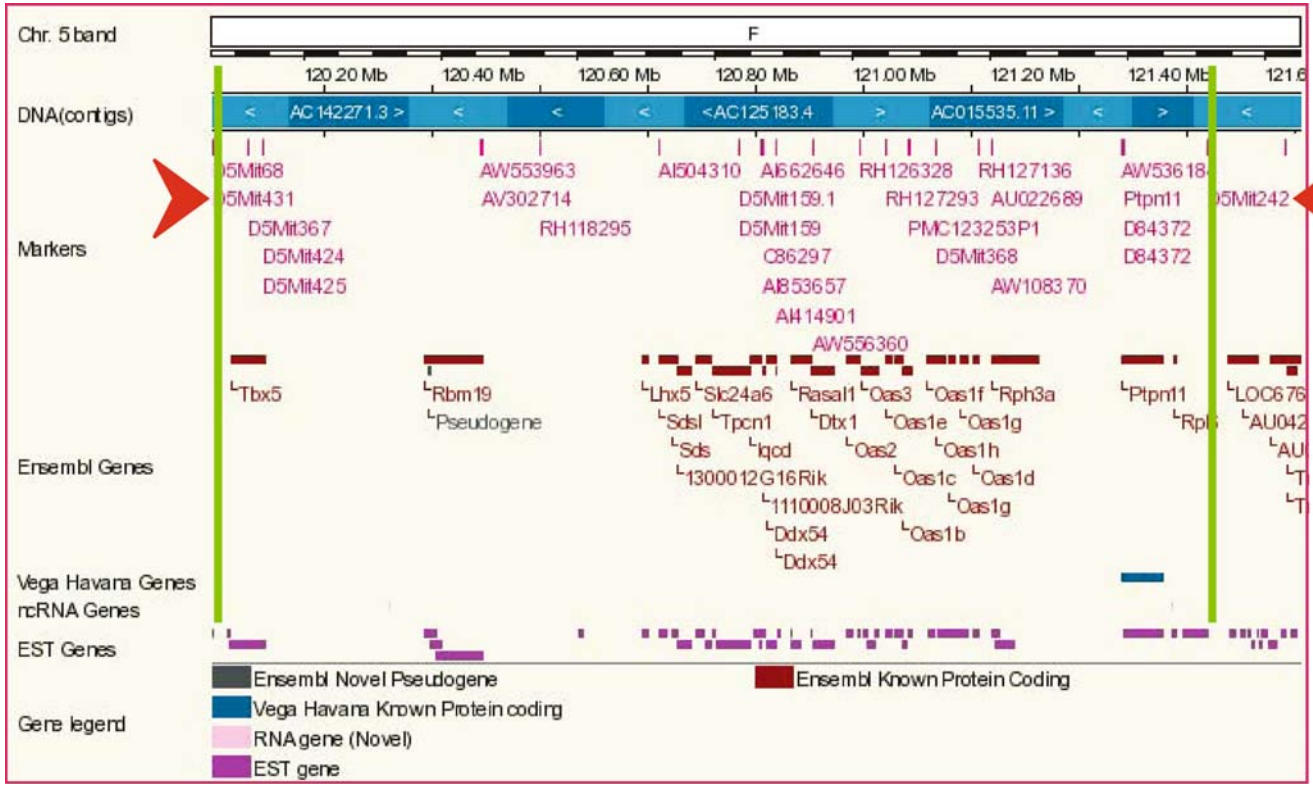
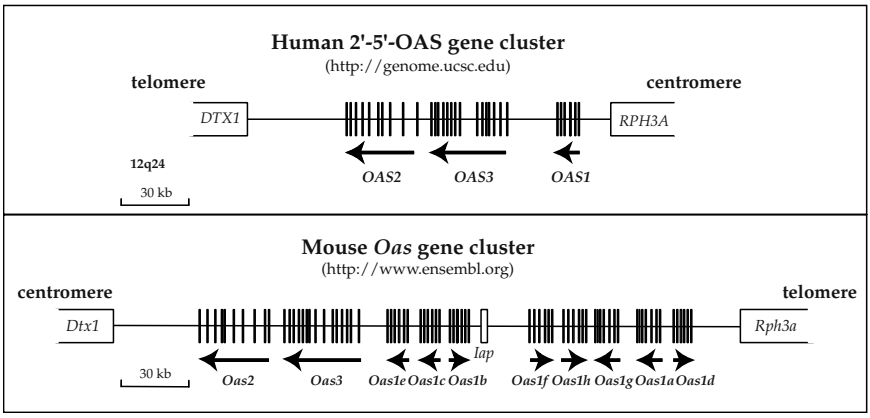


Fig. 2 This figure, from the *Ensembl* database, represents a segment of mouse Chr 5 in the critical region between markers *D5Mit431* and *D5Mit242* and shows the approx. 30 genes that are in the region. In this sequence (released after the positional cloning of *Flv*) the genes *Oas2*, *Oas3*, and the eight genes in the *Oas1* cluster are obvious. From the knowledge we have of their function, they appear as top-ranked candidates in the determinism of susceptibility to flaviviruses

the 2´-5´OAS unit, with one copy in *OAS1*, two copies in *OAS2*, and three copies in *OAS3*. This organization and sequence homology suggest that the *OAS1* gene is probably the ancestral gene, the other two genes being derived after duplication or triplication of this ancestral gene. The promoter region of the three genes contains an interferon-stimulated response element (ISRE), which is consistent with the fact that most of the 2´-5´OAS proteins are interferon (IFN)-inducible enzymes. *OAS1* is transcribed in four isoforms of 42, 44, 46, and 48 kDa respectively, depending on alternative splicing of exons 5 and 6. These isoforms, which have identical amino-termini but different carboxyl-termini, may have different functions. Formation of a human OAS1 tetramer is essential for the catalytic activity of the protein (Torshin 2005). *OAS2* is also spliced in two isoforms of 69 and 71 kDa that share a common amino-terminus of 683 residues, with extensions of 4 and 44 amino acids, respectively. *OAS3* encodes a unique protein of 100 kDa (Hovanessian and Justesen 2007; Justesen et al. 2000; Rebouillat and Hovanessian 1999; Fig. 3).

Aside from *OAS1*, *OAS2*, and *OAS3*, another gene—identified by screening a cDNA expression library with anti-OAS3 polyclonal antibodies (Rebouillat et al. 1998) and by screening an EST library (Hartmann et al. 1998)—with a sequence similar to the one of *OAS1*, although somewhat bigger, was discovered. This gene encodes a 56-kDa protein that differs from the other OAS proteins by an approx. 160-amino acid extension at its C-terminus, which has homology to the interferon-inducible protein ISG15 and also has a tandem repeat of two ubiquitin-like domains (Hartmann et al. 1998). This protein is devoid of 2´-5´OAS activity and accordingly was named OASL for "OAS like" protein. The human *OASL* gene exhibits a high degree of homology with the chicken *OAS* gene, but the latter encodes a highly active 2´-5´OAS (Torshin 2005).

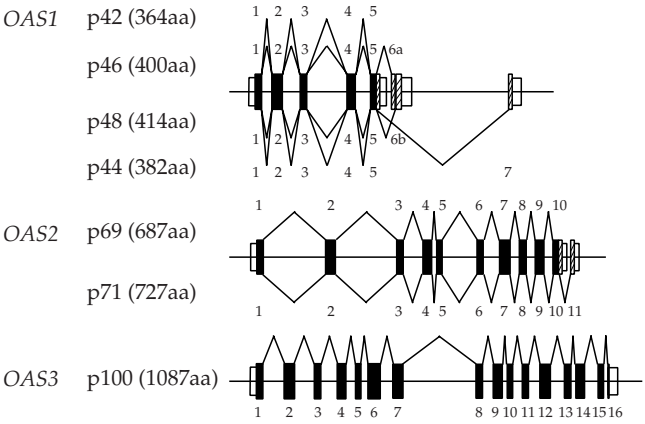


a

Fig. 3a A schematic organization of the OAS cluster in human and in mouse. Human *OAS1*, *OAS2*, and *OAS3* are paralogous copies of an ancestral gene. The mouse has at least eight orthologous copies of the human *OAS1*, some of them likely being nonfunctional pseudogenes (updated and modified from Mashimo et al. 2003)

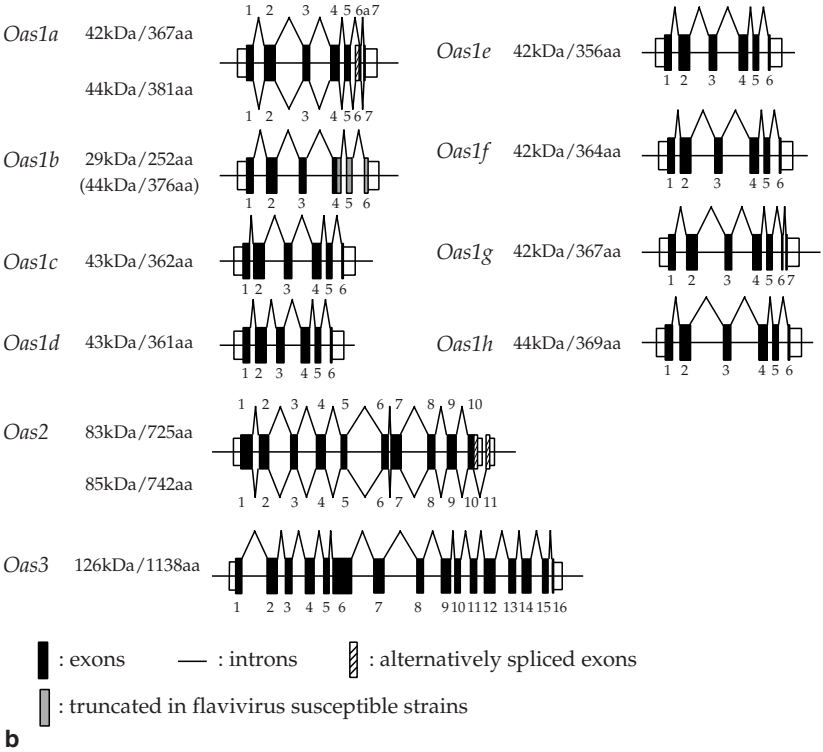
Human OAS transcripts

[Sarker *et al.* 1998, Rebouillat *et al.* 1999, Justesen *et al.* 2000]



Mouse Oas transcripts

[Mashimo *et al.* 2003]



b

Fig. 3 b The transcription pattern of the different genes in the mouse *Oas* cluster. All these genes are transcribed, but with minor tissue-specific variations. Among the different genes of the mouse *Oas1* cluster, only *Oas1a* is alternatively transcribed. *Oas1a* and *Oas1g* have an extra seventh exon.

The *mouse Oas* cluster is the second most well known after human. It contains one *Oas2* and one *Oas3* gene, and ten *Oas1* genes (or pseudogenes?) designated *Oas1a* to *Oas1j* (Eskildsen et al. 2002; Kakuta et al. 2002; Mashimo et al. 2003; Perelygin et al. 2006; Figs. 2 and 3). These 12 genes are arranged in tandem within a stretch of DNA that spans approximately 230 kb of mouse Chr 5. The segment harboring the *Oas* cluster (and a few other flanking genes) is inverted when compared to the human configuration (Mashimo et al. 2003). Mouse *Oas2/Oas3* genes have a genomic structure very similar to the one of human, and a very similar pattern of transcription even if the transcription products are somewhat bigger in the mouse (725 and 742 amino acids compared to 687 and 727 for OAS2; 1,138 amino acids compared to 1,087 for OAS3). Here again, based on their sequences and orientation, one can guess that these 12 genes result from the duplication, in several steps, of an ancestral copy after divergence of the primates and rodents phyla. RT-PCR performed with a set of specific primers on RNA extracted from several adult and fetal tissues, either before or after induction with double-stranded RNA (dsRNA), indicated that nine genes of the *Oas1* family (*Oas1a* to *Oas1i*) are transcribed and some variations can be observed in the different patterns of expression. According to Mashimo and coworkers (2003) each of the nine *Oas1* genes exhibits a unique battery of transcriptional regulatory elements, suggesting that each of these units has the potential to be differentially regulated. So far only *Oas1a* has been found to be alternatively spliced yielding two transcripts each including different parts of exon 6 (Kakuta et al. 2002; Mashimo et al. 2003; Fig. 3). Two genes, homologous to the human *OASL*, have also been identified in the mouse: *Oasl1* and *Oasl2*. Analysis of their structure indicates that they are probably nonfunctional pseudogenes.

Twelve 2′-5′OAS (*Oas*) genes were identified in the *rat* genome (Chr 12) (Mashimo et al. 2003), including eight *Oas1* genes (the orthologs of mouse *Oas1a* and *Oas1e* are missing in this species, while two additional isoforms are present: *Oas1k* and *Oas1l*). Two *Oas1* pseudogenes, a single *Oas2* and a single *Oas3*, and two *Oas*-like genes, *Oasl1* and *Oasl2*, are also present. The structure and organization of the rat cluster is very similar to the one of the mouse, which is no surprise.

The structure of the 2′-5′OAS cluster has also been investigated in detail in four other mammalian species (pig, dog, cow, and horse) and in chicken (Perelygin et al. 2005, 2006). Four *OAS* genes (*OAS1X*, *OAS1Y*, *OAS1Z*, and *OAS2*) plus a single copy of *OASL1* were detected in the cow genome. The cluster in the pig genome is similar to the *OAS* cluster of cattle although *OAS1Z* is absent. Remarkably, the orthologous copy of mouse/human *OAS3* is not found in either the pig or the cow genomes. The dog and horse clusters are also similar and are more like the human cluster than either the mouse or rat clusters. Dog and horse have three copies of the

Fig. 3 b (continued) Alignment of the predicted amino acid sequences for the proteins encoded by the eight *Oas1* genes indicates that *Oas1c*, *Oas1d*, *Oas1e*, *Oas1f*, and *Oas1h* are structurally similar and lack several functional domains. These observations suggest that these isoforms may actually be inactive pseudogenes rather than real isoforms. In contrast, *Oas1g* and *Oas1a* encode proteins that could be functional in the 2′,5′OAS/RNase L cascade. Note that for both **a** and **b** the scale for exons is five times larger than for introns

human/mouse *OAS3* ortholog (*OAS3C*, *OAS3M*, and *OAS3N*), two copies of *OAS2* (*OAS2N* and *OAS2C*) and a single copy of the human *OAS1* ortholog. However, while the dog has two copies of the *OASL* orthologous gene (*OASL1* and *OASL2*), the horse has only one. Two tandemly duplicated OAS-like (*OASL*) genes were identified in the dog genome but only a single *OASL* ortholog was found in both the cattle and the pig genomes. The bovine and porcine *OASL* genes contain premature stop codons and encode truncated proteins, which lack the typical C-terminal double ubiquitin-like domains. Evidence of concerted evolution of all these paralogous *2´-5´OAS* genes was obtained in rodents (*Rodentia*) and even-toed ungulates (*Artiodactyla*) (Perelygin et al. 2006).

The Functions of the OAS Molecules

The Antiviral Functions of OAS1 Molecules

The best known molecules among those that are encoded by the 2´-5´OAS cluster are described as interferon-induced enzymes that polymerize ATP into 2´-5´ oligomers of adenosine with the general formula pppA $(2´p5´A)_n$. These enzymes are activated by binding to double-stranded RNA, and their products, the 2´-5´ oligoadenylates, activate the latent endoribonuclease RNase L that finally degrades viral or cellular RNA molecules (Hovanessian and Justesen 2007). This is how the antiviral activity of these enzymes is generally explained. Several experiments have confirmed this crucial function in the innate, antiflavivirus mechanism of defense in the mouse (Kajaste-Rudnitski et al. 2006; Lucas et al. 2003) although some experiments suggest that RNase L activation is not a major component of the OAS1B-mediated flavivirus resistance phenotype (Scherbik et al. 2006).

Sequence alignment of human OAS1, OAS2, and OAS3 reveals the presence of highly conserved stretches of 7–14 amino acids among which the pentapeptide D-F-L-K_{199}-Q has been reported to represent a part of the ATP binding site, while K_{199} inside this pentapeptide seems to be essential for catalytic activity (Justesen et al. 2000; Rebouillat and Hovanessian 1999). In the mouse, interferon is also an inducer of the different OAS molecules, and alignment of the predicted amino acid sequences for the proteins encoded by *Oas1c*, *Oas1d*, *Oas1e*, *Oas1f*, *Oas1h*, *Oas1i*, and *Oas1j*—although structurally very similar—lack some essential functional domains, such as the LXXXPA motif (Ghosh et al. 1997), the highly conserved aspartic acid residues in exon 2 (Sarkar et al. 1999), and the CFK motif (Ghosh et al. 1997). These observations suggest that, although these isoforms have retained their binding activity to dsRNA, they have lost their Mg^{2+}-dependent catalytic activity and accordingly are most probably inactive pseudogenes rather than genes encoding a protein with 2´-5´OAS activity (Sarkar et al. 1999; Shibata et al. 2001). Another possibility that should be kept in mind would be that these isoforms, encoded in the above-mentioned genes, have acquired other functions. The three

genes, *Oas1g*, *Oas1a*, and of course *Oas1b*, encode proteins that have been proved (or are likely) to be functional in the 2´-5´OAS/RNase L cascade.

The suspected role of the OAS1B isoform in the innate mechanisms of defense in the mouse, hypothesized after experimental infection of mice with flaviviruses and positional cloning of the *Flv* locus, has been confirmed by the production of a knock-in, as already mentioned. It has also been confirmed by experiments performed in vitro in which stable neuroblastoma cell clones overexpressing either the mutant or wild-type OAS1B were infected with WN virus. These experiments indicated that viral replication is less efficient in cells that produce the normal copy of OAS1B than in those expressing the mutant form of the protein (Lucas et al. 2003). The experiments have been confirmed and reinforced by other experiments performed on genetically engineered fibroblasts that could upregulate OAS1B protein expression under the control of the *Tet-Off* expression system (Kajaste-Rudnitski et al. 2006).

The role played by the OAS1B isoform of 2´-5´OAS in the innate mechanisms of defense of the mouse against flavivirus infection now seems firmly established. In human and other mammalian species, however, the role of 2´-5´OAS in viral pathogenesis is much less clear. According to Perelygin and colleagues, the flavivirus-specific activity of the mouse OAS1B isoform on flaviviral replication might be correlated with a 4-amino acid deletion in the P-loop motif that is unique to this isoform and does not appear to exist in human (Perelygin et al. 2002). This 4-amino acid deletion (12 bp) might be of special importance for the OAS1B protein to specifically interact with the ATP substrate if one considers recent data from the crystalline structure of the porcine OAS1 enzyme (Hartmann et al. 2003). Even if this hypothesis is supported by other experiments in vitro (Urosevic et al. 1999), an alternative explanation for the specific activity of OAS1B on flavivirus replication might be found in its promoter sequence, where several binding sites [for NF-κB, GAS, and interferon (IFN)-stimulated specific response element (ISRE)] exhibit a unique organization. In particular, it is noteworthy that OAS1B is the only gene where the NF-κB and ISRE binding sites are closely associated in tandem, producing a genomic structure that has previously been reported as capable of triggering gene expression upon viral induction (Cheng et al. 1998). Sequencing the promoter regions of the OAS1B isoform in remotely related mouse species did not provide evidence that some particular structural changes in this promoter might be associated with the phenotype of resistance or susceptibility after flavivirus infection. This supports the hypothesis that the stop codon found in the OAS1B coding sequence of most laboratory strains, which is the only obvious structural difference between susceptible and resistant genotypes, indeed is directly related to this phenotype. The 4-amino acid deletion in the P-loop motif has not been found in the orthologous region of the rat OAS1B isoform but does occur in rat OAS1F. The rat, like wild mice, seems to be naturally resistant when naturally infected (Eldadah et al. 1967).

Two recent observations arguing in favor of genetic control of human susceptibility to flavivirus infections have been published recently. The first, by Bonnevie-Nielsen and colleagues, reports a significant correlation between the basal activity of OAS1 and an A/G SNP at the exon 7 splice-acceptor site (AG or AA) of the OAS1 gene (Bonnevie-Nielsen et al. 2005). According to these authors, in a cohort

of 83 families each containing two parents and two children, allele G had a higher frequency in people with high enzyme activity than in those with low enzyme activity, with the activity being related to this polymorphism in a dose-dependent manner across the GG, GA, and AA genotypes. Allele G generates the p46 enzyme isoform, whereas allele A ablates the splice site and generates a dual-function antiviral/pro-apoptotic p48 isoform and a novel p52 isoform. The discovery of this genetic polymorphism and of its influence on host susceptibility to flavivirus infections clearly underlines the likely importance of OAS1 in the innate mechanisms of defense.

The second observation was made in a survey performed on 33 individuals hospitalized with WN virus infection. The survey was designed to assess whether a structural change could be detected in the *OAS* genes of patients with a clinically severe form of the disease. Sequence comparisons between case patients and control subjects identified 23 SNPs, including a synonymous SNP in *OASL* exon 2 in which the reference allele occurred at a higher frequency in case patients ($p<.004$). According to the authors, the RNA transcripts generated from this allele may undergo increased splicing, resulting in a dominant-negative OASL isozyme similar to the nonsense/truncation mutant form of *Oas1b* in mice (Yakub et al. 2005). These two reports, although preliminary, are indicative of a possible role for the OAS1 molecules in human mechanisms of defense.

Although the role of the OAS1 molecules in innate immunity against flavivirus infections is now established, at least in the mouse, several experiments indicate that this resistance, unlike resistance to myxovirus associated with the *Mx* locus (Haller et al. 1998), does not require induction by interferon (Brinton and Perelygin 2003). This observation is totally consistent with the observation that plants transgenic for 2´-5´OAS family and for the gene encoding RNase L, were found to be resistant to experimental infections with a number of viruses such as tobacco mosaic virus, cucumber mosaic virus, and potato virus Y (Honda et al. 2003; Mitra et al. 1996; Ogawa et al. 1996).

Finally, and again concerning the *Oas1b* gene of the mouse, a likely hypothesis to account for the presence of the same stop codon in virtually all laboratory strains is that all these strains inherited the same segment of Chr 5 from a common ancestor. Such a situation is not uncommon among mouse laboratory strains and was also observed by Staeheli and colleagues when they investigated the genetic basis of susceptibility to orthomyxovirus infection (Staeheli et al. 1986). However, whether this occurred by chance only or under some sort of selective pressure is an open question. Nonetheless, it is also clear that the use of inbred strains derived from wild specimens of different species might be a rich source of information for investigating the genetic basis of resistance/susceptibility to infectious diseases.

Aside from their well-established role in the pathology generated by flavivirus infections, 2´-5´OAS molecules (and more specifically those encoded by *OAS1*) may be involved in the outcome of diseases generated by coronaviruses or hepaciviruses. Two independent surveys indicated that SNPs in the *OAS1* gene (more precisely in exons 3, 6 or in the 3´-UTR region) were associated with severe acute respiratory syndrome (SARS) susceptibility in Vietnamese or Chinese Han populations (Hamano et al. 2005; He et al. 2006). Another study suggested that a polymorphism

in the 3´-UTR of the *OAS1* gene was significantly associated with a higher frequency of self-limiting infection in patients with hepatitis C (Knapp et al. 2003). It is likely that with time, more associations between *OAS1* polymorphisms and resistance to viral infections will be discovered.

The Other Functions of OAS Molecules

Situations where different mammalian genomes harbor orthologous genes with a variable number of copies are not uncommon and it was suggested that such variations are the result of different selective environmental pressures experienced by the ancestors of modern rodents and primates. While infectious agents in natural environments certainly play an important role in natural selection (the "battle of two genomes"), however, the number and range of pathogens is not very different among the different mammalian species. It therefore makes sense to guess that the different OAS molecules have cellular functions other than the one made obvious by the accidental discovery of differential flavivirus resistance in the mouse species. In humans, for example, *OAS1*, *OAS2*, and *OAS3* appear to be differentially induced by interferon, induced in different types of cells, and for some of them expressed even in healthy individuals, which is indicative of an eventual role under physiological conditions and not only after infections. They are also characterized by different subcellular locations. Some OAS proteins might have as-yet-unidentified catalytic activities, suggesting that they may have distinct roles in the cell. In fact, 2´-5´OAS molecules have now been demonstrated to be involved in other cellular processes such as cell growth and differentiation, gene regulation, and apoptosis (Hovanessian and Justesen 2007).

Some polymorphisms at the *OAS1* locus have been reported to be associated with a variety of human pathologies. This is the case, for example, for a SNP generating an A/G splice-site in *OAS1*-exon 7, which was found to be associated with a protective effect against type 1 diabetes (Field et al. 2005). This observation was later disputed (Smyth et al. 2006) but another polymorphism in the same gene, generating a serine/glycine substitution resulting in a functional variant, was reported as a more likely cause for the observed association with type 1 diabetes (Tessier et al. 2006). Similarly, SNPs detected in exons 3 and 7 of *OAS1* demonstrated an association with risk for multiple sclerosis in 333 patients and 424 healthy controls, suggesting that OAS1 activity is involved in the etiology of this disease (Fedetz et al. 2006).

In the mouse species, with the unlimited possibilities of genetic engineering in embryonic stem (ES) cells in vitro, a comprehensive survey of the different functions of the OAS molecules should be undertaken in the forthcoming years, for example by knocking out each and every gene of the cluster. Although of importance, making alterations in the coding sequences of these genes in order to assess their function(s) would not necessarily require that the stop codon in *Oas1b* exon 4 be "repaired" in advance. Yan and colleagues, for example, demonstrated that mutant mice lacking OAS1D (*Oas1d*$^{-/-}$) displayed reduced fertility due to defects in ovarian follicle development,

decreased efficiency of ovulation, and arrest at the one-cell stage of fertilized eggs (Yan et al. 2005). This was indeed a totally unexpected function for a protein exhibiting a very high degree of similarity with OAS1B.

As we already noted, the *2´-5´OAS* family of genes exhibits both an evolutionarily ancient origin and wide variations in the number of copies between species. Experimental data collected after experiments on mouse flavivirus susceptibility and preliminary observations made in humans suggest that the cluster in question is important for the maintenance of cellular homeostasis since evolutionary (environmental) forces contribute to its "shaping" (Godfrey et al. 2004). Since no sequences related to *2´-5´OAS* genes could be identified in either *Caenorhabditis elegans* or in *Drosophila melanogaster*, however, it seems that the OAS cluster is either not absolutely fundamental for cell physiology or that it is replaced by another structure with similar functions in other developed organisms.

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Cmv1 and Natural Killer Cell Responses to Murine Cytomegalovirus Infection

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Contents

Abstract The dissection of genetic resistance to murine cytomegalovirus infection in inbred laboratory mouse strains led to the identification of a natural killer cell activation receptor that recognizes a virus-encoded protein. Herein, we summarize the genetic approach and findings that have provided novel insights into innate immune control of virus infections.

Abbreviations CMV: Cytomegalovirus; DAP12: DNAX activating protein of 12 kDa; Δm157: MCMV clone lacking m157 expression; ENU: *N*-Ethyl-*N*-nitrosourea; GFP: Green fluorescent protein; HCMV: Human CMV; Ig: Immunoglobulin;

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ILT: Ig-like transcripts; ITAM: Immunoreceptor tyrosine-based activation motif; ITIM: Immunoreceptor tyrosine-based inhibitory motif; KARAP:Killer associated receptor adapter protein; KIR: Killer Ig-like receptor; LCMV: Lymphocytic choriomeningitis virus; MCMV: Murine CMV; mAb: Monoclonal antibody; MHC: Major histocompatibility complex; NK: Natural killer; NKC: NK gene complex; ORF: Open reading frame; RI: Recombinant inbred; SCID-MCMV: MCMV clones isolated from *scid* mice; TLR: Toll-like receptor

Introduction

Natural killer (NK) cells constitute the third major population of lymphocytes that can be distinguished from other lymphocytes by the absence of the T and B cell antigen receptor complexes (Yokoyama 2008). Although initially discovered because of their "natural" ability to kill tumor cells without prior sensitization, NK cells are now known to participate in the early, innate immune response to infection. This is best illustrated by case reports of human patients with selective NK cell deficiency. These patients have in common a propensity to severe, disseminated, and recurrent herpesvirus infections, including cytomegalovirus (CMV) (Biron et al. 1989; Jawahar et al. 1996), strongly suggesting that NK cells play important roles in host defense against pathogens, especially herpesviruses.

CMV are β-herpesviruses (Mocarski and Courcelle 2001), which are large-enveloped, double-stranded DNA viruses. Due to species tropism, each mammalian host has a characteristic CMV; human CMV does not replicate in mice. The β-herpesviruses have diverged somewhat but are most closely related in sequence to each other than to other herpesviruses. Murine CMV (MCMV) has a 230-kb genome, similar in structure to human CMV, and characteristically contains many open reading frames (ORFs) for viral molecules that interact with the host (Rawlinson et al. 1996). Moreover, MCMV has similar properties to human CMV during in vivo infections and thus is a particularly useful model pathogen for study of host-pathogen interactions. After systemic inoculation (typically via intraperitoneal route), MCMV causes an initial acute systemic infection phase with readily detectable viral replication, and is characterized by innate immune responses (Biron 1994, 1999) followed by viral latency during which infectious virus is below detection limits. As with other herpesviruses, the host then sheds the virus for its lifetime during periods of viral reactivation (Mocarski and Courcelle 2001).

As for mouse NK cell responses to MCMV, classic studies involved depletion of NK cells in vivo by administration of a monoclonal antibody (mAb) against NK1.1—the most specific serological marker on $CD3^-$ NK cells in C57BL/6 mice—or other anti-NK cell antibodies (Bukowski et al. 1983; Bukowski et al. 1984; Welsh et al. 1990). When mice were then infected with MCMV, lethality and marked viral replication in internal organs was evident. Elevated viral titers were especially prominent in the spleen. If anti-NK cell antibody administration was delayed for a few days, there was no effect, indicating that NK cells are important in the early host immune response against viruses.

NK cells are activated by two major stimuli, cytokines and targets, and are capable of two major effector responses, cytokine production and target killing (Yokoyama 2008). In MCMV infections, NK cells can respond to interleukin (IL)-12 that is produced by macrophages and related cells, such as dendritic cells (DCs); IL-12 can stimulate NK cell production of interferon (IFN)-γ (Andoniou et al. 2005; Biron et al. 1999). In addition, through Jak/Stat pathways, other cytokines, such as IL-2, IFN-α, and IFN-β, stimulate NK cells to proliferate, produce IFN-γ and tumor necrosis factor (TNF)-α, and enhance their killing. Although cytokine-stimulated effector responses are important in MCMV infection (Orange et al. 1995; Orange and Biron 1996), most responses to cytokines are also seen in lymphocytic choriomeningitis virus (LCMV) infection even though NK cell depletion does not alter viral replication or survival (Biron et al. 1999; Bukowski et al. 1983, 1985). Thus, some of these effects may be bystander responses, suggesting that other effector mechanisms, especially killing, may be required for NK cell-mediated antiviral immunity.

Classically, NK cells kill their targets by the triggered release of preformed cytoplasmic granules containing perforin and granzymes, a process termed granule exocytosis (Henkart 1994), leading to target apoptosis. NK cells can kill certain targets through other means, including Fas and TNF-related apoptosis-inducing ligand (TRAIL), but the functional, especially in vivo, relevance of these pathways in antiviral innate defense is less clear (Zamai et al. 1998). In addition, resting NK cells apparently do not normally express Fas ligand, and must be triggered to mediate Fas-induced death (Bradley et al. 1998). Moreover, perforin-deficient mice have a defect in control of MCMV, implicating NK cell perforin-dependent killing (Loh et al. 2005; Tay and Welsh 1997). Thus, NK cell activation by their targets is a critical element, raising the issue that NK cell receptors specific for virus-infected cells may be important in infection control.

In this review, we will illustrate how a genetic approach was extremely valuable in elucidating the mechanisms by which NK cells recognize virus-infected cells.

Target Recognition by NK Cells

To further appreciate the possibility that NK cells may directly recognize virus-infected cells, it is useful to review the general topic of target recognition by NK cells that is regulated by target cell expression of major histocompatibility complex (MHC) class I molecules (Yokoyama 2008). Targets lacking MHC class I, occurring as a result of viral infection for example (Tortorella et al. 2000), are more susceptible to NK cell lysis than targets with normal levels of MHC class I molecules. This led Kärre to propose the "missing-self" hypothesis whereby NK cells survey tissues for normal expression of MHC class I that somehow prevents NK cell activation (Ljunggren and Kärre 1990). In the absence of MHC class I, NK cells are released to kill the target. This process is mediated through NK cell receptors that specifically bind MHC class I molecules and inhibit NK cell killing. There are two structural types of inhibitory receptors for MHC class I: (1) type I integral membrane proteins with immunoglobulin (Ig)-like domains, such as the human

killer Ig-like receptors (KIR) and Ig-like transcripts (ILTs); and (2) type II integral membrane proteins with external lectin-like domains, including the mouse Ly49 family, and mouse and human NKG2/CD94 receptor. Regardless of structural type, inhibition occurs through motifs in the cytoplasmic domains of the inhibitory receptors, termed immunoreceptor tyrosine-based inhibitory motifs (ITIMs). When the ITIMs are tyrosine phosphorylated, they recruit and activate the cytoplasmic tyrosine phosphatase SHP-1, which then dephosphorylates molecules involved in NK cell activation. It is important to note, however, that the absence of MHC class I (and thus no inhibition) does not automatically lead to NK cell activation against cellular targets because activation requires engagement of activation receptors. Thus, NK cell responses to cellular targets are regulated by an interplay between inhibitory and activation receptors.

Transcripts for many receptors related to the inhibitory receptors were identified by cross-hybridization in cDNA library screenings (Yokoyama 2008). Whereas the deduced receptors were highly related to the inhibitory receptors in terms of amino acid sequence homology and domain structure, these receptors could not be inhibitory because they lack cytoplasmic ITIMs. Instead, many were found to be activation receptors that have charged transmembrane residues allowing association with transmembrane signaling chains, such as DAP12 (Lanier et al. 1998), also known as KARAP (killer cell-activating receptor-associated polypeptide) (Olcese et al. 1997). These signaling chains contain immunoreceptor tyrosine-based activation motifs (ITAMs) similar to those in the signaling chains of the T and B cell receptor complexes, allowing the putative ligand-binding activation receptors to deliver positive signals for NK cell activation. The physiological function of most putative activation receptors, however, remained elusive.

NK cell activation receptors were likely to be physiologically important in antiviral defense because several viruses, notably members of the herpesvirus family, encode proteins that interfere with natural killing (Cohen et al. 1999; Cosman et al. 1997; Farrell et al. 1997; Ishido et al. 2000; Reyburn et al. 1997; Tomasec et al. 2000; Ulbrecht et al. 2000). For example, human CMV (HCMV) encodes an MHC class I-like molecule (UL18) that interacts with ILT-2 (also known as LIR-1, leukocyte inhibitory receptor-1), an Ig-like inhibitory receptor on NK cells. HCMV also encodes a peptide (UL40) that binds HLA-E, an MHC class Ib molecule that typically binds only leader peptides derived from MHC class Ia molecules. The HCMV peptide enhances expression of HLA-E that in turn is recognized by CD94/NKG2A, a C-type lectin-like NK cell inhibitory receptor. Thus, HCMV produces molecules that bind both structural types of inhibitory receptors.

NK cell inhibitory receptors prevent in vitro killing by blocking signals from activation receptors (Long 1999; Ravetch and Lanier 2000), and the transfected expression of the HCMV molecules blocks killing of tumor targets (Cosman et al. 1997; Tomasec et al. 2000; Ulbrecht et al. 2000). The inhibitory receptors, however, should not block cytokine receptor signaling. These viral evasion strategies therefore strongly suggested that NK cells could mediate in vivo antiviral defense with activation receptors that are related to those involved in tumor killing, but it had been especially challenging to identify such activation receptors until a genetic approach was undertaken.

Early Studies of Genetically Determined Resistance to MCMV

Differences in mouse strain-dependent resistance to lethal MCMV infection were demonstrated in studies by Selgrade and Osborn (1974). More systematic investigations by Chalmer and colleagues (1977) using *H2* congenic mice on the BALB/c genetic background indicated that *H2* loci, and in particular the $H2^k$ haplotype, conferred resistance to MCMV. The conclusion that the $H2^k$ haplotype contributed a major resistance phenotype to MCMV was corroborated by follow-up studies using *H2* congenic mice on the BALB/c, C57BL/10, C3H and A backgrounds (Grundy et al. 1981). In this same study Grundy (Chalmer) and colleagues also observed, based on studies with (C57BL×BALB/c)F_1 hybrids, that non-*H2* loci on the C57BL genetic background conferred dominant resistance to MCMV. An assessment of MCMV replication in the spleens of the relatively resistant C57BL/6 strain and the susceptible $H2^b$-matched BALB.B strain revealed greater than 100-fold higher titers at days 2 and 3 after infection in the BALB.B strain (Allan and Shellam 1984). This provided the first clue that non-*H2* loci play an important role in regulating viral replication early after acute infection. Other studies provided evidence implicating a role for non-*H2* loci in the control of early interferon production (Allan and Shellam 1985; Grundy et al. 1982; Quinnan and Manischewitz 1987) and NK cell responses (Bancroft et al. 1981; Bukowski et al. 1984; Shellam et al. 1981, 1985) following MCMV infection, but did not define the number or chromosomal locations of the genes involved.

Cmv1 and NK Cell Resistance to MCMV

Defining the Cmv1 Locus

The observations that C57BL non-*H2* loci both provided dominant protection against lethal MCMV challenge (Grundy et al. 1981) and contributed to a greater than 100-fold reduction in viral replication in vivo relative to the BALB/c genetic background (Allan and Shellam 1984) provided a powerful phenotypic "window" through which to explore the genetic basis of this host-determined resistance. Using this information, we investigated viral replication in the spleens and livers of BALB/c, C57BL/6, and (BALB/c×C57BL/6)F_1 hybrids (Scalzo et al. 1990). Levels of viral replication in the spleen during the early acute phase of infection of the F_1 hybrids were as low as in the parental C57BL/6 strain. This pattern was observed for both male and female mice, but did not extend to the liver. This indicated that regulation of MCMV replication in the spleen was mediated in an autosomal-dominant manner (Scalzo et al. 1990). To enumerate the number of genes affecting this trait, viral replication in the spleens of (BALB/c×C57BL/6)F_1×BALB/c backcross and (BALB/c×C57BL/6)F_2 intercross mice was assessed, and it was found that ratios of 1:1 and 3:1 for low versus high titers were obtained for progeny from each cross, respectively. This indicated that control of MCMV replication was

mediated by a single non-*H2* locus, which was named *Cmv1* (Scalzo et al. 1990). Resistant mouse strains, such as C57BL/6, are designated *Cmv1*^{r} and susceptible mouse strains, such as BALB/c, have the *Cmv1*^{s} allele. Mapping studies using the CXB set of recombinant inbred (RI) mouse strains derived from the BALB/c and C57BL/6 parental strains provided the first evidence that *Cmv1* mapped to the distal region of mouse chromosome 6 (Scalzo et al. 1990).

Distal Mouse Chromosome 6 Contains the NK Gene Complex

In the mouse, the majority of NK cell receptors are encoded in the NK gene complex (NKC) on distal chromosome 6. Initially described when the genes for the first putative NK cell receptors (*Ly49*, *Nkrp1*) were localized (Yokoyama et al. 1990, 1991), the NKC is now known to consist of several multigene families, many of which are selectively expressed by NK cells but also may be expressed by other hematopoietic cells (Yokoyama and Plougastel 2003). These molecules are typically type II integral membrane proteins with external C-type lectin-like domains. They are usually expressed as disulfide-linked dimers, either homo- or hetero-dimers. Both ITIM-containing inhibitory receptors and charged transmembrane residue-containing isoforms without ITIMs are typically found within a family of highly related molecules (~80% amino acid identity). Between families there is limited homology (~25% amino acid identity), but there is preservation of the main features of these molecules (type II, C-type lectin-like domains), indicating superfamily relationships. The NKC is conserved in other mammals, including humans (chromosome 12p13) and rat (chromosome 4) where the syntenic regions have been most extensively studied (Hao et al. 2006; Kelley et al. 2005; Kveberg et al. 2006). Moreover, several loci involved in resistance to other large DNA viruses also map to the NKC, including *Rmp1* (mousepox, ectromelia virus) and *Rhs1* (herpes simplex virus) (Delano and Brownstein 1995; Pereira et al. 2001; Yokoyama and Plougastel 2003) as well as *Cmv1* and *Cmv4* (CMV) (Adam et al. 2006; Scalzo et al. 1990).

For the purposes of this review, it is especially important to highlight the role of the mouse Ly49 family of molecules which is encoded by a cluster of genes in the NKC (Smith et al. 1994; Wong et al. 1991; Yokoyama and Plougastel 2003). The first identified NK cell inhibitory receptor for MHC class I was Ly49 (now known as Ly49A) (Karlhofer et al. 1992). Several other Ly49 family members, such as Ly49C, Ly49G, and Ly49I, are also inhibitory receptors for MHC class I (Hanke et al. 1999). By contrast, Ly49D and Ly49H are activation receptors that couple to DAP12 (Bakker et al. 2000; Smith et al. 1998). The Ly49D receptor mediates the effect of the *Chok* locus, which was defined based on the genetic ability of C57BL/6-derived NK cells to kill a xenogeneic target cell, Chinese hamster ovary (CHO) cells (Idris et al. 1999; Nakamura et al. 1999). The ability of Ly49D to recognize CHO cells was determined by a genetic approach and verified by gene transfer of Ly49D and monoclonal antibody (mAb) blockade of Ly49D. Whereas Ly49D recognizes a hamster MHC class I molecule (Furukawa et al. 2002), Ly49H remained an orphan receptor, as are many other receptors encoded in the NKC.

Cmv1 Resistance Is Mediated via NK Cells

The very rapid regulation of MCMV replication suggested that the *Cmv1* effect must be mediated through effector functions that are elicited early after infection. While type I interferons and $CD4^+$ and $CD8^+$ T cells were excluded as playing roles in the *Cmv1* effect (Scalzo et al. 1992), NK cells were found to be critically important since the depletion of NK cells from C57BL/6 mice or *Cmv1*^{r} CXB RI strains (CXBD, CXBE, and CXBJ), using an anti-NK1.1-depleting mAb (PK136), abrogated the early control of MCMV replication in the spleen, leading to levels of viral replication comparable to those seen in susceptible BALB/c mice (Scalzo et al. 1992). Coupled with the emerging concept of the NKC at that time (Yokoyama et al. 1991), these data strongly suggested that the *Cmv1* effect may be linked to the NKC itself.

By analyzing viral replication in the spleens of the larger set of BXD RI strains, derived from the susceptible DBA/2 and resistant C57BL/6 progenitor strains, we confirmed the chromosomal map location of *Cmv1* and refined it to a distal region of chromosome 6 (Scalzo et al. 1992). The strain distribution pattern of *Cmv1* phenotypes in the BXD RI set was identical to that for *Ly49* and *mNKR-P1*, i.e., the NKC, with one exception being that the phenotype in the BXD-8 strain was *Cmv1*^{s}. Originally these data were interpreted as suggesting that *Cmv1* might be a distinct gene from *Ly49* and *mNKR-P1* (Scalzo et al. 1992), but subsequent genetic analysis of this strain indicated it had a C57BL/6-like genotype in the entire region of the NKC, suggesting it may harbor a germ-line mutation in the candidate gene for *Cmv1* (Scalzo et al. 1995a). Hence, the *Cmv1*^{s} phenotype of the BXD-8 RI strain later became a critical factor in the successful identification of the gene encoding the protein that mediates the effects of the *Cmv1* locus.

High-Resolution Genetic and Physical Mapping of Cmv1

The initial localization of *Cmv1* to the NKC was confirmed by low-resolution mapping studies using (BALB/c×C57BL/6)F_1×BALB/c backcross mice and by the construction of a congenic mouse strain in which the *Cmv1*^{r} allele and NKC alleles from the C57BL/6 mouse were introduced onto the BALB/c background to create the BALB.B6-*Cmv1*^{r} strain (Scalzo et al. 1995a, b). Subsequent studies then focused on fine genetic mapping of the locus and its physical mapping. Two high-resolution genetic mapping studies were performed using large cohorts of over 1,000 backcross mice. In one study no segregation was found from the *Ly49a* locus, indicating very close linkage to the *Ly49* multigene family (Depatie et al. 1997). In the other study the map position of *Cmv1* was determined to be approximately 0.2 cM distal to *Ly49a* (Forbes et al. 1997). In the latter study, however, it should be noted that several mice that were critical for assignment of the map location of *Cmv1* possessed titers intermediate between those of the parental strains. Nonetheless, *Cmv1* was in close proximity to the NKC if not in the NKC itself.

Following determination of a low-resolution physical map of the NKC region (Brown et al. 1997), high-resolution mapping of the physical location of *Cmv1* on chromosome 6 was performed. One study localized *Cmv1* to an approximately 1.6 Mb region between the markers *D6Ott8* and *D6Ott115* that encompassed the entire *Ly49* multigene cluster (Depatie et al. 2000), whereas the other study provided evidence that the gene encoding *Cmv1* should physically reside between *Ly49b* and *Prp* (Brown et al. 1999). The reasons for differences in the fine positioning of *Cmv1* between these genetic and physical mapping studies may have been due to differences in the viral strain used (Smith versus K181), equivocal phenotyping of animals representing critical recombination events in the study by Forbes et al. (1997), or the contribution of modifying genes affecting the principal gene mediating the *Cmv1* effect (Scalzo et al. 2003). Nevertheless, abundant genetic mapping data from three different genetic approaches (RI, backcross panels, congenic strains) indicated that *Cmv1* is linked to the NKC, and subsequent investigations then focused on assessment of candidate genes in the distal NKC region.

Cmv1 Is Ly49h

The mapping of *Cmv1* in close proximity to the NKC and its dependence on NK cells for MCMV resistance strongly suggested that an NKC-encoded receptor on NK cells was responsible for its effect. However, identification of *Cmv1* remained challenging for several reasons including: (1) the high density of closely related genes in the NKC; (2) the already known allelic polymorphism for many of these genes; and (3) a recombination "hotspot" with corresponding absence of additional informative recombination events in the NKC (discussed in Scalzo et al. 2003; Yokoyama and Plougastel 2003). Point Nos. 1 and 2 indicated that a direct sequencing approach would not be informative because it was unlikely to yield a specific candidate gene, and issue No. 3 made it difficult to narrow the genetic interval by further breeding experiments. Therefore, identification of *Cmv1* required clues from several other related lines of work.

In studies originally aimed at identifying NK cells in situ during MCMV, it became important to analyze the Ly49 repertoire on NK cells during the course of MCMV infection (Dokun et al. 2001a). In parallel, a mAb was generated that was specific for Ly49H, and it was used to demonstrate that Ly49H was an orphan activation receptor expressed only on a subset of NK cells from C57BL/6 mice (Smith et al. 2000). Subsequent studies revealed a marked and selective increase in the Ly49H subset during the latter stages (day 6) of infection (Dokun et al. 2001b), prompting an experiment to assess the effect of anti-Ly49H mAb administration on the course of MCMV infection (Brown et al. 2001). Interestingly, anti-Ly49H treatment adversely affected survival and led to increased viral titers, similar to depletion of all NK cells in C57BL/6 mice with mAb PK136 (anti-NK1.1). Similar results were obtained with an anti-Ly49 mAb with broader reactivity, including for

Ly49H, but these effects were not seen with other mAbs reactive with only Ly49 molecules other than Ly49H, such as anti-Ly49D (Brown et al. 2001; Daniels et al. 2001; Tay et al. 1999). Anti-Ly49H treatment did not appear to deplete the Ly49H subpopulation of NK cells and $F(ab')_2$ fragments of anti-Ly49H demonstrated similar effects, suggesting that anti-Ly49H was blocking recognition of MCMV infected cells.

Armed with a specific candidate gene (*Ly49h*) in mind for *Cmv1*, we obtained genetic evidence by analysis of susceptible BXD-8 mice which displayed an absence of $Ly49H^+$ NK cells even though they possess the C57BL/6 haplotype for the NKC (Brown et al. 2001). Other Ly49 receptors were normally expressed, the results being confirmed by specific absence of *Ly49h* transcripts (Brown et al. 2001; Lee et al. 2001). Southern blot analysis revealed a deletion in the 5′ proximal portion of *Ly49h* in BXD-8 mice whereas other *Ly49* genes were intact. Finally, *Ly49h* was not present in $Cmv1^s$ mice, such as BALB/c. These data firmly established that *Ly49h* is responsible for the *Cmv1* effect.

Subsequent corroborating evidence was provided by other laboratories examining transgenic and knockout mice. By transgenesis, a bacterial artificial chromosome containing the *Ly49h* gene from C57BL/6 genome was able to confer resistance to mouse strains that were otherwise susceptible to MCMV (Lee et al. 2003). Moreover, mice on the C57BL/6 background having a Tyr-to-Phe mutation in the ITAM of DAP12 displayed a nonfunctional Ly49H receptor (Tomasello et al. 2000). These mice were susceptible to MCMV infection (Sjolin et al. 2002). Taken together, these data firmly established that the Ly49H NK cell activation receptor is responsible for genetic resistance to MCMV infection.

Ly49H Recognizes m157 Encoded by MCMV

Identification of m157

The search for the ligand for Ly49H was aided by the generation of Ly49H reporter cells based on an approach pioneered by Nilabh Shastri (University of California, Berkeley) who produced a cell line (BWZ.36) by stably transfecting a derivative of the T cell hybridoma fusion partner, BW5147, with a reporter construct for nuclear factor of activated T cells (NFAT)-dependent expression of β-galactosidase in response to ITAM-dependent signaling (Sanderson and Shastri 1994). BWZ.36 was transfected with cDNAs for Ly49H and its ITAM-containing signaling chain DAP12 (Smith et al. 2002). The subsequent reporter cell line (HD12) produced β-gal when cross-linked with immobilized anti-Ly49H. Moreover, it responded to MCMV-infected but not uninfected cells and required cell–cell contact. Supernatants from infected cells did not stimulate nor did high doses of innate cytokines such as IFN-α/β. By contrast, stimulation occurred even when the infected cells were fixed. A wide variety of cells infected with MCMV stimulated HD12 cells, but when

infected with other herpesviruses (HSV-1, γHV68) no stimulation occurred, suggesting that ligand expression was not due to herpesvirus infection per se. Thus, we concluded that the ligand for Ly49H was expressed on the cell surface, and was probably encoded by MCMV itself.

A bioinformatics approach was then undertaken for identification of ORFs in the MCMV genome encoding putative transmembrane proteins (Smith et al. 2002). Inasmuch as other Ly49 receptors recognize MHC class I molecules, MHC class I-like proteins were also considered, but only one ORF (m144) had basic local alignment search tool (BLAST) sequence homology to MHC class I. However, m144 is thought to be a ligand for an NK cell inhibitory receptor (Farrell et al. 1997; Kubota et al. 1999). Nonetheless, 11 other ORFs were identified with putative MHC class I folds with a structural prediction strategy (3D-PSSM, http://www.sbg.bio.ic.ac.uk/~3dpssm/index2.html) (Kelley et al. 2000). Most were also dispensable for in vitro viral replication as assessed by the study of MCMV mutants with large deletions covering these ORFs (Cavanaugh et al. 1996; Kleijnen et al. 1997; Thale et al. 1995). Thus, these ORFs are likely to interact with the host.

PCR primers were generated for all 40 ORFs encoding putative transmembrane proteins or MHC class I folds (Smith et al. 2002). Of them, 29 were readily amplifiable from a MCMV-infected cell cDNA library and were expressed in target cells with retroviral expression vectors. Only one, m157, stimulated HD12 reporter cells; m157 has a predicted MHC class I-like structure. Transduced expression of other ORFs did not stimulate and m157 did not stimulate another Ly49 activation receptor reporter cell. m157 transfectants also specifically and selectively stimulated primary Ly49H$^+$ NK cells to produce IFN-γ in vitro, and IFN-γ production was associated with downregulation of Ly49H expression. Finally, anti-Ly49H blocked m157 stimulation of Ly49H reporter cells and primary Ly49H$^+$ NK cells.

A related approach was undertaken by the Lanier laboratory (Arase et al. 2002) with a Ly49H transfectant with ITAM-dependent expression of green fluorescent protein (GFP). They refined their considerations to ORFs absent in MCMV deletion clones that failed to stimulate the Ly49H reporter cell. They further used an m157-Ig fusion protein to determine that m157 directly binds Ly49H-transfected cells and to the Ly49I inhibitory receptor on approx. 10% of NK cells from the 129/J strain mice. Thus, Ly49H recognizes m157 that may have originated as an MCMV ligand for an inhibitory receptor on NK cells.

Further validation that m157 is the ligand for Ly49H came from studies of an MCMV clone with a deletion in m157 (Δm157) (Bubic et al. 2004). This virus showed enhanced virulence in C57BL6 mice that was unchanged when NK cells were depleted. Furthermore, Δm157-infected cells did not stimulate Ly49H$^+$ NK cells in vitro and Ly49H expression was not downregulated. When m157 was ectopically replaced in the Δm157 clone, characteristics of wildtype (wt) MCMV infection were restored. Moreover, the Δm157 clone displayed a slight attenuation of virulence in BALB/c mice that lack any NK cell receptor for m157 (Arase et al. 2002). These studies provide in vivo evidence for the interaction between Ly49H and m157 and suggest m157 has another immune evasion role.

A mouse mAb was generated that is specific for m157, i.e., it bound m157 transfectants but not parental cells, and stains MCMV-infected cells but not Δm157-infected cells, indicating that it reacts exclusively with m157 on virus-infected cells (Tripathy et al. 2006). m157 is expressed with glycophosphatidylinositol linkage since a stop codon is present in the midst of its otherwise canonical transmembrane domain, and its expression is diminished with phosphatidylinositol phospholipase C treatment. m157 is readily expressed 12 h after infection, consistent with the expression of its transcript as an early gene. m157 is expressed upon in vitro MCMV infection of a wide variety of tumor and primary cells, including macrophages and hepatocytes, but is expressed much less on fibroblasts, suggesting that some infected cells may be better recognized by Ly49H$^+$ NK cells. Despite its putative MHC class I-like structure, m157 is expressed on β2m$^{-/-}$ and Tap1$^{-/-}$ bone marrow (BM)-derived macrophages infected with MCMV. Moreover, m157 is expressed equivalently on cells infected with an MCMV mutant lacking all ORFs (m04, m06, m152) that downregulate host cell MHC class I molecules. Finally, anti-m157 blocks recognition by Ly49H$^+$ NK cells and Ly49H-reporter cells. Thus, m157 is not subject to viral regulation of MHC class I and is specifically recognized by Ly49H.

MCMV Escape Mutants

Interestingly, the K181 strain of MCMV (containing *m157*) is typically passaged through BALB/c mice that lack *Ly49h*. When K181 was passaged through a BALB/c mouse strain congenic for the C57BL/6 allele of *Ly49h*, mutant MCMV clones arose with mutations in *m157* that affect m157 expression (Voigt et al. 2003). These clones essentially replaced viruses containing intact *m157* and evidence was obtained for *m157* mutation occurring by the third passage. Thus, selection pressure in immunocompetent C57BL/6 mice is sufficient to result in the emergence of MCMV clones that can escape Ly49H-mediated control.

Meanwhile, the early innate control of MCMV by Ly49H-dependent NK cell responses suggested that mice deficient in B and T cells should control the virus initially. Indeed, severe combined immunodeficiency (SCID) and recombination activation gene (RAG)-deficient mice initially survived infection, unlike early lethality in mice lacking NK cells or injected with anti-Ly49H, all of which die within 7 days post-infection (Brown et al. 2001). Immunodeficient mice succumbed, however, about 3–4 weeks after infection. There was early control of viral replication in SCID mice but subsequently viral titers increased. When viruses emerging from SCID mice at 3 weeks were cloned (termed SCID-MCMV), they produced early lethality in naïve SCID and wt mice, mostly occurring by day 7 post-infection. Also, SCID-MCMV isolates showed higher viral titers at day 3 compared to wt MCMV. Thus, these data indicated that MCMV clones with enhanced virulence emerge rapidly during infection of mice deficient in adaptive immunity.

The SCID-MCMV clones had similar properties to the Δm157 clone, i.e., SCID-MCMV-infected cells failed to stimulate Ly49H$^+$ NK cells (French et al. 2004) and Ly49H reporter cells (French et al. 2005) in vitro, suggesting that the SCID-MCMV isolates had mutations in *m157*. Indeed, sequence analysis revealed mutations in m157 in more than 95% of isolates; most should disrupt expression. By contrast, no mutations were identified in the other ORFs tested. Furthermore, the original MCMV preparation was re-cloned and all clones contained intact m157. When one of these MCMV clones with verified intact m157 was expanded only in tissue culture and then inoculated into SCID mice, there was again late emergence of viruses that were cloned. Of them, 100% had m157 mutations. Thus, the innate immune system provides selection pressure on MCMV resulting in emergence of escape viruses.

Host-Pathogen Lessons from Ly49H and m157

The interaction of Ly49H with m157 is likely to reflect an ongoing "arms" race between the pathogen and the host, but this co-evolution story is likely to be complex. While more work is certainly required, the available data suggest that m157 may be an immune evasion molecule, allowing MCMV to avoid detection from NK or other immune cells. For example, m157 can be recognized by the 129 (mouse strain) allele of the Ly49I inhibitory receptor (Arase et al. 2002). Inasmuch as Ly49I is expressed on only a small fraction of NK cells, and there is no known activation receptor in 129 mice for recognition of MCMV, the significance of this finding is unclear, but it may provide a clue to further analysis. [There is abundant evidence that MCMV devotes several ORFs to target ligands for the NKG2D activation receptor, which is expressed in 129 mice (Hasan et al. 2005; Ho et al. 2002; Krmpotic et al. 2002, 2005; Lenac et al. 2006; Lodoen et al. 2004), suggesting that producing an inhibitory receptor ligand, i.e., m157, could be another means to affect NK cell activation through NKG2D.] In addition, the Δm157 clone replicates less well in BALB/c mice that do not have an NK cell receptor that binds m157, suggesting that m157 may be involved in evasion of other immune cells (Bubic et al. 2004).

On the other hand, Ly49H may be the host evolutionary response to the inhibitory effects of m157 (Arase et al. 2002). Moreover, it is important to recall that MCMV undergoes an acute replication phase during which NK cell control operates, followed by the establishment of latency (Biron et al. 1999). It is possible that an MCMV clone that is too virulent during this phase will kill the host, and not allow latency to be established. This might be detrimental to long-term survival of the virus at the population level, as noted in the co-evolution of hosts and poxvirus (rabbits and myxoma virus) (Fenner 1983). Thus, viruses can evolve mechanisms to attenuate their virulence, including addition of ORFs to enhance host responses with another example being the soluble IL1-R in poxviruses (Alcami and Smith 1992).

The rapid loss of m157 in *Ly49h* hosts (French et al. 2004; Voigt et al. 2003) nonetheless suggests that an NK cell activation receptor response can exert enough selection pressure to result in m157-deleted escape mutants, suggesting that m157

will become eventually absent in MCMV. So why is it still present in K181? It should be noted that C57BL/6 mice represent the minority of inbred mouse strains that demonstrate early innate resistance to MCMV (day 3 viral titers, for example) (Scalzo et al. 2005). The MA/My strain also shows early resistance but this is not due to *Ly49h* (Desrosiers et al. 2005; Dighe et al. 2005). Indeed, most inbred mouse strains lack *Ly49h*. Furthermore, early results indicate significant variability in wild mice with respect to control of MCMV (Scalzo et al. 2005). Most strains are unable to show resistance to the K181 strain of MCMV, suggesting that they lack *Ly49h*. Microsatellite marker data also suggest that they have different NKC haplotypes. It will therefore be informative to survey wild strains of mice, the majority of which are typically infected with MCMV, and determine the relationship between *Ly49h* and *m157* in these host-pathogen pairs.

Role of Ly49H in Host Resistance to MCMV: The Bigger Picture

The critical role of Ly49H in MCMV resistance strongly suggested that Ly49H$^+$ NK cells are specifically triggered during MCMV infection. During an early phase of infection, however, nonspecific NK cell activation occurs without regard to Ly49H expression (Dokun et al. 2001b). This was manifested in two ways, IFN-γ production at 36 h (time of maximal production) as detected by intracellular staining, and proliferation at 2 days. Subsequently, there was preferential proliferation of Ly49H$^+$ NK cells peaking in days 4–6. Ly49H-specific proliferation was virus-specific and inhibited by anti-Ly49H treatment, indicating that Ly49H itself is specifically stimulated in MCMV infection, and providing an explanation for the early clue to Ly49H involvement in the *Cmv1* effect. These studies indicate that NK cells undergo two distinct phases of activation during MCMV infection, an early generic response, presumably to cytokines, and a later specific response triggered through a virus-specific NK cell receptor.

Ly49H ligation by m157 on transfected cells also results in coordinated release of five cytokines/chemokines, [IFNγ, ATAC (lymphotactin), MIP-1α, MIP-1β, RANTES] from Ly49H$^+$ NK cells (Dorner et al. 2004). Whereas other cytokines (IL-2, IL-12, IL-15, IL-18) also triggered release of the five cytokines/chemokines, stimulation was not confined to Ly49H$^+$ cells. At the single cell level, production of all five mediators showed strong positive correlation with each other. NK cells were a major source of the five cytokines/chemokines in vitro and in vivo, whereas infected macrophages produced only limited amounts of MIP-1α, MIP-1β, and RANTES. These findings suggest that both virus-specific and nonspecific NK cells play crucial roles in activating other inflammatory cells during MCMV infection.

Ly49H engagement by itself does not lead to NK cell proliferation (French et al. 2006). NK cells require IL-15 and IL-15Rα for in vivo growth and survival. For example, mice deficient in IL-15 or the IL-15 receptor complex [IL15Rα, IL2/15Rβ (CD122), or common γ chain (γc)] fail to develop NK cells. Although IL15Rα is

expressed by NK cells, IL15Rα can present IL-15 in *trans* (from another cell) to the NK cell (Dubois et al. 2002; Koka et al. 2003; Prlic et al. 2003). Regardless, IL-15 can supply the necessary growth factor to allow specific proliferation of Ly49H$^+$ NK cells after Ly49H engagement whereas IL-18 is not required in contrast to early reports (French et al. 2006).

Recent studies indicate that other molecules are required for Ly49H-dependent MCMV control. For example, the *Unc13d* gene encodes a molecule that is required for efficient granule exocytosis (Crozat et al. 2007). *Jinx* mice, with an abnormal splice donor site in *Unc13d*, are extremely susceptible to MCMV. Moreover, it is intuitive that other molecules involved in Ly49H-dependent responses, such as DAP12, perforin, and granzymes, should also affect MCMV control, as has been shown, though it is sometimes difficult to isolate these effects to Ly49H and NK cells (Loh et al. 2005; Sjolin et al. 2002; Tay and Welsh 1997; van Dommelen et al. 2006).

On the other hand, the Ly49H-dependent responses alone are insufficient to control MCMV infection. The early nonspecific stimulus involves, at least in part, virus-induced activation of plasmacytoid DCs (pDCs, also known as interferon-producing cells, IPCs) via Toll-like receptor 9 (TLR9) whereas NK cells are not triggered through TLR9. This results in signaling through MyD88 leading to type I interferon responses that are critical in innate immune responses. In TLR9-deficient mice resulting from deliberate targeting of TLR9 or *N*-ethyl-*N*-nitrosourea (ENU)-mutagenesis, MCMV replication is poorly controlled (Krug et al. 2004; Tabeta et al. 2004). Interestingly, these responses are required even when the Ly49H pathway is intact, and they affect nonspecific activation of NK cells, indicating that TLR9/MyD88 activation of pDCs is upstream of NK cell responses.

These studies highlight the complexities of the immune response to MCMV. Although preexisting genetic variation was related to only a relatively small number of polymorphic loci (~2, i.e., H-2 and *Cmv1* as discussed previously) (Grundy et al. 1981; Scalzo et al. 1990), this was limited by the numbers of inbred laboratory mouse strains that were compared and analyzed, as well as the inherent relatedness of inbred strains of the same species (Tsang et al. 2005). On the other hand, ENU mutagenesis of a single inbred strain (C57BL/6) and subsequent analysis of MCMV resistance suggest that nearly 300 genes play nonredundant roles in control of MCMV (Beutler et al. 2005). Thus, how these genes relate to Ly49H-mediated resistance will be of major interest in the near future.

Emerging Role of Other NK Cell Activation Receptors in Recognition of Virus-Infected Cells

The identity of Ly49H as an activation receptor that recognizes virus-infected cells prompted a broader search for other NK cell activation receptors involved in recognition of virus-infected cells. Some mouse strains resist MCMV in a Ly49H-independent manner. In genetic analysis, Ly49P from MA/My mice appears to

recognize MCMV infection (Desrosiers et al. 2005; Dighe et al. 2005; Xie et al. 2007). In contrast to Ly49H and m157, however, a specific MHC allele, $H2D^k$ is required, suggesting that Ly49P may recognize either altered $H2D^k$ or peptides presented by $H2D^k$ in the context of MCMV infection.

In humans, a notable example is the recognition of influenza hemagglutinin by human NKp46, an Ig-like receptor coupled to DAP12 (Mandelboim et al. 2001; Sivori et al. 1997). Although it has been difficult to define the basis for specificity since hemagglutinin recognition apparently requires sialic acid residues on NKp46 (sialic acid residues are ubiquitous), recent studies in NKp46-deficient mice indicate that mouse NKp46 is required for resistance to influenza (Gazit et al. 2006). Thus, these data indicate that NKp46 is important in control of influenza virus.

Finally, with respect to human NK cell recognition of HCMV, emerging data indicate a role for the lectin-like heterodimer CD94/NKG2C (Guma et al. 2006a, b). Whereas CD94 can partner with NKG2 family members (except NKG2D, which forms homodimers and probably should not be considered as a member of the NKG2 family despite its name), the NKG2 partner chain dictates the function of the heterodimer. NKG2A has a cytoplasmic ITIM and is inhibitory whereas NKG2C lacks an ITIM and has a charged transmembrane residue for association with DAP12 for signaling. Both CD94/NKG2 heterodimers recognize the nonclassical MHC class I molecule HLA-E (Qa1 in mouse) that binds peptides derived from the signal peptides of classical MHC class I molecules (Braud et al. 1998; Lee et al. 1998; Vance et al. 1998). The apparent preferential expansion of NK cells expressing CD94/NKG2C both in vitro and in vivo strongly suggests that activation through CD94/NKG2C itself is responsible (Guma et al. 2006a, b). Thus, it will be of interest to determine if human CD94/NKG2C molecules on NK cells recognize HLA-E molecules that have been altered during CMV infections.

Emerging data therefore strongly support a direct role for additional NK cell activation receptors in recognition of virus-infected cells.

Summary

The genetic dissection of mouse resistance to MCMV provided our first detailed insights into a physiologically important way by which NK cells recognize virus-infected cells. This approach was challenging at several levels, but the advances derived from it have continued to impact our understanding of NK cell activation receptors, recognition of virus-infected cells, and, more broadly, innate immunity, host-pathogen interactions, and pathogen evolution.

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Genetic Dissection of Host Resistance to *Mycobacterium tuberculosis*: The *sst1* Locus and the *Ipr1* Gene

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Contents

Abstract Genetic variation of the host significantly contributes to dramatic differences in the outcomes of natural infection with virulent *Mycobacterium tuberculosis* (MTB) in humans, as well as in experimental animal models. Host resistance to tuberculosis is a complex multifactorial genetic trait in which many genetic polymorphisms contribute to the phenotype, while their individual contributions are influenced by gene–gene and gene–environment interactions. The most epidemiologically significant form of tuberculosis infection in humans is pulmonary tuberculosis. Factors that predispose immunocompetent individuals to this outcome, however, are largely unknown. Using an experimental mouse model of infection with virulent MTB for the genetic analysis of host resistance to this pathogen, we have identified several tuberculosis susceptibility loci in otherwise immunocompetent mice. The *sst1* locus has been mapped to mouse chromosome 1 and shown to be especially important for control of pulmonary tuberculosis. Rampant progression of tuberculosis infection in the lungs of the *sst1*-susceptible mouse was associated with the development of necrotic lung lesions, which was

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prevented by the *sst1*-resistant allele. Using a positional cloning approach, we have identified a novel host resistance gene, *Ipr1*, which is encoded within the *sst1* locus and mediates innate immunity to the intracellular bacterial pathogens MTB and *Listeria monocytogenes*. The *sst1* locus and the *Ipr1* gene participate in control of intracellular multiplication of virulent MTB and have an effect on the infected macrophages' mechanism of cell death. The Ipr1 is an interferon-inducible nuclear protein that dynamically associates with other nuclear proteins in macrophages primed with interferons or infected with MTB. Several of the Ipr1-interacting proteins are known to participate in regulation of transcription, RNA processing, and apoptosis. Further biochemical analysis of the Ipr1-mediated pathway will help delineate a mechanism of innate immunity that is especially important for control of tuberculosis progression in the lungs.

Pathogenesis of MTB Infection and Heterogeneity of Host Populations

The year 2007 marked the 125th anniversary of Robert Koch's discovery of *Mycobacterium tuberculosis* (MTB) and his demonstration of its causative role in human tuberculosis, for which he received the Nobel Prize in Physiology or Medicine in 1905. Koch's studies of tuberculosis became a cornerstone for the studies of pathogenic microorganisms (Kaufmann and Schaible 2005). In his classical work Koch firmly established the infectious nature of tuberculosis, and formulated postulates that defined how disease causality could be established for any microbial pathogen. Not only did he demonstrate the association of the acid-fast bacillus, MTB, with tuberculous lesions, but also proved that it was the causative agent of the disease by demonstrating that pure bacterial culture isolated from the tuberculosis patients caused similar disease in experimentally infected animals and that the bacilli could be re-isolated from their organs. Since Koch's truly pioneering work, animal models have played a pivotal role in tuberculosis research.

In the nineteenth century about 12% of all deaths were due to tuberculosis and Koch's discovery of its infectious nature and identification of the etiological agent was hailed as a major medical discovery of the century and was anticipated to bring about rapid victory over the "white plague" (Gradmann 2006). Indeed, within 100 years after Koch's discovery, lethality from tuberculosis was tremendously reduced, achieved through the development of specific antibacterial drugs, diagnostic tests, and a live attenuated vaccine, all made possible by Koch's discovery. Despite global efforts to control tuberculosis, however, it remains the second leading cause of death from any infectious disease, with approximately 8.9 million new cases and 1.7 million deaths in the year 2004 (WHO 2006). The number of new cases and deaths from this infection worldwide has not decreased globally for over a decade (Bleed et al. 2000). This alarming trend suggests that existing methods of prevention and treatment of the disease are reaching their limits and that novel strategies are urgently needed (Kaufmann et al. 2005; Orme 2006). Rational design of those strategies, however, requires new insights

into the pathogen's virulence strategy, which it has developed over thousands of years of co-evolution with the human host (Gagneux et al. 2006).

What makes tuberculosis an especially formidable pathogen is the extent of its spread in the human population: 1.7 billion people, approximately one-third of the world's population, are infected with MTB. With a 50% lethality rate in untreated human tuberculosis cases, the pathogen likely exerted selective pressure in the pre-antibiotic era, explaining why a great majority of modern humans are initially resistant to tuberculosis infection. Indeed, the lifetime risk of developing clinical disease following infection is about 10%, and development of clinical disease within a year after primary infection occurs in less than 5% of cases (Fig. 1).

Among those who do develop the disease after initial exposure, the rate of the disease progression and gravity of clinical manifestations vary significantly. Pulmonary tuberculosis, the only epidemiologically significant form of the disease,

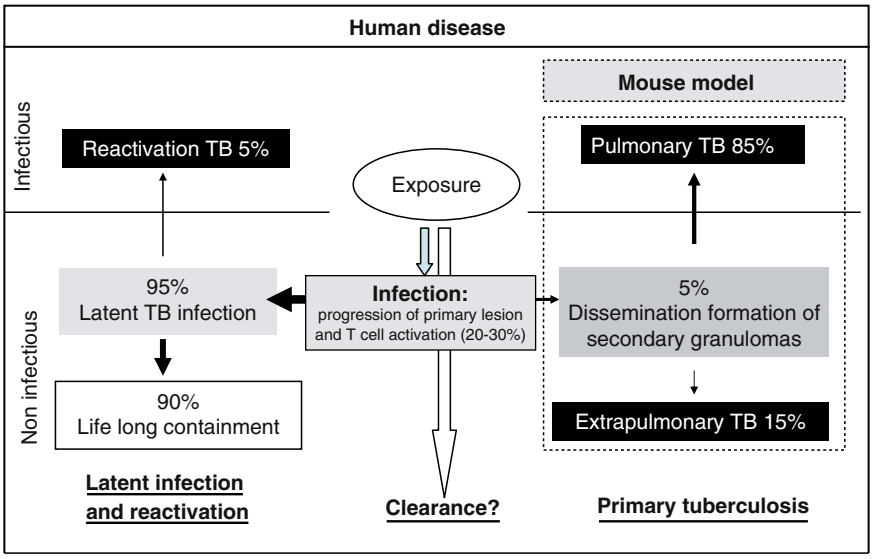

Fig. 1 Outcomes of tuberculosis infection in humans and in the mouse model. It is estimated that approximately one-third of the world's population is exposed to virulent *M. tuberculosis* during their lifetime. Of those, 20%–30% become infected and develop a T cell-mediated immune response to the bacteria, which is documented by a positive skin test (delayed-type hypersensitivity) to the purified protein derivative (PPD) of *M. tuberculosis*. More than 90% of people infected do not develop clinical manifestations of disease and are thus latently infected. They are at risk of the disease for their lifetime, however, and may develop reactivation disease as their immune system is weakened, which happens in approximately 5% of the latently infected individuals. The risk of reactivation of latent tuberculosis infection is dramatically increased by concomitant infection with human immunodeficiency virus (HIV). In contrast, in about 5% of infected individuals disease develops within the first year after exposure to the pathogen, i.e., primary tuberculosis. Of those, approximately 85% manifest pulmonary tuberculosis and become potentially infectious. The mouse model (delimited by the *dashed lines*) recapitulates primary progressive tuberculosis only

develops in 85% of all cases of clinical tuberculosis (Onyebujoh and Rook 2004). Unlike infectious agents that can be transmitted within populations by latent carriers, i.e., infected individuals that do not develop disease, MTB is an obligatory pathogen for transmission. Active pulmonary tuberculosis with extensive lung tissue damage is necessary for MTB transmission via the respiratory route.

The typical tuberculosis lesion is called a granuloma. It contains myeloid cells, mostly macrophages and their derivatives, the epithelioid and multinucleated Langhans giant cells, and varying proportions of T and B lymphocytes. A characteristic feature of those lesions is the development of a dense central necrotic area, caseous necrosis. Immunocompetent cells are organized in concentric layers surrounding the necrotic core. The bacteria in the granulomas are located extracellularly within the necrotic area as well as inside macrophages that constitute the granuloma wall. Stable granuloma formation is a hallmark of chronic persistent infection as it protects both the host from the pathogen and the pathogen from the host. This status quo, however, can be broken, which leads to an increased inflammatory reaction, bacterial multiplication, liquefaction of the necrotic masses, and erosion of the granuloma wall, giving the pathogen access to airways. Necrotic masses that contain vast numbers of the pathogen are spread via fine aerosolized particles by individuals that often remain undiagnosed for extended periods of time, during which they may expose hundreds of contacts to the pathogen. This stealth strategy enables continuous transmission even within a population in which susceptible hosts are rarely encountered, making MTB a very successful human pathogen (Flynn and Chan 2005; Hingley-Wilson et al. 2003; Rodrigo et al. 1997). Formation and subsequent decomposition of tuberculous granulomas in the lungs of immunocompetent but susceptible individuals is a key element of this strategy.

Although disseminated extrapulmonary tuberculosis is a life-threatening disease, this clinical form is a dead end from the pathogen's evolutionary perspective, because patients that develop fatal disseminated mycobacterial infections and rapidly succumb to the infection do not transmit the pathogen efficiently. Therefore, understanding host and pathogen factors that allow preferential colonization and destruction of lung tissue by the pathogen in the face of effective host immunity is necessary for the development of effective control measures.

Genetic Predisposition to Tuberculosis

Initially, Koch's discovery of MTB overturned the prevailing theory of tuberculosis pathogenesis championed by the prominent German pathologist Rudolf Virchow, that tuberculosis was a noninfectious disease or "diathesis" resulting from a heritable malfunction of host cells (Rich 1951). In the subsequent years, however, substantial heritable variation in host resistance to tuberculosis infection was demonstrated in humans as well as in animal models. In humans, the first convincing evidence was obtained in epidemiological studies of twins (Comstock 1978; Kallmann and Reisner 1943). Although the authors were able to clearly demonstrate

significant heritability of susceptibility to tuberculosis by comparing concordance rates of monozygotic and dizygotic twins, they concluded that the genetic control was complex and did not follow Mendelian laws. In experimental animal models heritability of tuberculosis susceptibility or resistance was demonstrated directly by analyzing outcomes of standardized tuberculosis infection in several generations of progeny of resistant and susceptible animals. In his classical studies, Max Lurie used a rabbit model of airborne infection with virulent MTB to demonstrate using quantitative analysis that not only did individual rabbits vary in their native resistance to the infection, but their susceptibility to the infection was a heritable polygenic trait that could not be explained according to a simple Mendelian formula (Lurie et al. 1952). Early studies using inbred mouse strains and their hybrids also demonstrated complex genetic control (Lynch et al. 1965).

From the genetic perspective host resistance to tuberculosis is a complex multifactorial genetic trait in which many genetic polymorphisms contribute to the phenotype, while their individual contributions are influenced by gene–gene and gene–environment interactions (Bellamy et al. 2000; Hill 2006). It was also proposed that the genetic component of tuberculosis susceptibility in human populations is heterogeneous, representing a spectrum ranging from rare cases of monogenic control (Mendelian susceptibility to mycobacterial disease, MSMD) to truly polygenic control (Abel and Casanova 2000; Alcais et al. 2005). MSMD mutations in genes involved in the central pathway of antituberculosis immunity [interferon (IFN)-γ-mediated activation of macrophages] confer a high degree of susceptibility to mycobacteria. In MSMD patients, normally apathogenic mycobacterial species or an attenuated vaccine strain of *M. bovis* bacillus Calmette-Guerin (BCG) cause disseminated, often fatal disease due to severe disruptions in the essential pathway of antimycobacterial immunity, namely the interleukin IL-12–IFN-γ–signal transducer and activator of transcription (STAT)1 axis (Jouanguy et al. 1996; Levin et al. 1995; Newport et al. 1996; reviewed in Casanova and Abel 2002; Fortin et al. 2007)). Remarkably, the same genes were found earlier to be essential for resistance to tuberculosis infection in mice (reviewed in Flynn 2006). In fact, extreme susceptibility to MTB of IFN-γ, INF-γ receptor, and IL12p40 knockout mice provided clues for identification of the MSMD genes in humans. From an epidemiological point of view mutations causing MSMD are very rare in human populations (Casanova and Abel 2005) and arguably account for a small fraction of the tuberculosis burden (Hill 2006).

As discussed above, the most epidemiologically significant form of tuberculosis infection in humans is pulmonary tuberculosis. However, factors that predispose immunocompetent individuals to this outcome are largely unknown. Even after infection with virulent MTB, immune mechanisms in susceptible hosts in most of the cases allow for systemic control of the infection, but fail specifically in the lungs. If host genetic polymorphisms do contribute to pulmonary tuberculosis in humans, they neither confer general immunodeficiency, nor compromise host resistance to environmental and attenuated vaccine strains of mycobacteria. Identification of such virulent mycobacteria-specific genes in humans would reveal mechanisms that the pathogen has evolved to exploit in the majority of

immunocompetent hosts. Genetic heterogeneity, weak effects of individual genes, and the significant impact of environmental factors, however, have made the genetic analysis in human populations especially difficult.

From this perspective, attempts to use animal models for the genetic analysis of tuberculosis susceptibility provide several important advantages: uniform environment, equal exposure to identical pathogen (no variation in virulence), limited genetic heterogeneity of the host, and the possibility of controlled breeding and repeated testing of animals that bear the same genotype at individual loci or combinations of allelic variants at several loci. Intraspecies variation in host susceptibility to tuberculosis infection appears to be a rule—it has been observed in laboratory mice (Buschman et al. 1988; Medina and North 1998), rats (Sugawara et al. 2004), guinea pigs (Cohen et al. 1987; Wright and Lewis 1921), rabbits (Dorman et al. 2004; Lurie et al. 1955), red deer (Mackintosh et al. 2004), and primates (Capuano et al. 2003; Flynn 2006). Although rabbits and guinea pigs represent a popular model for studying the pathomorphology of pulmonary tuberculosis and testing vaccines and drugs, tools for their genetic analysis have yet to be developed (Helke et al. 2005; Orme 2005).

Genetic Analysis of Tuberculosis Resistance Using Experimental Mouse Model

The mouse is by far the most popular experimental animal model in which to study tuberculosis infection because powerful immunological and genetic tools are available for this species. Genetically engineered mouse strains represent a useful tool for dissecting roles of individual genes in complex phenotypes. Studies of knockout mice greatly contributed to our knowledge of essential mechanisms of host resistance to tuberculosis (reviewed in Cooper and Flynn 1995; Flynn 2006; North and Jung 2004; Salgame 2005). This “reverse genetic” approach was extremely successful in demonstrating an essential role of T cell-mediated immunity and production of T helper (Th)1-type cytokines, e.g., IFN-γ, as well as IL-12 and TNF-α signaling and nitric oxide production (MacMicking et al. 1997) for host resistance to virulent MTB. It appears that many of the essential pathways of antituberculosis immunity are similar in man and mouse. Mutations that disrupt the IFN-γ pathway in humans were shown to be responsible for extreme susceptibility to mycobacterial infections (reviewed in Casanova and Abel 2002; Ottenhoff et al. 2005), and neutralization of TNF-α led to relapses of latent tuberculosis infection (Gardam et al. 2003; Keane 2005). To date, the utility of this “reverse genetic” approach has been mostly limited to identification of essential pathways of tuberculosis immunity, but it has not illuminated lung-specific aspects of the disease.

As comprehensively discussed by North and Jung (2004), the mouse model may faithfully recapitulate major aspects of antituberculosis immunity and pathogenesis, including the particular vulnerability of the lung. However, the utility of the

mouse for dissecting the pathogenesis of human tuberculosis has been debated. Indeed, the mouse is not a natural host of MTB, the pathogen is not transmitted among the murine species, and properly organized tuberculous lung granulomas containing necrotic centers have not been previously described in the experimental mouse model. In inbred mice, infection with virulent MTB invariably leads to progressive primary disease, while this outcome of infection is usually observed in less than 5% of humans (Fig. 1). Thus, mice cannot be used to model reactivation in pulmonary disease.

To address this dilemma, one should consider the "mouse model of tuberculosis" in light of host genetic heterogeneity. In mice, as in humans, host resistance to tuberculosis is a multigenic trait in which epistatic gene interactions play a significant role in shaping the phenotype. The genetic variation among standard inbred mouse strains, however, is much narrower compared to human populations, and therefore not all aspects of the human disease are easily recapitulated using several standard inbred mouse strains. Nevertheless, as evidenced by several studies of natural variation in resistance to tuberculosis infection among inbred mouse strains, conspicuous phenotypic diversity representing various manifestations of clinical tuberculosis exists in murine species, including phenotypes relevant to the human disease (Kramnik et al. 2000; Lavebratt et al. 1999; Mitsos et al. 2003; Watson et al. 2000). Those phenotypes can be systematically dissected using a forward genetic approach, which is uniquely suited for the identification of the molecular basis of complex phenotypes in vivo, regardless of whether they are controlled by a single or multiple genes (Casanova et al. 2002; Fortier et al. 2005b). The attractiveness of this laborious and time-consuming analysis is its potential to uncover novel gene functions and molecular pathways that are particularly important for disease pathogenesis in vivo.

Three independent forward genetic studies of mouse resistance to infection with virulent MTB have been performed to date using different pairs of resistant and susceptible parental strains and different strains of virulent mycobacteria, varying the doses, routes of infection, and readouts. In a study by Apt and colleagues tuberculosis progression in progeny of A/Sn (relatively resistant) and I/St (susceptible) strains was monitored by weight loss and survival after i.v. infection with the MTB strain H37Rv (Lavebratt et al. 1999; Nikonenko et al. 2000). Both phenotypes were controlled by three major loci, which were mapped to distal mouse chromosome 3 (designated tuberculosis severity 1, *tbs1*), proximal chromosome 9 (*tbs2*) and to chromosome 17 in the vicinity of the H-2 complex (Lavebratt et al. 1999; Sanchez et al. 2003). Gros and co-workers mapped four tuberculosis-resistance loci (Mitsos et al. 2000, 2003). Survival after the i.v. challenge was controlled by loci on chromosomes 1, 3, and 7, while multiplication of MTB in the lungs after aerosol challenge was controlled by two loci on chromosomes 7 and 19. Importantly, the location of the chromosome 7 peak in both experiments was very similar, suggesting that the same locus played an important role in controlling tuberculosis infection irrespective of the route and dose of MTB infection. The third study, which utilizes the same resistant parental strain as Gros and co-workers but a different susceptible strain (C3HeB/FeJ), is discussed in detail below.

Identification of the *sst1* Locus

These studies began with the observation that the standard inbred mouse strain C3HeB/FeJ was extremely susceptible to tuberculosis (Kramnik et al. 1998). After systemic intravenous infection with 10^5 colony-forming units (CFU) of MTB Erdman strain, C3HeB/FeJ mice died abruptly within 25–28 days, while other standard inbred mouse strains, including other substrains of C3H, survived for 10–40 weeks after infection with the same dose of MTB, the C57BL/6J (B6) mice being among the most resistant. Although the C3HeB/FeJ substrain of the C3H mice had no known immunodeficiency, its survival time was similar to that of the immunodeficient severe combined immunodeficient (*scid*) mice and T cell receptor knockout mice, and even shorter than that of the CD4 and inducible nitric oxide synthase (iNOS) knockouts (Fig. 2). Only the immunodeficient IFN-γ, STAT1, and tumor necrosis factor receptor (TNFR)-I knockout mice were more susceptible to MTB. The survival time of C3HeB/FeJ mice in our studies was much shorter than that of another susceptible strain, DBA/2, described by North, Gros, and colleagues (Mitsos et al. 2000). After i.v. infection with a similar dose of MTB H37Rv, the DBA/2 mice survived for about 100 days. The major distinction of tuberculosis progression in the C3HeB/FeJ mice was the development of very unusual macroscopic lesions in their lungs starting 3 weeks after systemic i.v. infection. Those lesions resembled abscesses rather than the typical diffuse lung lesions observed in various other strains of immunocompetent inbred mice.

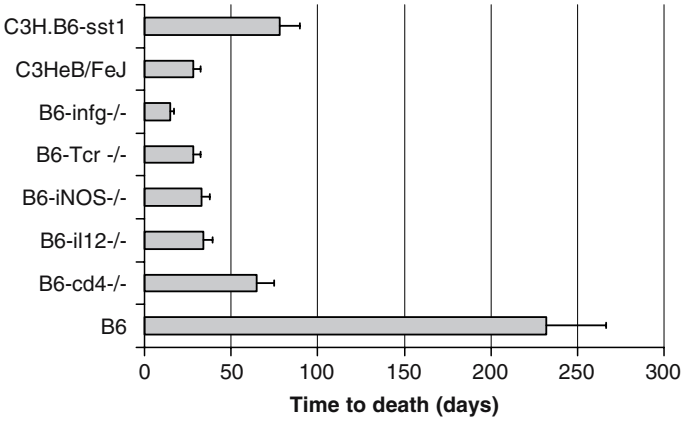

Fig. 2 Effect of the *sst1* locus on mouse survival after infection with virulent MTB. Time to death of the *sst1* susceptible and resistant congenic mouse strains (C3HeB/FeJ and C3H.B6-*sst1*, respectively) is compared to the resistant inbred mouse strain C57BL/6J (B6) as well as to mice with knockout of genes essential for systemic antituberculosis immunity on the B6 genetic background. Mice were infected intravenously with 50×10^3 CFU of virulent MTB strain Erdman

Acid fast fluorescent staining, however, of the lung tissue sections with auromine O and rhodamine, a standard method of identifying mycobacteria, demonstrated that the abscess-like lesions were in fact loaded with mycobacteria localized both extracellularly within central necrotic masses and airways, as well as inside macrophages of the inflammatory lesions. Importantly, no necrotic lesions were observed in other organs. These findings were of particular interest because formation of necrotic lung lesions resembling human lesions had not been previously reported in immunocompetent mice, although it was a hallmark of experimental tuberculosis infection in guinea pigs, rabbits, and monkeys (Helke et al. 2005). In fact, the absence of necrotic centers in tuberculosis granulomas was considered a major limitation of the mouse model of tuberculosis, decreasing its utility for testing new vaccines and antituberculosis drugs. Consequently, we chose a forward genetic approach to dissect this phenotype, which held the promise of revealing yet unknown mechanism(s) underlying the exceptional vulnerability of lung tissue to tuberculosis infection.

Time to death after systemic i.v. infection with MTB was used for linkage analysis of tuberculosis susceptibility in the F_2 hybrid progeny of C57BL/6J and C3HeB/FeJ mice. The shortest survival correlated with formation of the necrotic tuberculosis lesions in the mouse lungs, which were identical to those observed in the parental C3HeB/FeJ mice. We used DNA of these extremely susceptible F_2 mice, which represented approximately 15% of the F_2 population, for a whole genome scan with microsatellite markers and found that all F_2 hybrid mice that developed necrotic lung lesions within 4 weeks post-infection were homozygous for a C3H-derived segment in the central region of mouse chromosome 1. This indicated that the recessive C3H-derived allele of the gene(s) responsible for this phenotype was encoded within that locus, which was termed *sst1* (for *s*uper*s*usceptibility to *t*uberculosis) (Kramnik et al. 2000). We have generated the *sst1* congenic mouse strain by introgression of the C57BL/6J-derived 20-cM region of mouse chromosome 1 encompassing the *sst1* locus into the susceptible C3HeB/FeJ background and demonstrated that the presence of the *sst1*-resistant allele was sufficient to prevent formation of necrotic lung lesions after either i.v. or aerosol infection with MTB (Fig. 3), and to increase the survival of the *sst1* congenic mice under both conditions of infection (Pan et al. 2005). Analysis of the disease progression demonstrated that the *sst1*-susceptible animals ($sst1^S$) were capable of controlling multiplication of MTB in spleen and liver, and that all the bacteria were intracellular, confined to small clusters of macrophages, and no necrosis associated with the infection was observed. The specific effect of the *sst1* locus on progression of lung tuberculosis made it an especially attractive target for further analysis. Our subsequent research focused on (1) revealing functional activity of the *sst1*-encoded genes in host resistance and (2) identification of the *sst1*-encoded candidate genes using positional cloning. The functional and genetic studies were performed in parallel, which was important for subsequent prioritization and validation of the *sst1*-encoded candidate genes.

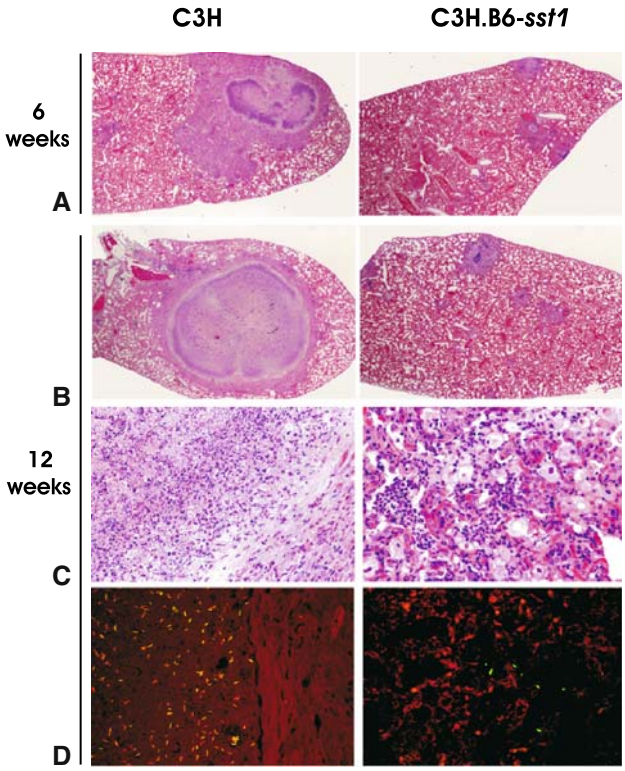

Fig. 3 A–D Progression of pulmonary tuberculosis in the *sst1* congenic inbred mice after aerosol infection with MTB. **A**, **B** Tuberculous lesions in the lungs of the C3HeB/FeJ (C3H) and the *sst1*-resistant congenic mouse strain C3H.B6-*sst1* at 6 weeks (**A**) and 12 weeks (**B**) after aerosol challenge with MTB. Extensive inflammation resembling caseous pneumonia develops in the lungs of the *sst1*-susceptible mice 6 weeks after infection with 15–30 CFU of *M. tuberculosis* Erdman via aerosol. Areas of necrosis are formed within the inflammatory lesions, which tend to merge and expand (**A**, *left panel*). At 12 weeks post-infection, large areas of necrosis occupy a significant portion of the lung lobe and are surrounded by a fibrotic capsule (**B**, *left panel*). Lung lesions in the *sst1*-resistant congenics were much smaller, lacked necrosis, and did not progress much between the 6th and the 12th week of infection (**A** and **B**, *right panels*). **C** Tuberculosis granuloma wall adjacent to central necrosis in the lung lesions of C3H ($sst1^S$) mice (*left panel*) and lung granuloma of the C3H.B6-*sst1* ($sst1^R$) mice (*right panel*) 12 weeks after aerosol challenge with MTB. H&E, 200×, original magnification. **D** Auromine-rhodamine staining of the acid fast bacteria (MTB), 400×, original magnification. Central necrosis with fibrous capsule is seen in lesions of the susceptible C3H mice, with numerous extracellular bacilli seen under fluorescence microscopy within necrotic masses. Lung lesions of the *sst1*-resistant congenic mice (C3H.B6-*sst1*) showed minimal necrosis, abundant lymphocytes and foamy macrophages with scant bacilli upon fluorescence microscopy

Role of the *sst1* Locus in Antituberculosis Immunity

The *sst1*-susceptible ($sst1^S$) C3HeB/FeJ and *sst1*-resistant ($sst1^R$) C3H.B6-*sst1* congenic mouse strains were compared for their ability to control multiplication of virulent MTB and *M. bovis* as well as avirulent attenuated vaccine strain *M. bovis* BCG. The effect of the *sst1* locus on survival and control of mycobacterial multiplication was significant only when the mice were infected with virulent mycobacteria. Multiplication of the attenuated mycobacteria BCG was controlled similarly in both the $sst1^R$ and $sst1^S$ mice, in which the bacterial load rapidly decreased. After infection with virulent TB, the survival of the $sst1^R$ congenic mice was significantly longer (10–12 weeks) as compared to the parental $sst1^S$ mice (3–4 weeks). The C57BL/6J parental strain, however, was much more resistant than the C3H.B6-*sst1* both in terms of survival and the ability to control multiplication of MTB in lungs, spleens, and livers. This was observed after MTB infection either via systemic or respiratory routes, indicating that the *sst1* locus was only partially responsible for the genetically controlled difference between the parental strains (Yan et al. 2006).

Comparing immune responses to tuberculosis infection of C57BL/6J and C3HeB/FeJ mouse strains, Behar and co-workers found a greater proportion of IFN-γ-producing CD4-positive T cells in the lungs (Chackerian et al. 2001) and earlier dissemination of MTB to peripheral organs after respiratory infection (Chackerian et al. 2002) in the resistant C57BL/6J mice. These findings suggested a mechanism of resistance in which earlier trafficking of the pathogen to lymphoid organs resulted in more rapid priming of adaptive immunity and better control of the infection. Defects in recruitment of the mycobacteria-specific IFN-γ-producing Th1 cells to the lungs seemed to be a plausible explanation for the dramatic differences in progression of lung tuberculosis between the parental strains. Later this group tested a set of the H-2 congenic mouse strains and found that, indeed, the C57BL/6J haplotype at the major histocompatibility complex ($H\text{-}2^b$) was responsible for greater CD4-positive T cell responses to mycobacterial antigens, IFN-γ production, and T cell recruitment to the lungs (Kamath et al. 2004). Survival and lung pathology, however, was controlled by non-MHC loci.

To define a specific role for the *sst1* locus in the control of tuberculosis pathology and progression in the lungs, we generated and tested reciprocal bone marrow chimeras between the $sst1^S$ and $sst1^R$ congenic mice. Although the *sst1*-susceptible phenotype during tuberculosis infection was expressed predominantly in the lungs, we found that the *sst1* phenotype was carried exclusively by the bone marrow-derived cells (Pan et al. 2005). However, neither the priming nor recruitment of the IFN-γ-producing CD4- and CD8-positive T cells to the lung was *sst1*-dependent. Production of IFN-γ by T cells isolated from tuberculous lung lesions of the *sst1* congenic mice was also similar (Yan et al. 2007). Adoptive transfer of naïve lymphocytes obtained from the *sst1* congenic mice into C3H.*scid* recipients did not reveal any functional differences between the *sst1*-resistant and -susceptible lymphocytes: both were equally efficient in transfer of protective immunity to tuberculosis. Considering the small contribution of humoral immunity to overall resistance to MTB infection, these experiments suggested that the

sst1-mediated effect was most likely expressed by nonlymphoid bone marrow-derived cells.

To test this hypothesis a pair of immunodeficient *sst1* congenic mice was generated. Both the $sst1^S$ and $sst1^R$ mice carried a mutation in the *Prkdc* gene, which leads to severe combined immunodeficiency. In this setting, effects of the *sst1* locus could be studied in the absence of an adaptive immune response. In contrast to immunocompetent mice, after i.v. infection with a standard dose of MTB, the spread of infection in *scid* mice was fastest in the spleens and not the lungs, and the effect of the *sst1* locus on multiplication of the bacteria and survival of the mice was statistically significant but minimal—both $sst1^S$ and $sst1^R$ strains of *scid* mice succumbed within 1 month post-infection, although the $sst1^R$ mice survived several days longer. The *Prkdc* mutation, which ablated the adaptive immune response, acted epistatically, such that the *sst1*-dependent differences were almost completely masked in immunodeficient hosts. This effect is consistent with the requirement for a T cell-mediated immune response and IFN-γ production to control MTB infection: immune defects leading to compromise of the Th1 immune response result in severe disseminated mycobacterial infections both in humans and in mice.

In a separate experiment we reconstituted adaptive immunity in the *sst1* congenic *scid* mice via adoptive transfer of mycobacteria-specific T cells shortly after the infection. The CD4-positive T cells were isolated from the BCG-immunized $sst1^R$ mice and transferred into either the $sst1^S$ or $sst1^R$ *scid* mice. In this setting, we bypassed the initial stages of T cell activation and delivered identical mycobacteria-primed CD4-positive $sst1^R$ T cells to *scid* animals that differed only at the *sst1* locus. On both $sst1^S$ and $sst1^R$ genetic backgrounds the mycobacteria-specific T cells significantly suppressed multiplication of MTB. This effect was especially prominent in the spleens. After an initial delay, however, necrotic lesions started to appear in the lungs of the $sst1^S$ *scid* mice, and the bacterial burden in the lungs, but not other organs, rapidly increased. Thus, nonlymphoid bone marrow-derived cells appeared to be responsible for the *sst1*-mediated effect on progression of pulmonary tuberculosis. Taken together, these studies demonstrate that T cell-mediated immunity is necessary but not sufficient for protection, especially in the lungs, where the *sst1*-mediated mechanism of innate immunity appears to play a prominent role in the presence of active T cell-mediated immunity. During tuberculosis infection in vivo, *sst1*-mediated immunity is relatively organ-specific as compared to complete lack of T cell-mediated immunity, which results in systemic failure of antituberculosis host defense.

A distinctive lung-specific feature of the *sst1*-susceptible phenotype is formation of necrotic lesions. To address the effect of the *sst1* locus on cell death within the tuberculous lung lesions, we used a terminal transferase dUTP nick end labeling (TUNEL) assay to detect cells containing fragmented DNA. Within the lung lesions of the *sst1*-resistant C3H.B6-*sst1* congenic mice, cells containing TUNEL-positive nuclei (indicative of apoptosis) represented a major fraction of all TUNEL-positive cells. Meanwhile, extensive TUNEL staining in the lungs of the *sst1*-susceptible C3HeB/FeJ mice was cytoplasmic in more than 90% of TUNEL-positive cells, which is indicative of necrotic death (Yan et al. 2007). Previously, formation of

necroses in the mouse lungs has been observed at terminal stages of tuberculosis progression as a result of very high bacterial loads (Flynn 2006; Mitsos et al. 2000). In contrast, in our studies the first necrotic microfoci started to appear in the tuberculous lung lesions of *sst1*-susceptible mice as early as 2 weeks post-infection. At that time the bacterial loads in the lungs of the $sst1^S$ and $sst1^R$ congenic mice were similar and relatively low, suggesting that the necrosis was not due to higher bacterial loads in the lungs of the $sst1^S$ animals, but was a result of higher sensitivity of their lung tissue to virulent mycobacteria. The results of these experiments are consistent with the concept that apoptotic death of infected cells limits local inflammation, bacterial multiplication, and spread and is therefore beneficial for the host (Chen et al. 2006; Henson 2003; Kornfeld et al. 1999; Watson et al. 2000; Zamboni et al. 2006). Our findings suggest that the *sst1*-mediated control of progression of pulmonary tuberculosis in vivo may be directly or indirectly involved in control of cell death in the tuberculous lung lesions.

Subsequent experiments in vitro demonstrated that the *sst1* locus affects multiplication of MTB in bone marrow-derived macrophages, and death of the infected macrophages (Pan et al. 2005). In addition, innate resistance to infection with another intracellular pathogen, *Listeria monocytogenes*, was also shown to be mediated by the *sst1* locus both using infection of the *sst1* congenic *scid* mice in vivo and infection of their bone marrow-derived macrophages in vitro (Boyartchuk et al. 2004). Taken together, our experiments establish that the *sst1*-dependent susceptible phenotype is expressed by macrophages in a cell-autonomous manner.

Identification of the Candidate Gene *Ipr1*

The C57BL/6J-derived resistant allele of *sst1* is dominant. For positional cloning we therefore analyzed median survival time (MST) of backcross progeny of males that carried recombinant chromosome 1 on the C3HeB/FeJ genetic background. After three backcrosses on the susceptible background, the *sst1* effect was converted into a monogenic trait. The backcross progeny that carried the C57BL/6J-derived resistant allele displayed an MST within the range of 8–12 weeks, while the *sst1*-susceptible homozygous mice succumbed within 4 weeks after i.v. infection.

Initial mapping of the *sst1* locus to a 30-cM interval in the middle part of mouse chromosome 1 raised the possibility that the *sst1* candidate gene was identical to a previously identified host resistance gene, *Nramp1* (natural resistance-associated macrophage protein 1), the first bacterial resistance gene identified by positional cloning by Gros and co-workers (Vidal et al. 1993). This gene controls host resistance to several taxonomically unrelated intracellular macrophage parasites: avirulent vaccine strain *M. bovis* BCG (*Bcg*), *Salmonella typhimurium* (*Ity*), and *Leishmania donovani* (*Lsh*). The phenotypic expression of *sst1* locus, however, was distinct from *Bcg/Ity/Lsh*, as it was specific for virulent MTB and did not control host resistance to the *Nramp1*-dependent microorganisms avirulent *M. bovis* BCG, and *L. donovani* (A.R. Satoskar, unpublished). Using genetic recombination we

reduced the *sst1* region to a 2-cM interval approximately 10–12 cM apart from the *Nramp1* gene (Kramnik et al. 2000).

Our attempts to further reduce the interval were complicated by the presence of an unusually large homogeneously stained repeat (HSR) region. This region was first described in some populations of wild mice from Western Europe (Traut et al. 2001; Weichenhan et al. 2001) and Siberia (Agulnik et al. 1993). It was estimated that this long-range repeat cluster may contain between 60 and 2,000 repeat units and extend over 6–200 Mb of *Mus musculus* chromosome 1, to make up as much as 0.1%–5% of the haploid genome. Weichenhan et al. identified component genes encoded within the HSR region and determined that it appeared only 1–2 million years ago by duplication, gene fusion, and amplification in the genus *Mus* and hypothesized that it might have become fixed in a 'selective sweep' (Traut et al. 2001; Weichenhan et al. 2001). We determined that the *sst1* critical interval encompassed the distal part of the HSR repeat region and a flanking region between the repeat region and the *NppC* gene. This region, in addition to the transcripts encoded within the repeat itself, contained a total of 22 known and predicted genes. The presence of the HSR repeat within the *sst1* candidate region discouraged further attempts to reduce the *sst1* critical region by genetic recombination, since the great majority of recombination events occurred within the repeat region. Thus, our subsequent work focused on functional evaluation of individual genes within the minimal candidate region and relied on our understanding of the specific effect of the *sst1* locus on immunity to infection described above.

All genes encoded within the candidate region were prioritized based on their expression pattern, which was established using RNA isolated from the lungs of the *sst1* congenic mouse strains during the course of tuberculosis infection in vivo, as well as from the bone marrow-derived macrophages infected with MTB in vitro. Our criteria for selecting candidate genes were based on their expression in the lung tissue and in macrophages, regulation of gene expression levels during the course of tuberculosis infection, and possible differential expression of the candidate gene(s) between the $sst1^S$ and $sst1^R$ mouse strains. Gene expression was analyzed using RT-PCR for known or predicted genes within the candidate region, GeneChip microarrays, and interval-specific arrays that we have constructed using clones isolated from the mouse genome bacterial artificial chromosome (BAC) libraries RPCI-23 and RPCI-24. In addition, rapid analysis of cDNA ends (RACE) analysis was performed for all *sst1*-encoded candidate genes, in which expression was detected in MTB-infected lung tissue and macrophages. The BAC arrays were included in our studies because by the time of our experiments, the HSR repeat region was represented by a gap in the mouse genome assembly due to difficulties associated with sequencing repeat regions, which suggested that our knowledge of the *sst1*-encoded genes might be incomplete. The BAC clones were isolated during our unsuccessful attempt to produce a contiguous physical map of the candidate region, which would include the HSR repeat. Nevertheless, we were able to identify BAC clones that covered the flanking regions and bridged the flanking and the HSR repeat region, as well as multiple clones containing the HSR-encoded sequences. A subset of those clones was digested with restriction enzymes and, after separation

in agarose gels, transferred to membranes, which were used for comparative hybridizations with radioactively labeled probes prepared from mRNA isolated from the tuberculous lung lesions of the *sst1*^{S} and *sst1*^{R} congenic mice. This analysis produced the first indications that transcripts differentially expressed between the *sst1*^{R} and *sst1*^{S} mice were encoded within the HSR repeat.

Subsequently, the *ifi75* (interferon-inducible-75) gene encoded within the HSR repeat region emerged as a top candidate. Expression of this gene in the lungs of the *sst1*^{R} congenic mice was significantly upregulated during MTB infection in vivo, but was undetectable by Northern blot analysis in the lungs of the *sst1*^{S} C3HeB/FeJ mice either prior to or after the infection (Pan et al. 2005). This candidate gene was also upregulated in the *sst1*^{R}, but not *sst1*^{S}, bone marrow-derived macrophages after infection with MTB in vitro. *Ifi75* was also upregulated in *sst1*^{R} macrophages primed with type I and type II interferon, most prominently by IFN-β and -γ. These data suggested that *ifi75* function may be associated with the innate mechanism of antituberculosis immunity, which is consistent with the characteristics of the *sst1* locus.

Since multiple copies of *ifi75* are encoded within the HSR repeat region (Weichenhan et al. 2001), we wanted to determine whether specific *ifi75* isoforms are expressed in tuberculous lung lesions. We performed RACE on mRNA isolated from the lungs of the MTB-infected *sst1* congenic mouse strains to reconstruct full-length *ifi75* expressed in tuberculosis lesions. One isoform of *ifi75* was predominantly expressed in the lungs of *sst1*^{R} mice, and expression of this isoform was also strongly induced in *sst1*^{R} macrophages by infection with MTB or *M. bovis* BCG, or by macrophage activation with type I and type II interferon. Meanwhile, the *sst1*^{S} macrophages weakly expressed multiple aberrant forms of the *ifi75* transcripts under the same activation conditions. Although some of the aberrant transcripts were also present in the lung tissue of the *sst1*^{R} animals, the majority of the *Ifi75*-related transcripts in the tuberculous lung lesions were represented by a single isoform, which was 92% identical to the *Mus caroli ifi75* described by Weichenhan et al. (2001). The cDNA encoding the *sst1*^{R}-specific isoform isolated from tuberculous lung lesions and IFN-activated macrophages was subsequently named *Ipr1* (intracellular pathogen resistance 1) to differentiate it from other *ifi75*- related sequences (*ifi75-rs*) encoded within the HSR repeat that have been or may be identified in other genetic backgrounds or cell types, or induced by different activating stimuli.

A key experiment confirming the role of *Ipr1* in host resistance to intracellular pathogens was expression of an *Ipr1* transgene in *Ipr1*-negative macrophages. Transgenic mice were generated directly on the C3HeB/FeJ background. A modified human scavenger receptor A (hSR-A) promoter was used to drive expression of the *Ipr1* transgene specifically in mature macrophages of the *sst1*^{S} (and *Ipr1*-negative) genetic background. We observed that expression of *Ipr1* improved macrophage ability to control multiplication of two species of pathogenic intracellular bacteria, MTB and *L. monocytogenes*. Although both *Ipr1*-positive and *Ipr1*-negative bone marrow-derived macrophages eventually died after infection with either pathogen, we noticed that monolayers of C3HeB/FeJ macrophages were destroyed

more rapidly than transgenic macrophages. Membrane damage in C3HeB/FeJ cells could be detected by flow cytometry even in the absence of signs of apoptosis. *Ipr1*-expressing bone marrow-derived macrophages controlled bacterial multiplication better and displayed signs of apoptotic cell death, such as annexin V staining of plasma membranes and mitochondrial membrane permeability transition (Pan et al. 2005).

Genetic Basis of the *Ipr1* Variation

The fact that the candidate gene was encoded within the repeat region posed several questions: How many copies of this gene are present within the repeat region? Are all copies similar in terms of their sequence, tissue-specific regulation, and function? What specific genetic lesion differentiates the *sst1*-resistant and -susceptible alleles?

Previously, the genome of another murine species, *M. caroli,* was shown to contain single copies of genes that constitute the HSR repeat in *M. musculus* (Traut et al. 2001; Weichenhan et al. 2001). Weichenhan and co-workers determined the genomic structure of *M. caroli ifi75* and found that it contained 12 exons. To compare the number of individual exons in *ifi75* in $sst1^R$ versus $sst1^S$ mice, we used quantitative PCR to amplify genomic DNA from the *sst1* congenic strains using primer pairs specific for individual exons. *M. caroli* genomic DNA was used as a standard for quantitation. We determined that in both strains the number of copies of each of the 12 individual exons of *ifi75* ranged from 5 to 40. Thus, approximately 40 copies of *ifi75* were present in both genetic backgrounds. Most of the HSR repeat units, however, encoded incomplete *ifi75* copies; we subsequently referred to them as *ifi75*-related sequences (*ifi75-rs*). In addition, the *sst1*-susceptible genome contained fewer copies of exons 1, 2, and 4 of the *ifi75* gene. This was confirmed using single strand conformation polymorphism (SSCP) analysis (see supplemental Figs. 3c and d in Pan et al. 2005). Taken together, our data demonstrated that individual copies of *ifi75-rs* are nonidentical within the same genetic background as well as between strains.

We also found that the lack of *Ipr1* expression in C3HeB/FeJ mice was due to a recent mutation, because other substrains of C3H tested in our experiments still expressed the *Ipr1* gene. The ability of several C3H substrains to resist experimental tuberculosis infection was quantitatively compared to that of the C3HeB/FeJ and the C3H.B6-*sst1* congenic mice. With the exception of the C3HeB/FeJ, all other C3H mice were similar to the *sst1*-resistant congenic C3H.B6-*sst1* mice in terms of their survival and control of MTB multiplication in the lungs. The C3HeB/FeJ was the only substrain that developed necrotic lung lesions within the first month of tuberculosis infection. Given the high degree of genetic similarity between the C3H substrains, correlation between *Ipr1* expression and the *sst1*-resistant phenotype provided independent evidence for the candidacy of the *Ipr1* gene.

The C3HeB/FeJ substrain of C3H was derived by Fekete (Fe) in the 1950s using transfer of C3H embryos into C57BL/6J females (designated as eB). The C3H

inbred mice are highly susceptible to mouse mammary tumor virus (MMTV), which is transmitted with milk from mothers to their progeny. The C57BL/6J mice are resistant to MMTV and do not transmit the virus. The litters were raised by the C57BL/6J mothers, which prevented transmission of the milk-borne MMTV and rendered the C3HeB/FeJ mice MMTV-free. The genetic defect that resulted in reduction of *Ipr1* expression might have been a random event that occurred after separation of C3HeB/FeJ from other stocks of C3H in the 1950s, or it might have been triggered by the embryo transfer. Another intriguing possibility is that the presence of MMTV might have exerted selective pressure to preserve functional copy(s) of the *Ipr1* gene in C3H inbred mice, which was lost after eliminating the virus. This speculation is consistent with reports demonstrating interactions between the human homolog of the Ipr1 protein, SP110, with viral proteins in yeast two-hybrid screens (discussed in the following section). Unusual expansion of the HSR repeat to up to 2,000 copies per genome in some populations of wild mice may also suggest that the high number of repeat elements within the HSR is maintained due to selective pressure exerted by natural infectious agents. Perhaps the interferon-inducible *Ipr1*-mediated mechanism plays a broader role in host immunity and possibly in adaptation to other environmental stressors. This hypothesis may also provide a tentative explanation for decreased numbers of functional HSR-encoded sequences in laboratory mouse strains, which are not exposed to the everyday dangers of free living.

Recent studies revealed that structural variation within normal genomes is much greater than previously estimated. While initial analysis of genetic variation in mammals focused primarily on single nucleotide polymorphisms (SNPs), regions of structural variation (deletions, insertions, and segmental genome duplications) were less studied, primarily due to difficulties associated with their sequence assembly. Application of novel high-resolution methods to genomic analysis demonstrated appreciable amounts of variation in copy numbers between normal genomes (Sebat et al. 2004). Redon et al. identified 1,447 copy number variation (CNV) regions in humans, estimated that in total those regions covered approximately 12% of the human genome, and revealed marked variation in CNVs in human populations (Redon et al. 2006). Copy number variation was also detected among inbred mouse strains (Adams et al. 2005; Lakshmi et al. 2006). It has been recently demonstrated that CNVs had significant impact on gene expression variation, suggesting their important contribution to phenotypic diversity, evolution, and disease (Stranger et al. 2007). The CNV regions contain hundreds of genes, including disease loci (Redon et al. 2006).

It has been noted that regions of segmental genome duplications are enriched for genes involved in immunity. For example, macrophage-mediated resistance to *Legionella pneumophila* in mice is controlled by the *Naip5/Birc1* gene (Fortier et al. 2005a; Ren et al. 2006), a putative cytosolic microbe sensor, which is encoded within a complex region containing a cluster of closely linked paralogs generated by duplications from a single progenitor gene (Endrizzi et al. 2000). Interestingly, an independently evolved orthologous genomic segment exists in humans, where it is involved in a noninfectious disease phenotype, spinal muscular atrophy (Growney

et al. 2000). In another example, segmental duplications containing the *CCL3L1* gene (CCR5 ligand) were linked to HIV-1 infection in humans. Possession of *CCL3L1* in lower copy number than the population average was associated with markedly enhanced susceptibility to acquired immunodeficiency syndrome (AIDS) (Gonzalez et al. 2005). In this case, the benefit of increased copy number may simply be due to a higher level of CCL3L1 protein production, which reduces viral cell entry via binding to the HIV-1 co-receptor CCR5. It is possible, however, that closely related genes that evolve within duplicated regions of the genome may generate functional diversity and thus facilitate recognition or elimination of pathogens. Further analysis of functional and structural variants of proteins encoded by the *Ipr1* gene and its human homolog SP110 in genetically diverse individuals may provide insight into mechanisms associated with CNVs that mediate host resistance to pathogenic microorganisms and, possibly, other immune-related phenotypes.

Structure and Function of the Mouse Ipr1 Protein and Its Human Homolog SP110

In humans, the most structurally related homolog of the predicted mouse Ipr1 protein is SP110 (41% identity), a member of the Speckled protein 100-kDa (Sp100) protein family. Three related nuclear proteins, SP100, SP110, and SP140 are encoded within a single cluster on human chromosome 2q37. This region is highly conserved between mice, rats, and humans, although no long-range repeat similar to the mouse HSR repeat was reported in humans and rats. In humans, *SP110* is a single gene represented by three isoforms, a, b and c, that are products of alternative splicing. Expression of both *Ipr1* and *SP110* is regulated by interferons, suggesting their possible role in immunity in both species (Grotzinger et al. 1996; Kadereit et al. 1993). Using a yeast two-hybrid screen, the SP110b protein has been found to interact physically with two viral proteins, Epstein–Barr virus SM protein (Nicewonger et al. 2004) and hepatitis C virus core protein (Watashi et al. 2003), suggesting that SP110b may also play a role in host response to viral infections.

The SP110b isoform is the closest human homolog of mouse Ipr1. Both proteins contain a putative Sp100-like protein–protein interaction domain, chromatin-associated SAND domain, a bipartite nuclear localization signal (NLS) and a single LXXLL nuclear receptor co-activator motif. All other members of the Sp100 family also contain the Sp100 and SAND domains, and may contain known chromatin association domains, such as plant homeobox domain (PHD), high mobility group (HMG), and Bromo domains, which indicate their involvement in transcriptional control and chromatin remodeling. In humans, SP100, SP110, and SP140 produce multiple isoforms as a result of differential mRNA splicing. They are called Speckled proteins (SP) because in the nucleus they are not distributed uniformly, but form aggregates, which are reported to colocalize with structures called nuclear bodies (NBs) (Bloch et al. 2000). NBs contain two permanent components, the PML (promyelocytic leukemia) and SP100 proteins. Many proteins that play an

important regulatory role in cell activation, division, and apoptosis (Hofmann and Will 2003) dynamically localize to NBs (Hofmann and Will 2003; Maul et al. 2000; Regad and Chelbi-Alix 2001; Zhong et al. 2000).

The SAND domain (named after Sp100, AIRE-1, NucP41/75, DEAF-1) is a conserved approx. 80-residue region found in a number of nuclear proteins, many of which function in chromatin-mediated transcriptional regulation. These include Sp100, NUDR (nuclear DEAF-1 related), GMEB (glucocorticoid modulatory element binding) (Bottomley et al. 2001; Surdo et al. 2003), and AIRE-1 (autoimmune regulator 1) proteins (Purohit et al. 2005). The DNA binding surface of the SAND domain has been mapped to a conserved KDWK motif (Bottomley et al. 2001), which is also present in the Ipr1 and SP110b proteins. Proteins containing the SAND domain have a modular structure, and the SAND domain can be associated with a number of other modules, which have been implicated in interactions with chromatin or transcription factors. Most frequently the SAND domain is associated with the N-terminal Sp100 domain.

The Sp100-like domain is approximately 100 amino acid residues in length and rich in hydrophobic residues. The Sp100-like domain was found to be required for dimerization of Sp100 and its localization to the NBs (Sternsdorf et al. 1999). We found that deletion of the Sp100-like domain of Ipr1 prevents its association with nuclear speckles and produces a diffuse nuclear localization pattern. Perhaps this domain is required for the interactions of Ipr1 with the nuclear matrix or other structural elements in the nucleus. Indeed, AIRE-1 also contains the Sp100 domain and was found to be tightly associated with nuclear matrix (Tao et al. 2006).

The LXXLL nuclear receptor binding motif was originally identified in nuclear receptor co-activators (reviewed in Savkur and Burris 2004). This motif, however, was also found in nuclear receptor co-repressors (e.g., NcoR) (Horlein et al. 1995), other transcription regulators (e.g., PIAS3, protein inhibitor of activated STAT3) (Jang et al. 2004), and chromatin proteins (e.g., SCC3, Sister chromatid cohesion protein-3) (Lara-Pezzi et al. 2004). Interestingly, PIAS3 suppressed nuclear factor (NF)-κB-mediated transcription by interacting with the p65/RelA subunit via its LXXLL motif (Jang et al. 2004), while SCC3 had NF-κB coactivator activity that also depended on one of its LXXLL motifs (Lara-Pezzi et al. 2004). Watashi et al. showed that Sp110b is a transcriptional cofactor negatively regulating retinoic acid receptor α-mediated transcription (Watashi et al. 2003). Perhaps this interaction also involves the LXXLL motif. The vitamin D receptor is an attractive candidate to interact directly with Ipr1/SP110b via LXXLL motifs and control macrophage interactions with MTB. Indeed, vitamin D receptor activity was recently found to mediate antimycobacterial activity of human macrophages stimulated via a Toll-like receptor (Liu et al. 2006), and its transcriptional activity was shown to be dependent on interaction with coactivators via an LXXLL motif (Pike et al. 2003).

An exciting picture of the Ipr1/Sp110b function begins to emerge from these studies. Ipr1/Sp110b might connect structural components of the nucleus with chromatin, participate in its reorganization, and help recruit transcription factors and other proteins to specific nuclear domains. This may affect transcriptional activity via facilitation or interruption of communication between promoters,

enhancers and distant *cis* regulatory elements, and other components of chromatin. Regulation of this protein by interferons and infection suggests that its specific functional activity is important for adjustment of nuclear processes to challenges imposed by infectious agents and, possibly, other stressors.

A recent publication by Roscioli et al. demonstrated that in humans rare inactivating mutations in the *SP110* gene were associated with a specific autosomal recessive primary immunodeficiency, hepatic veno-occlusive disease with immunodeficiency (VODI) (Roscioli et al. 2006). Patients homozygous for the mutant allele of Sp110 presented between 3–7 months of age with a combined T and B cell immunodeficiency, and were very susceptible to a number of bacterial, viral, and fungal infections, although no mycobacterial infections were observed in that study. Macrophage function has not been studied in these patients.

To date, four studies investigated the impact of common *SP110* polymorphisms on tuberculosis susceptibility. In family-based association studies in three West African countries, two SNPs were found to associate with tuberculosis (Tosh et al. 2006). However, population-based association studies in Ghana, Russia, and South Africa failed to detect significant association between common sequence variations of the *SP110* gene with tuberculosis (Babb et al. 2007; Szeszko et al. 2007; Thye et al. 2006). In the mouse, the *sst1* locus controls not susceptibility to MTB per se, but the type of progression of pulmonary tuberculosis. Lack of *Ipr1* expression is associated with formation of extensive necroses within tuberculous lung lesions. This phenotype, however, has not been incorporated into the design of human association studies. Obviously, the role of the *SP110*-mediated pathway in progression of pulmonary tuberculosis, as well as other infectious diseases, deserves further experimental, biochemical, and genetic analysis.

Multigenic Control of Tuberculosis Resistance and Susceptibility

Our studies indicate that the *sst1*/Ipr1-mediated pathway of immunity is necessary but not sufficient to protect host from virulent MTB. Indeed, the C3H.B6-*sst1* mice that carry the *sst1*-resistant allele on the C3H genetic background are significantly more susceptible than the resistant parental C57BL/6J strain. In addition, the reciprocal congenic strain B6.C3H-*sst1* that carries the *sst1*-susceptible allele on the resistant genetic background is also more resistant than the C3H.B6-*sst1*, indicating that the *sst1* locus is not a sole determinant of susceptibility to MTB in our genetic model (Kramnik et al. 1998; Kramnik et al. 2000). To identify non-*sst1* loci involved in susceptibility, we crossed the *sst1*-resistant congenic strain C3H.B6-*sst1* with the parental C57BL/6J strain to eliminate the strong effect of the *sst1*-susceptible allele. This allowed easier mapping of four additional tuberculosis-resistance loci on chromosomes 7, 12, 15, and 17 (Yan et al. 2006). The C57BL/6J-derived allele of the chromosome 7 locus had the highest linkage scores in these experiments. To study this locus further, we have generated a chromosome 7 consomic strain, which carries a C57BL/6J-derived chromosome 7,

while the rest of the genome, including the *sst1*-susceptible allele, is from C3H. In these mice, the isolated effect of the C57BL/6J-derived chromosome 7 on survival after tuberculosis infection was very small—the median survival time increased by less than a week as compared to the susceptible parental C3HeB/FeJ mice. However, interactions of the *sst1* and chromosome 7 loci produced a remarkable synergistic effect on survival, control of bacterial replication, and lung pathology. The survival time of the C3HeB/FeJ mice that carried the C57BL/6J-derived resistant alleles at both the *sst1* and the chromosome 7 loci increased from 4 weeks to approximately 20 weeks. Thus, the synergistic interactions of the two C57BL/6J-derived resistance loci identified in our crosses accounted for more than a half of the dramatic difference in tuberculosis susceptibility between the C3HeB/FeJ and C57BL/6J inbred mouse strains.

We observed that mice carrying the $sst1^S$ allele developed necrotic lesions in their lungs, albeit at different rates. The most resistant B6.C3H-*sst1* mice carrying the $sst1^S$ allele on a C57BL/6J genetic background developed lung necrosis about 10–12 weeks post-infection. Progression of the necrotic lung lesions in these mice was limited by thick granuloma wall and resulted in chronic disease similar to pulmonary tuberculosis in adult humans. Thus, in our model, formation of lung necroses was controlled by the *sst1* locus irrespective of other genes, while the rate of pulmonary disease progression, the extent of necrotic inflammation, and the survival of animals were controlled by *sst1* interactions with other genes. Further dissection of the genetic architecture of tuberculosis susceptibility using the mouse model will include: (1) identification of specific functional effects of individual non-*sst1* loci; (2) discovery of causal genetic variants encoded within each locus; (3) identification of molecular pathways of immunity that these polymorphisms affect; and (4) understanding how individual host resistance loci interact to produce tuberculosis-resistant or susceptible phenotypes.

Conclusions

Tuberculosis susceptibility, even in a simple genetic model produced by breeding of two immunocompetent mouse strains, is a multigenic trait in which epistatic gene interactions play a prominent role in shaping the resistant phenotype. This genetic architecture reflects complex interactions that develop during the course of tuberculosis infection in immunocompetent individuals, and no single mutation accounts for a failure of host immunity. Identification of genes and polymorphisms that contribute to the outcome of tuberculosis infection in vivo, using forward genetics in an experimental mouse model of infection, will help reconstruct the biochemical and cellular pathways that those genes control and determine how they fit into a complex hierarchy of regulatory networks at the whole organism level.

Although genetic and phenotypic variation in human hosts is much greater than in laboratory mice, genetically defined mouse models allow in-depth analysis of a key element of the MTB virulence strategy—lung colonization and destruction in

genetically susceptible, but immunocompetent, individuals. This is useful not only to reach deeper understanding of tuberculosis pathogenesis, but also for testing novel antituberculosis vaccine candidates and drugs to identify those that protect or cure the lung most efficiently.

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Part II
Self-Reactivity

Scurfy, the *Foxp3* Locus, and the Molecular Basis of Peripheral Tolerance

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Contents

Abstract The ability to rapidly and efficiently recognize and eliminate pathogens while sparing normal self tissue is a hallmark of the mammalian immune system. When it fails, however, autoimmune disease results. The genetic and environmental factors that control the process of making such distinctions, not to mention the specific targeted tissues, are extraordinarily complex in the human population; only now are we characterizing the candidate genes responsible for these responses to pathogens. The examination of specific traits in murine models of disease has led to the identification of many of the candidate genes for human disease. The study of mouse mutations (both induced and spontaneous) has also greatly advanced our understanding of the immune responses and autoimmune disease. Here, we describe the use of classical mouse genetics to identify one gene centrally involved in the control of immune responses. Furthermore, although mutations in the orthologous human gene result in a virtually identical phenotype to that seen in the mouse, it is unlikely that studying the human disease populations alone would have successfully identified this gene. Thus, despite the complete sequencing of the human and mouse genomes, the examination of murine mutations remains a powerful and unbiased tool to connect genotype and phenotype.

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Introduction

The process of scientific discovery is often thought of as a continuum in which our understanding of a system is advanced through the integration of knowledge acquired over a series of incremental steps. Under appropriate circumstances, however, it is possible for scientific understanding to take a more substantial leap forward and in recent years the exploitation of phenotype-driven gene discovery programs has afforded us one such route to rapid discovery. Thus by working with disease populations it has been possible to shed new light on fundamentals of biology by uncovering the molecular basis of normal processes that have gone awry. The murine immune system represents particularly fertile ground for the exploitation of these phenotype-driven approaches: phenotypic abnormalities that compromise immune development are seldom sufficiently devastating to result in embryonic lethality, and genetic abnormalities compromising immune function are generally well tolerated in the typical pathogen-free environment that the laboratory mouse calls home. But such phenotypic abnormalities can often be revealed at the developmental level by flow cytometric analysis of the immune system and at the functional level by experimental challenge. As a result, inherited mutations affecting the murine immune system have proved to be a valuable source for the identification of novel genes that have shaped our thinking of immune function, including Fas and Fas ligand revealed by *lpr* and *gld* mouse strains and the Tlr signaling pathway uncovered by the *Lps* locus.

A number of years ago we became interested in a novel mutant mouse strain, the *scurfy* mouse, that had originated at Oak Ridge National Laboratory (ORNL) in the 1940s. The mouse exhibited a profound phenotype characterized by massive lymphoproliferation, extensive multiorgan immune infiltration, and the overproduction of multiple cytokines, which resulted in early juvenile lethality (Russell et al. 1959; Godfrey et al. 1991a). We reasoned that the phenotype present in these animals was reflective of massive T cell dysregulation and that the identification of the gene mutated in these animals might help uncover a key regulator of immune function. We also reasoned that mutations in this gene product would likely affect the human population, but that the rapid and aggressively fatal nature of the *scurfy* mutation might make their identification using traditional familial human genetics difficult. With this in mind we set about identifying the molecular basis of the *scurfy* mouse mutation.

Scurfy: The Early Years

The *scurfy (sf)* mouse mutation arose spontaneously in 1949 at the ORNL in Oak Ridge, Tennessee. The Laboratory itself had something of a spontaneous origin, being built in secret in 1943 as part of the Manhattan project, on farmland in the mountains of east Tennessee. The wartime race to develop the atomic bomb opened a number of scientific avenues for subsequent exploration in the post World War II

arena. Included in these was the pressing need to understand the human consequences of exposure to ionizing radiation encountered either as fallout from nuclear weapons tests or from the domestic use of nuclear power. In order to do this a mouse genetics program devoted to the investigation of the effects of radiation was initiated at ORNL under the guidance of Dr. Bill Russell. In 1951 Dr. Russell published the first results of these experiments, involving the analysis of more than 85,000 animals generated either from irradiated or control parents (Russell 1951). Dr. Russell also developed an elegant mechanism for facilitating the study of germ cell mutations, which allowed the detection of recessive mutations in first generation offspring. This method, known as the specific locus test, took advantage of an established tester strain of mice that had been bred to be homozygous at a number of autosomal recessive visible loci. Mutagen-treated animals were then mated to these tester strain mice: under normal circumstances the progeny from these crosses would receive one functional allele at each of the autosomal recessive visible loci from the mutagenized parent and one mutant allele from the tester strain. But a germ cell mutation in one of these visible marker loci would be uncovered when mutagenized animals were crossed onto the tester strain, allowing the detection of mutations in this first generation (Russell et al. 1958; Silver 1995). The construction of this tester strain represented a substantial breeding exercise, requiring the breeding to homozygosity of all seven recessive alleles, and ironically the *scurfy* mutation arose spontaneously from this program.

As the name suggests the original *scurfy* animals were characterized by an early scaling of the skin that was particularly apparent on the ears, tail, and feet and which was followed by a generalized wasting, with most animals dying around the time of weaning (Russell et al. 1959). Early studies at ORNL focused on the mechanism of inheritance of this mutant phenotype. Studies conducted by Russell and colleagues showed that female animals occasionally exhibited the *scurfy* phenotype and that these animals had an X^{sf}/O genotype. This led Lee Russell and colleagues to propose in 1959 that the Y chromosome was the male determining factor in mice, and that, by inference, the Y chromosome might confer maleness in other mammals including man (Welshons and Russell 1959).

Despite the seminal nature of these initial observations and their implications for our understanding of sex determination, the *scurfy* mutant mouse drifted into PubMed obscurity. Any additional early characterization of the *scurfy* mutation was limited to observations of the animal's external appearance and comments on the animal's early mortality. Analogies were drawn between the characteristic scaling and other skin abnormalities observed in *scurfy* and those present in X-linked ichthyosis of humans (Lyon et al. 1990; Godfrey et al. 1991b).

Scurfy: A Novel Immunoregulatory Gene

A more systematic evaluation of the *scurfy* phenotype and a dismissal of this ichthyosis association had to wait until the early 1990s, when studies by Lyon and colleagues (1990) and Godfrey and colleagues (1991a) demonstrated substantial

differences at the pathophysiological and clinical level between *scurfy* and ichthyosis. *Scurfy* mutant mice developed bloody diarrhea, and hematological studies indicated that platelet and erythrocyte counts were reduced in *scurfy* animals and that these mice were severely anemic. By histopathology, *scurfy* animals exhibited splenomegaly and enlarged lymph nodes, with lympho-histiocytic proliferation and infiltration of peripheral lymph nodes, spleen, liver, and skin. It thus appeared as if the *scurfy* phenotype had an underlying immune basis that distinguished it from X-linked ichthyosis. The unique nature of the *scurfy* mutation was further confirmed by mapping studies, which placed the *scurfy* locus near the centromere of the X chromosome, separate from the steroid sulfatase gene that had been implicated in ichthyosis (Lyon et al. 1990).

A handful of publications then followed that further emphasized the immunologic nature of the *scurfy* phenotype. It became apparent that this was not a classic X-linked immunodeficiency but rather was a disease characterized by immune dysregulation with an absolute requirement for T cells. Studies from Oak Ridge demonstrated that *scurfy* mice that had been subjected to neonatal thymectomy had a prolonged life and less severe disease when compared to euthymic *scurfy* controls, while the transfer of the *scurfy* mutant allele onto either a nude or severe combined immunodeficiency (SCID) background resulted in abolition of disease (Godfrey et al. 1991b, 1994). Disease progression also exhibited a requirement for the exposure of developing T cells to endogenous antigen. When the *scurfy* mutation was bred onto a T cell receptor (TCR) transgenic line, the resultant *scurfy*/TCR transgenic animals (in which 75%–95% of the T cells expressed TCR for an exogenous antigen) had ameliorated disease. This disease could be blocked entirely in a *scurfy*/TCR-transgenic/Rag1 knockout background in which only T cells reactive to the exogenous antigen develop (Zahorsky-Reeves and Wilkinson 2001). Disease progression appeared to exhibit an absolute requirement for $CD4^+$ T cells: monoclonal antibody depletion of $CD4^+$ T cells but not $CD8^+$ T cells resulted in a retardation of *scurfy* disease, and life expectancy was also extended when *scurfy* animals were crossed onto a CD4-null but not CD8-null background (Blair et al. 1994a). $CD4^+$ T cells from *scurfy* animals were also able to transfer disease when transplanted into H-2 compatible nude mice, unlike $CD8^+$ T cells. In addition the $CD4^+$ T cells present in the *scurfy* animal appeared to have a dysregulated phenotype, expressing activation markers and demonstrating a hyper-responsiveness to stimulation through the TCR and a decreased requirement for costimulation (Blair et al. 1994a; Clark et al. 1999). Thus the *scurfy* gene product appeared to play a key role in the generation of a functional and appropriately regulated population of $CD4^+$ T cells.

The *Scurfy* Phenotype Is Caused by a Mutation in *Foxp3*

The *scurfy* locus was initially mapped to a 1.7-cM interval in the proximal region of the mouse X chromosome (Lyon et al. 1990; Blair et al. 1994b). Today, the completion of the mouse and human genome initiatives would make the identification

of genes within this region [real as well as predicted open reading frames (ORFs)] a simple computational exercise using publicly available resources. In the late 1990s, however, such resources were at a primitive stage, making this a considerable logistical challenge. Nevertheless, the compelling nature of the phenotype persuaded us that further mapping and gene identification efforts were warranted.

Through an intersubspecific backcross, our group, led by Mary Brunkow, was able to further localize the *scurfy* locus to a 0.3-cM region (Brunkow et al. 2001). By using a combination of high resolution genetic and physical mapping and large scale sequence analysis, Brunkow and colleagues identified 20 candidate genes within this genomic region, including several genes predicted based on sequence analysis only. Initial prioritization of these candidate genes based on T cell-specific mRNA expression was not informative: no mutations were detected in those genes restricted to lymphocyte expression. Further prioritization based on drugability characteristics (wishful thinking) was similarly unsuccessful. Ultimately, DNA sequencing of all genes from both normal and *scurfy* mice identified a single gene (the last gene on our prioritization scale) with a mutation in the coding sequence. This ORF had strong similarity with the DNA-binding domain of the forkhead family of proteins and contained a 2-bp insertion in the predicted coding region. This insertion was contained in affected *scurfy* animals but absent from non-*scurfy* siblings, absent from seven other mouse strains, and absent from the analogous human cDNA. This insertion resulted in a frameshift and the subsequent generation of a truncated gene product lacking the carboxy terminal forkhead domain. Confirmation that this insertion was causal of the *scurfy* mutant phenotype came from transgenesis, when a 30.8-kb genomic fragment was used to rescue the mutant phenotype (Brunkow et al. 2001). In compliance with accepted nomenclature standards for the forkhead family, the gene mutated in *scurfy* mice was given the mundane designation *Foxp3*. Since there is no required/accepted nomenclature for the protein, we adopted a more descriptive name for the protein: scurfin (Brunkow et al. 2001).

Mutations in *Foxp3* Cause Disease in Humans

Given the severe nature of the *scurfy* mutant phenotype one might expect that mutations in the human gene would be equally catastrophic. A number of X-linked human immunodeficiencies and immunodysregulatory disorders have been described, and studies in the late 1980s mapped one of these, Wiskott-Aldrich syndrome (WAS), to a *scurfy* syntenic region (Lyon et al. 1990). The gene responsible for WAS was cloned by Derry and colleagues and shown to be a 502 amino acid-containing protein containing putative SH3 binding domains and a nuclear localization signal (Derry et al. 1994). However, the subsequent identification of the mouse homolog of the WAS gene followed by sequence analysis and transcript analysis failed to reveal any disease-specific alterations between normal and *scurfy* mice (Derry et al. 1995).

Several investigators have reported isolated cases of infant males with a variety of immune-related symptoms culminating in early lethality, including enteropathy, type I diabetes, eczema, and hyperthyroidism. That these individuals might be suffering from a common disorder with an underlying genetic component was first proposed by Powell et al., working with a family in which multiple males across three generations (connected through females) had various combinations of intractable diarrhea, eczema, hemolytic anemia, diabetes mellitus, or thyroid autoimmunity (Powell et al. 1982). Linkage analysis of this and other families suggested that this disease locus lay close to the centromere of the X chromosome, close to (but not allelic with) the WAS locus and thus close to the *Foxp3* locus (reviewed in Wildin and Freitas 2005). The multiplicity of symptoms associated with this disorder was summarized in the name conferred upon it: IPEX, for immune dysfunction/polyendocrinopathy/enteropathy/X-linked. Over time, a small number of familial clusters of IPEX were reported (e.g., Peake et al. 1996; Levy-Lahad and Wildin 2001).

While this familial disease had been recognized for years, little progress beyond the association with WAS had been made in the identification of the gene responsible. We were intrigued by the complex immune dysfunction seen in IPEX patients and by its map location, which placed it close to *Foxp3*, and the cloning of the gene responsible for *scurfy* enabled us to establish collaborations to test for the presence of mutations in this gene. In a multinational effort led by Dr. Bob Wildin and working with collaborators in Italy, Australia, France, and Israel we were able to identify five unrelated and ethnically diverse families, each of which contained at least two males affected with IPEX. Sequence analysis of DNA from one male from each of these families indicated that four of these five males carried mutations in exons 10 or 11, within the winged-helix domain of *Foxp3*. Where material was available we were able to show that carriers were heterozygous for these mutations and that an unaffected male sibling lacked the mutation. To determine the normal spectrum of variation in exons 10 and 11 these two exons were sequenced in 240 unaffected ethnically diverse individuals. No sequence variation was found in any of these individuals (Wildin et al. 2001). At the same time Bennett et al. were able to take advantage of an extended family with a history of IPEX disease over four generations: a G to A transition, resulting in an Ala to Thr substitution at residue 384 of the winged-helix domain, segregated with disease in all members of the family tested, with two affected males being hemizygous for the mutation. The significance of this substitution was confirmed by sequence analysis of 500 control X chromosomes, none of which showed the same polymorphism. Analysis of a second family of Japanese origin also revealed a *Foxp3* mutation, in this case a 2-bp deletion within the *Foxp3* termination codon, resulting in a predicted addition of 25 amino acids (Bennett et al. 2001). Thus the cloning of a genetic mutation responsible for a lethal mouse disorder led directly to the identification of the gene responsible for a lethal human disorder, reaffirming the utility of using the mouse as a genetic tool to support human genetic studies. In addition to *Foxp3*, mutations in several other forkhead family proteins have been shown to result in autoimmune disease in mice. Mutations in Foxn1 in mice and humans result in a relatively

diminished T cell response, whereas mutations in Foxo3a and Foxj1 can lead to a progressive autoimmune disease that progresses with age. It remains to be definitively determined whether these family members have a role in human disease.

Tolerance

The original rationale for choosing to work on the *scurfy* mutation was based on the autoimmune nature of the phenotype of the mouse (Godfrey et al. 1991a). It seemed apparent that defining the mutation would provide a significant insight into the mechanism(s) by which the immune system developed or was controlled. There were several examples of mutations in genes that led to disorders of immunological development (most notably several mutations that led to a SCID-like phenotype), but no specific mutations in which a defect in tolerance had been established, and the *scurfy* mouse seemed like it might represent such an example. It has been known for some time that there are multiple processes to maintain tolerance within the immune system. Pioneering studies by Kappler and Marrack (Kappler et al. 1987; MacDonald et al. 1988), among others, indicated T cells that recognized "self" antigens could be eliminated during their development. A similar phenomenon occurs for B cells during their development (Goodnow et al. 1990; Basten et al. 1991). This process of clonal deletion was postulated to be a major mechanism by which the immune system removed those cells that could react to self tissues, but maintain a diverse repertoire to respond to pathogens. Ultimately, the use of TCR [and B cell receptor (BCR)] transgenic mice, in which the antigen-specific receptor was predetermined on all T (or B) cells, allowed for a dramatic visualization of this process (Kisielow et al. 1988; Sha et al. 1988; Nemazee and Burki 1989). In these types of studies it was possible to control the dose of antigen, the timing of antigen presence, and even the nature of the cell that presented the antigen, to understand more fully the process of clonal deletion.

A major issue for the clonal deletion model, however, was how tolerance to antigens not expressed within the thymus during T cell development was induced. It seemed apparent that there were tissue-specific antigens (TSA) that would not be present within the thymic microenvironment, and that there must be a mechanism to prevent or control reactivity to these tissues. While many models have been proposed, experimental evidence has supported two major mechanisms to maintain tolerance to TSA, the first being the "promiscuous" expression of TSA within the thymus and the second model being the presence of "suppressor" cells within the peripheral milieu that prevent autoreactivity. As is commonly the case in immunology, both theories have ultimately proved to be correct.

The expression of TSA within the thymus could derive either from the transport of such antigens to the thymus or from specific gene expression within the thymus. Several studies demonstrated that the genes for at least some TSA are in fact expressed in thymic stromal cells during thymic development (Klein and Kyewski 2000; Derbinski et al. 2001). Further studies showed that the transcription factor

AIRE (autoimmune regulator) is in part responsible for this expression, resulting in the deletion of T cells reactive to antigens that are normally expressed only in specific tissues of the body (Anderson et al. 2002, 2005). The process of thymic clonal deletion could thus ensure that as T cells developed they were exposed to normal self antigens. Those cells that reacted strongly with these antigens were then programmed to undergo apoptosis and thus be eliminated from the pool of potentially reactive cells that entered the peripheral immune system. Importantly, in the absence of AIRE, an organ-specific autoimmunity called APECED (autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy) develops (Aaltonen et al. 1997; Nagamine et al. 1997). While this disease is not as severe as IPEX, this nonetheless reflects the importance of antigen-specific clonal deletion in maintaining tolerance.

This system of clonal deletion is not perfect at eliminating self-reactive cells. It is clear that normal mice and humans, as well as autoimmune individuals, possess T cells capable of reacting with self antigens. There have been a number of alternative models described for controlling these self-reactive cells. Two predominant theories are the functional inactivation of T cells (loosely termed anergy for the current discussion) and the generation of a population of T cells capable of suppressing self-reactive cells. These suppressor cells are today more commonly referred to as regulatory T cells (T_R). Although there are numerous in vitro and in vivo model systems in which anergy can be demonstrated, the data suggesting that this is a significant component of normal tolerance in vivo is not strong at present. In part, this may be because the anergic state is difficult to measure (due to the relative lack of a response) and because it may be transient in nature. Thus, while anergy may be important for maintaining tolerance to self in vivo, this has been difficult to prove experimentally.

T_R cells to a great extent represent the reincarnation of the suppressor T cell populations described in the 1970s and 1980s. Although this population of cells has a somewhat "checkered" history and fell out of research favor for many years (Shevach 2000), seminal studies from Sakaguchi and colleagues established that a minor population of $CD4^+$ T cells (those co-expressing CD25) was capable of inhibiting the response of autoantigen-specific cells (Sakaguchi et al. 1995). The $CD4^+25^+$ subset of T_R cells is most often referred to as nT_R cells or natural regulatory T cells. These comprise approximately 10% of all $CD4^+$ T cells in the mouse. Thymectomy of mice within the first 3 days of life dramatically reduces the number of these cells and also results in the development of a broad-spectrum, tissue-specific autoimmunity (largely directed at endocrine tissues) (Sakaguchi et al. 1985). Similarly, antibody-mediated elimination of $CD4^+25^+$ cells causes a pathology that resembles that observed following thymectomy (Sakaguchi et al. 1995). In vitro studies have demonstrated that when purified, the addition of $CD4^+25^+$ cells could inhibit proliferation and interleukin (IL)-2 production by the $CD4^+25^-$ subset of cells (Thornton and Shevach 1998). Finally, in vivo studies demonstrated that the co-transfer of $CD4^+25^+$ cells could inhibit an autoimmune-induced inflammatory bowel disease in SCID animals (Mottet et al. 2003). It has been historically far more challenging to demonstrate a corresponding subset of cells in humans,

although in vitro data for such activities does exist (Baecher-Allan and Hafler 2006). In addition, there are also descriptions of subtypes of T_R cells with somewhat different properties in terms of their phenotypes and mechanisms of action (for a review see Shevach 2002). Thus, the data indicate that both in vitro and in vivo, such $CD4^+25^+$ T_R cells could act to suppress the activities of $CD4^+25^-$ cells, and one possible explanation for the break in tolerance observed in the *scurfy* mice could be the loss of nT_R cells.

Foxp3 and Tolerance

The nature of the pathology observed in *scurfy* mice suggested that the etiology of the disease might be due to a defect in tolerance. The nature of the mutation suggested that this defect would lie within the T cell compartment since this was the major population of cells that appeared to express the gene. We thus began to analyze the various forms of tolerance that had been described for T cells to determine what process(es) might have been affected by the mutation. Our earlier studies had suggested, in fact, that clonal deletion was operative within *scurfy* mice. This was based on examination of the repertoire of TCRs in mice carrying the *scurfy* mutation and indicated that those specific TCR families expected to be deleted were, in fact, deleted normally. More conclusive studies by Zahorsky-Reeves and Wilkinson demonstrated that *scurfy* mice carrying only an ovalbumin (OVA)-specific TCR (due to a RAG-1^{null} mutation) did not succumb to disease and that thymic deletion of these cells appeared normal in the presence of antigen (Zahorsky-Reeves and Wilkinson 2001). This suggested that, at least for the TCR–antigen combinations tested, deletion appeared normal. Because mice in which T cells could only recognize OVA failed to develop disease, the data further indicated that the T cells within the *scurfy* mice must recognize endogenous antigens in order to initiate pathology.

Since $CD4^+25^+$ T_R cells appeared to be critical for maintaining tolerance, Roli Khattri in our group examined the expression of *Foxp3* in $CD4^+$ T cell subsets. It was immediately apparent that the $CD4^+25^+$ subset was highly enriched for *Foxp3* mRNA expression compared to virtually all other cell types (Khattri et al. 2003). This suggested that the disease seen in *scurfy* mice was due to an inability to generate nT_R cells. Two other pieces of data supported this theory. First, we were unable to identify any suppressor activity in $CD4^+25^+$ cells from *scurfy* mice. Because CD25 is expressed on all activated cells, and many $CD4^+$ cells in *scurfy* mice are activated, we further attempted to isolate subsets of $CD25^+$ cells in an effort to identify suppressor activity, without success. The second, and more important, piece of data to suggest that *Foxp3* was involved in the generation of nT_R cells derived from a more detailed analysis of the *Foxp3* transgenic mice generated previously. These animals were developed to prove that the mutation in *Foxp3* was in fact the causal mutation in the *scurfy* strain as discussed. In five independent lines, each expressing a distinct amount of *Foxp3* message, crossing of the transgenic animal to *scurfy* mice

resulted in the prevention of disease in genotypically *sf*/Y animals. However, when all of these lines were bred onto an otherwise wildtype background, the resulting animals displayed a reduction in the number of peripheral T cells. Interestingly, the reduction in T cells was directly proportional to the amount of transgene expression. A variety of in vitro and in vivo analyses indicated that there was also a functional defect in the T cells from these transgenic mice. The more transgene expressed, the less responsive these cells were to stimulation. This was particularly obvious when IL-2 production was examined. Thus, the cells were virtually unresponsive in vitro, very much like nT_R cells. When tested in suppressor assays, however, it was discovered that the transgenic cells (even those that failed to express CD25) could mediate suppression toward normal T cells. This result was confirmed by others using retroviral transduction of *Foxp3* into $CD4^+25^-$ cells and demonstrating suppressor activity (Hori et al. 2003; Fontenot et al. 2003) in the transduced cells. Further studies using both the transgenic animals and the retrovirally transduced cells demonstrated that *Foxp3*-expressing cells could act as suppressor cells in vivo.

Thus, the cloning of the mutation in *scurfy* mice had identified a transcription factor involved in the generation of nT_R cells. The presence of *Foxp3* within most tissues (as assessed by Northern blot and PCR techniques) suggested that nT_R cells were also present within these tissues. Analyses of *Foxp3*–green fluorescent protein (GFP) knockin mice confirmed the presence of *Foxp3*-positive cells in a variety of nonlymphoid tissues. Initially it was this presence of *Foxp3* message in most tissues that led us to think that *Foxp3* was unlikely to be the gene responsible for disease in *scurfy* mice, as we had reasoned (incorrectly) that the mutated gene would be most highly expressed in lymphoid tissues. The data, however, support a model in which nT_R cells reside within peripheral tissues and are critical for preventing autoimmune responses. The data also demonstrate that this is a dominant form of tolerance in which autoreactive T cells are maintained in a quiescent state due to the inhibitory effects of nT_R cells. Unlike clonal deletion, suppression by nT_R cells can act to inhibit autoimmune responses that arise as a consequence of pathogen-specific T cells inadvertently recognizing normal self tissues due to similarities between pathogen and tissue antigens.

One of the most controversial and highly studied areas of research involves the induction of *Foxp3* expression. In the thymus there are a number of interactions that are required for selection of nT_R cells, including TCR-MHC II, CD28, and IL-2. The specific regions of the *Foxp3* promoter are just now being characterized, and the integration of factors that regulate expression is not yet fully determined (Mantel et al. 2006). While continued *Foxp3* expression in peripheral cells is critical to maintain tolerance, it remains unclear whether transient induction of *Foxp3* in responding T cells is necessary for tolerance, particularly in humans. The autoimmune pathology observed in day 3 thymectomized mice or in mice depleted of $CD25^+$ T_R cells is substantially less severe than that observed in *scurfy* mice. This could reflect the fact that these manipulations result in only partial depletion of nT_R cells, or the need to generate *Foxp3*$^+$ T_R cells subsequent to thymic development. The absolute requirement for *Foxp3* expression in peripheral T cells could support

either model. Nonetheless, the induced expression of *Foxp3*, its stabilization in peripheral T cells, or controlling both together presents a unique opportunity for therapeutic intervention in autoimmune disease. Regardless of whether this leads directly to the generation of T_R cells or simply a reduced responsiveness to stimulation, it could result in a diminished T cell response and thus diminished disease.

A final point emphasizes the utility of such mouse mutational analyses for biological annotation: when first identified, *Foxp3* was not found within the public EST database and was not present on any of the commercial microarray chips. Genes whose function is restricted to a particular cellular subset, especially a rare or not easily accessible subset, may be very difficult to functionally annotate if starting from expression data alone. Even when the relevant cellular distribution is unknown in advance, such as with *scurfy*, the existence of a demonstrable phenotypic effect of the mutation enables such annotation.

Mechanism of Action

How *Foxp3* acts to generate or maintain nT_R cells is still unclear. The original model suggested that *Foxp3* might act in much the same way that T-bet and GATA-3 are involved in the commitment of cells to specific differentiation pathways (Th1 and Th2, respectively). Recent data from two groups, however, suggest that *Foxp3* is not required to initiate the process of nT_R cell development (Lin et al. 2007; Gavin et al. 2007). In these studies, cells that express a nonfunctional *Foxp3* can still develop a number of nT_R characteristics. These cells, however, do not mediate suppressive activity. The data also support previous conclusions that *Foxp3* expression must be maintained in nT_R cells in the periphery in order to maintain tolerance. This followed from the observation that mice that expressed *Foxp3* only in the thymus (via a transgene) remain susceptible to disease in a manner very similar to *scurfy* mice. The overall conclusions to date suggest that during T cell development, some factor drives a subpopulation of T cells toward a regulatory lineage. One consequence of this process is the induction of *Foxp3*, the expression of which is absolutely required for the maintenance and suppressive activity of nT_R cells, which are in turn absolutely necessary for the normal control of the immune system. The factors responsible for the initial commitment to the nT_R lineage may involve TCR affinity (Picca et al. 2006), the nature of the antigen-presenting cell, the type and magnitude of costimulatory molecule engagement, and the cytokines present within the thymic microenvironment during development (Watanabe et al. 2005). Data from Aschenbrenner et al. (2007) suggest that medullary thymic epithelial cells have the ability to determine this fate. Since the ability to control and induce *Foxp3* (and more importantly, the suppressive state) is of great potential therapeutic value, characterizing the factors that induce and maintain *Foxp3* expression will remain a very active area of interest for some time to come.

From a more mechanistic perspective, whether *Foxp3* is involved in epigenetic or acute transcriptional events is yet to be determined. For T-bet and GATA-3, a key

component of their function appears to be the ability to remodel chromatin structure, although their overall mechanism of action is still not completely understood (Murphy and Reiner 2002). Most studies on *Foxp3* have focused on direct transcriptional activity, although one report suggests that chromatin remodeling via histone deacetylase binding and activation may be involved (Chen et al. 2006). As noted earlier, there is no apparent transcriptional activation domain for *Foxp3*, and preliminary data suggested that the protein is capable of inhibiting IL-2 transcription directly (Schubert et al. 2001). Recent data suggest that the *Foxp3* protein can dimerize with other transcription factors, notably NFATc (nuclear factor of activated T cells, cytoplasmic), and can thus alter the interactions of NFATc with AP-1 to inhibit IL-2 production (Wu et al. 2006). This is similar to the manner in which both GATA-3 and T-bet can interact with NFAT. Studies also indicate that *Foxp3* interacts with other transcriptional elements, including AML1, and can affect transcription of IL-2 through this pathway in an apparently independent manner (Ono et al. 2007). In addition to IL-2, chromatin immunoprecipitation (ChIP) assays have recently been performed and suggest that *Foxp3* binds to a number of relevant promoter sequences, including Ctla-4, CD25, and glucocorticoid-induced TNFR-related gene (GITR), among many others (Marson et al. 2007; Zheng et al. 2007). Combined with mRNA analyses, the data suggest that *Foxp3* binding (likely in a multimeric form with other factors) can both augment and inhibit transcription. This has been observed with other Fox-family members, and can depend upon the homo- or heterodimerization of these proteins.

The structure of the *Foxp3* protein predicts a number of functional domains, including zinc finger, leucine zipper, and forkhead binding domains. It would be predicted that the leucine zipper and zinc finger domains are involved in dimerization. Indeed, both *Foxp3* homodimerization and heterodimerization with NFAT and nuclear factor (NF)-κB have been described (Bettelli et al. 2005; Lopes et al. 2006). The proline-rich N-terminal portion of *Foxp3* is important for modulating gene expression as well, apparently independent of *Foxp3* dimerization with NFAT and possibly by recruitment of transcriptional corepressors, coactivators, or both (Ono et al. 2007; Lopes et al. 2006). In humans, an additional splice variant lacking exon 2 is also expressed at the protein level. At this point, no significant functional distinctions between the full-length protein and the variant lacking exon 2 have been identified, although this exon codes for sequences within the N-terminal domain of *Foxp3*. Emerging data clearly suggest that *Foxp3* binds to and forms a stable complex with NFATc that results in a pattern of gene expression distinct from AP-1:NFATc complexes. Determining which other factors are involved in the control of gene expression awaits further experimentation.

While the mutation discovered in the *scurfy* mouse results in a truncation and complete loss of function, there has been a variety of mutations identified throughout the *Foxp3* gene from human patients. These mutations include insertions, deletions, and missense mutations in the forkhead domain as well as other regions of the protein. There are also several examples of noncoding mutations that significantly affect mRNA levels. Changes within the forkhead domain have been shown to alter DNA binding (or intracellular localization) whereas mutations within other

domains, such as the leucine zipper, can alter dimerization. The individual mutations are now being examined for their specific effect on functional activity to better understand the mechanism(s) by which *Foxp3* controls immune responses. To date there have been approximately 20 distinct IPEX families with identified *Foxp3* mutations. It is interesting to note that all coding polymorphisms identified within the human *Foxp3* gene have resulted in IPEX disease. At present, no studies have demonstrated a convincing association between *Foxp3* and any autoimmune disease (other than IPEX), suggesting that this pathway is tightly regulated and that even minor alterations in expression or function lead to catastrophic disease.

Scurfy: Prospects

A number of aspects of *Foxp3* identification merit comment. The mutation arose spontaneously at ORNL from among a large-scale mouse genetics program. The fact that it was identified and subsequently established for future generations is a testament to the enthusiasm of the ORNL staff. We ourselves were fortunate, both in learning of this fascinating but little-studied animal and in being able to make the leap between the murine disease phenotype and human disease syndrome. The identification of the *scurfy* gene in the mouse led to a new understanding of the basic principles of immune regulation and tolerance. At the same time it brought resolution to a long-running series of family disease studies that had been initiated in the early 1980s and importantly offered a diagnostic tool for the identification of IPEX carriers. In the absence of the mouse mutation this identification of the human gene would have been a logistical challenge.

The study of the *Foxp3* mutant mouse provides a clear illustration of the power of using monogenic diseases in a phenotype-first approach. The compelling biology of the *scurfy* mutation and the phenotypic similarity to knockouts of two key immunoregulatory molecules, CTLA-4 and TGF-β, convinced us that the underlying mutation might reveal an equally important immunoregulatory gene. The resultant identification of the gene responsible for this disorder provided a transformative insight into a fundamental mechanism for controlling immune function. As evidenced by the number of PubMed citations (nine in 2001, greater than 1,000 by 2007), this result represents the type of substantial leap in understanding possible through a phenotype-driven discovery program.

Monogenic diseases in both the mouse and in humans have played an important historical role in revealing the mechanistic basis of normal and disease processes. Yet despite considerable successes in disease mapping and cloning, a sizeable proportion of the genome remains to be annotated. Furthermore, the existing collection of spontaneous mouse mutations is limited and there remains a gulf between the spectrum of phenotypes afflicting these animals and those which one might expect to find based on the spectrum of genetic diseases in humans: classical mouse mutations are representative of only a subset of those diseases afflicting the human population. This disparity (the “phenotype gap”) likely reflects a historical bias

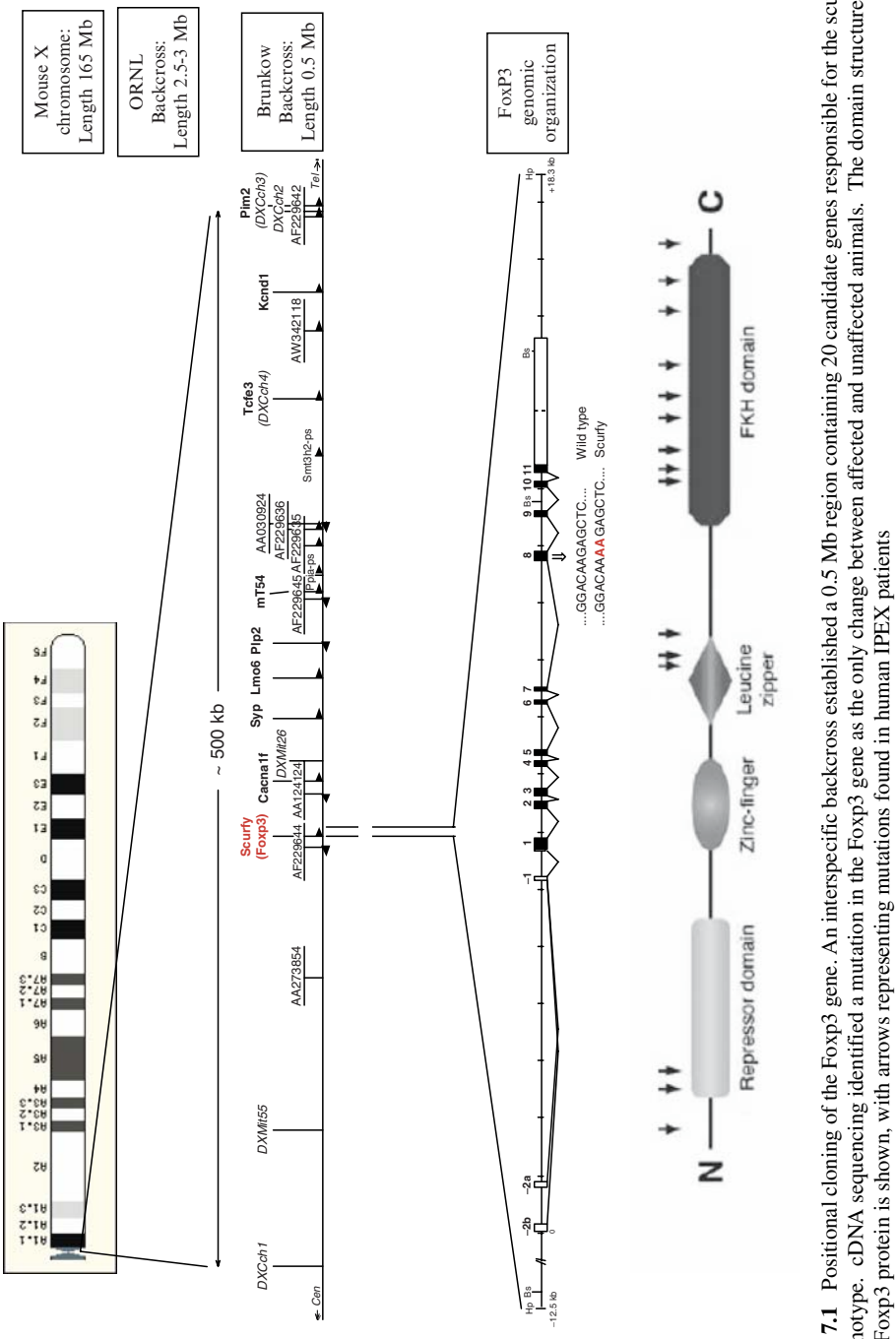

Fig. 7.1 Positional cloning of the Foxp3 gene. An interspecific backcross established a 0.5 Mb region containing 20 candidate genes responsible for the scurfy phenotype. cDNA sequencing identified a mutation in the Foxp3 gene as the only change between affected and unaffected animals. The domain structure for the Foxp3 protein is shown, with arrows representing mutations found in human IPEX patients

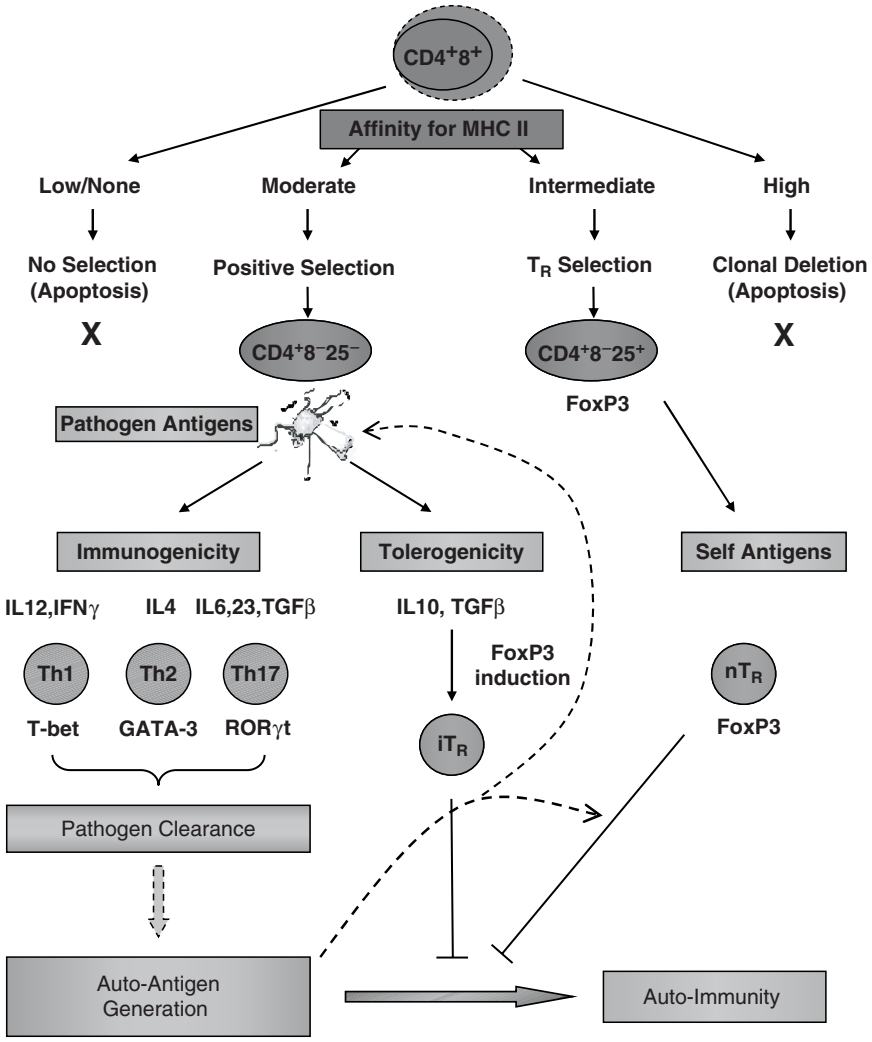

Fig. 7.2 A model for the continuum of development of Regulatory T Cell development in the thymus (top section) and periphery (lower section). Multiple factors control the expression of Foxp3, as well as other genes required for regulatory T cell development and activity (see text)

toward the identification of easily discernible visible mutant phenotypes (Brown and Peters 1996). A number of groups have set out to close this phenotype gap by combining traditional mouse mutagenesis, based on that pioneered by ORNL, with high throughput phenotyping (Beutler et al. 2006; Cook et al. 2006). Just as the *scurfy* mutation arose spontaneously from the directed mutagenesis activities at ORNL, we can only hope that these latest genome-wide efforts will spawn equally elucidative phenotypes.

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Fevers, Genes, and Innate Immunity

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Contents

Abstract The characterization of patients with recurrent inflammatory syndromes into distinct clinical phenotypes provided early clues to the mode of inheritance of these conditions and facilitated the subsequent identification of causative gene mutations. The prototype autoinflammatory syndrome, familial Mediterranean fever, is characterized by self-limiting episodes of localized inflammation. Hallmarks of the classical autoimmune response are largely absent. The use of positional cloning techniques led to the identification of the causative gene, *MEFV*, and its product pyrin. This previously unrecognized protein plays an important role in modulating the innate immune response. Cryopyrin, the protein encoded by *CIAS1*, is mutated in a spectrum of autoinflammatory conditions, the cryopyrinopathies. In response to a wide range of potential pathogens, it forms a macromolecular complex termed the "inflammasome," resulting in caspase-1 activation and subsequent release of the active proinflammatory cytokine interleukin-1β (IL-1β). The role of an established biochemical pathway in regulating inflammation was uncovered by the discovery that the hyperimmunoglobulin D with periodic fever syndrome (HIDS) results from mutations in *MVK*, which encodes an enzyme in the isoprenoid pathway. The discovery that mutations in the gene encoding tumor necrosis factor (TNF) receptor 1

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(TNFR1) cause a proinflammatory phenotype was unanticipated, as it seemed more likely that such mutations would instead have resulted in an immunodeficiency pattern. This review describes the clinical phenotypes of autoinflammatory syndromes, the underlying gene mutations, and current concepts regarding their pathophysiology.

Introduction

The study of Mendelian recurrent inflammatory syndromes, through the identification of new proteins and the recognition of novel roles for established biochemical pathways, has led to a deeper understanding of the innate immune response. Each of these syndromes is characterized by episodic inflammation without a clear antigenic stimulus. In addition, features typical of the autoimmune diseases are notable by their absence, namely autoreactive antibodies and T cells. The term "autoinflammatory diseases" was proposed to encompass this new group of disorders (McDermott et al. 1999). These conditions result from the aberrant activation of the innate, rather than the adaptive, immune system. In the case of familial Mediterranean fever, the discovery of the causative gene resulted in the identification of a novel protein called pyrin. The discovery that hyperimmunoglobulin D with periodic syndrome resulted from a mutation in an enzyme in the isoprenoid biosynthesis pathway has suggested a novel role for this established biochemical pathway. In this chapter we will discuss the Mendelian autoinflammatory diseases, the discovery of causative genes, and current concepts regarding their pathophysiology. In addition, we will describe how knowledge of the basic science underlying these disorders has led to targeted therapies that have proven life altering for many patients with these diseases.

Familial Mediterranean Fever

Familial Mediterranean fever (FMF) [OMIM 249100 (Online Mendelian Inheritance in Man 2008)] is the prototype autoinflammatory disease. Early reports highlighted a preponderance of individuals of Mediterranean ancestry with this condition, leading to the adoption of the term FMF. It is the most common of the autoinflammatory diseases, with a modest male preponderance, and is most frequently observed in Jewish, Armenian, Arab, Turkish, and southern Italian populations (Aksentijevich et al. 1999). Carrier rates in certain high-risk populations can reach 1 in 3.

FMF is characterized by acute attacks of fever, and localized inflammation of the peritoneum, pleura, joints, or skin, sometimes in combination. Typically, episodes last 12–72 hours. Episodes may vary in nature; childhood episodes may be manifested by fever alone, while other features may develop progressively with time. Abdominal symptoms range from mild discomfort to severe pain and abdominal rigidity. The most serious complication of FMF is the development of AA amyloidosis. Prior to the advent of effective therapy for FMF, in many patients the development of amyloidosis led to chronic renal failure by age 40 (Samuels et al. 1998).

In the 1960s, segregation analysis in Israeli families manifesting typical FMF symptoms established FMF as a single-gene recessive disorder with incomplete penetrance. Attempts to identify the FMF gene by functional hypothesis-driven approaches were not productive and positional cloning was ultimately employed. Linkage studies in 1992 placed the FMF susceptibility locus on the short arm of chromosome 16, and this area of interest was narrowed by analyses of genetic recombinations in families and conserved haplotypes in populations. All genes within a refined 200-kb interval were screened for disease-associated mutations; and two independent consortia identified *MEFV* (The International FMF Consortium 1997; The French FMF Consortium 1997). An online database (Infevers 2008) has been established and provides an updated list of mutations and polymorphisms in *MEFV* and other autoinflammatory diseases. Over 50 disease-associated *MEFV* mutations have been described, with many clustered on exon 10 (Infevers 2008).

MEFV (*ME*diterranean *FE*ver) consists of 10 exons, and covers approximately 15 kb of DNA. It encodes a 781-amino acid protein named pyrin (to denote fever) or marenostrin (from the Latin for the Mediterranean sea). Pyrin is expressed in skin, peritoneal fibroblasts (Matzner et al. 2000), synovial fibroblasts, granulocytes, dendritic cells, and monocytes (Diaz et al. 2004). Pyrin is a member of the tripartite motif (TRIM) family of proteins, and is composed of four domains. At the N-terminal end of pyrin is the pyrin domain (PYD), a 92-amino acid motif, encoded by exon 1 of *MEFV*. This domain bears structural homologies to caspase-recruitment (CARD) domains, death domains, and death effector domains, and together these four motifs constitute the death fold family (Fairbrother et al. 2001). Variants of the pyrin domain are present in approximately 20 proteins, each of which plays a role in modulating the innate immune response. The PYD of pyrin engages in homotypic interactions with an adaptor protein called ASC (apoptosis-associated speck-like protein with a CARD) influencing the activation of interleukin (IL)-1β. Deletion of the PYD of pyrin abolishes its interaction with ASC (Richards et al. 2001; Yu et al. 2006).

The C-terminal end of pyrin contains a B-box zinc-finger domain (B-box), an α-helical coiled-coil domain (CC), and a B30.2 (PRYSRPY) domain. The B-box domain is both necessary and sufficient for pyrin's interactions with proline serine threonine phosphatase interacting protein (PSTPIP1) (Shoham et al. 2003). The B-box has recently been shown to interact with the PYD and thereby to block its interaction with ASC, thus serving as an intramolecular inhibitor (Yu et al. 2007).

The CC domain of pyrin mediates the formation of a homotrimer, a process required for pyrin's recently demonstrated induction of ASC oligomerization and subsequent caspase-1 activation (Yu et al. 2007) . The CC domain is also necessary, but not by itself sufficient, for pyrin's interaction with PSTPIP1 (Shoham et al. 2003).

The B30.2 domain of pyrin is responsible for interactions with the NACHT (Koonin and Aravind 2000) domain of NALP3, a component of the inflammasome (Papin et al. 2007). The B30.2 domain interacts, albeit weakly, with pro-caspase-1, and more avidly with active cleaved caspase-1 (Chae et al. 2006; Papin et al. 2007). There is also speculation that the B30.2 domain of pyrin acts as an intracellular

pathogen-associated molecular pattern (PAMP) sensor, and that mutations in pyrin may bind PAMPs more avidly and thereby confer a heightened immune response to potential pathogens with possible survival benefit (Yepiskoposyan and Harutyunyan 2007). In support of this theory is the recognition of the role for members of the TRIM family proteins in control of retroviral infections (Yap et al. 2004). Interestingly, the amino acid changes that cause FMF are often present as wildtype in other species. For several human mutations, the mutant represents the reappearance of an ancestral amino acid state. Studies in primates suggest that these mutations are indeed counter-evolutionary changes selected to cope with a sporadically encountered pathogen (Schaner et al. 2001).

There is a general consensus that pyrin plays a role in modulating caspase-1 activity and subsequent IL-1β release; there is disagreement, however, as to its net effect on levels of IL-1β. Findings in keeping with a net negative effect include the demonstration that pyrin competitively binds with ASC, via PYD, preventing ASC binding to caspase-1 (Chae et al. 2003). Pyrin also binds caspase-1, via its B30.2 domain, thus reducing caspase-1 activation (Chae et al. 2006; Papin et al. 2007). In addition, pyrin's competitive interaction with ASC may prevent the formation of the NALP3 inflammasome. The "sequestration hypothesis" has been proposed to encompass pyrin's net negative effect on IL-1β release. Further support for an inhibitory role for wildtype pyrin comes from mouse constructs. Mice expressing truncated pyrin produce increased amounts of activated caspase-1 and IL-1β in response to stimuli (Chae et al. 2003). Most mutations in patients with FMF affect the B30.2 domain, which is responsible for protein–protein interactions. Recent data that common mutations in pyrin result in impaired binding of pyrin to caspase-1 imply that these mutations may lead to clinical disease by impairing pyrin's antiinflammatory interactions with caspase-1 (Chae et al. 2006). However, the impact of mutations in the B30.2 domain has been variable (Papin et al. 2007).

A net positive effect of pyrin on IL-1β levels is suggested by data that fresh human monocytes have elevated pyrin protein and mRNA compared to monocyte-derived macrophages, a finding that parallels their ability to release active IL-1β in response to lipopolysaccharide (LPS) stimulation (Seshadri et al. 2007). In contrast to the competitive binding described previously, it has been suggested that pyrin's interaction with ASC modulates the formation of the "pyroptosome," a protein complex involved in "pyroptosis," a recently described caspase-1-dependent form of inflammatory cell death. The adaptor protein ASC contains an N-terminal PYD and C-terminal CARD. In response to various stimuli, including LPS and potassium flux, ASC self associates via its PYD domain, and forms a supramolecular assembly, termed the pyroptosome (Fernandes-Alnemri et al. 2007). This ASC supramolecular assembly activates caspase-1, thus leading to elevated IL-1β. This process is independent of the recently described inflammasomes formed by members of the NALP family. Moreover, data using THP-1 human monocytic cell lines suggest that PSTPIP1 binding to pyrin may lead to PYD interaction with ASC, pyroptosome assembly, and procaspase-1 activation (Yu et al. 2006, 2007).

Colchicine has been the mainstay in the therapy of FMF since the 1970s (Goldfinger 1972). Clinical trials support the role of colchicine in the treatment of

acute episodes, in the prevention of FMF attacks (Zemer et al. 1974), and in reducing the risk of AA amyloidosis. The induction of *MEFV* mRNA by the addition of a combination of colchicine and interferon (IFN)-α suggests a role for *MEFV* in the antiinflammatory actions of these agents (Centola et al. 2000). Pyrin's close association with actin filaments and microtubules suggests a role for pyrin in directed migration that can be modulated by colchicine (Mansfield et al. 2001). Colchicine displays a dose responsive effect on microtubule function and structure. At high concentrations colchicine disrupts microtubules by inhibiting polymerization at the "plus" or growing terminus. Low concentrations inhibit tubulin exchange at microtubule ends without affecting polymerization. High doses of colchicine inhibit the processing of caspase-1, a finding replicated with nocodazole, a compound with microtubule-inhibiting properties (Yu et al. 2007). The low concentrations resulting from the usual doses given to patients with FMF, however, are insufficient to disrupt microtubule arrangement patterns, but may instead affect microtubule dynamics, resulting in defective trafficking of cell adhesion molecules. As not all patients benefit from colchicine, in some, adjunctive therapy with anakinra, an IL-1 receptor antagonist, has proved beneficial, supporting the role of IL-1β in the pathogenesis of FMF (Calligaris et al. 2007; Chae et al. 2006).

Cryopyrin-Associated Periodic Syndromes

The cryopyrinopathies or cryopyrin-associated periodic syndromes (CAPS) correspond to a spectrum of dominantly inherited disorders. They include familial cold autoinflammatory syndrome (FCAS)/familial cold urticaria, Muckle–Wells syndrome (MWS) and neonatal onset multisystem inflammatory disease (NOMID)/chronic infantile neurologic cutaneous and articular syndrome (CINCA). All three may present with fever and urticaria-like skin rash and varying degrees of joint and neurologic involvement. FCAS (OMIM 120100) is generally considered the mildest, with distinct cold-induced episodic attributes. NOMID (OMIM 607115) is typically the most severe, with nearly continuous symptoms that may fluctuate in severity. Persistent central nervous system inflammation may result in intellectual impairment and loss of vision. NOMID is associated with a deforming arthropathy (Prieur 2001). MWS (OMIM 191900) is intermediate, with urticarial rash that is not cold-induced, and some patients develop sensorineural hearing loss. The clinical boundaries of these conditions have become blurred and a greater degree of overlap is now recognized. Patients with CAPS may develop AA amyloidosis and subsequent renal failure.

Independent linkage studies placed the susceptibility locus for both MWS and FCAS on chromosome 1q (Cuisset et al. 1999; Hoffman et al. 2000). In 2001, mutations in a 9-exon gene were identified in both FCAS and MWS families (Hoffman et al. 2001); the next year mutations in the same gene were identified in patients with NOMID (Aksentijevich et al. 2002; Feldmann et al. 2002). The gene named *CIAS1* (for cold-induced autoinflammatory syndrome-1) (NALP3/PYPAF1/NLRP3, OMIM

606416) encodes the protein cryopyrin. Cryopyrin is composed of an N-terminal pyrin (PYD) domain, a NACHT/nucleotide-binding oligomerization domain (NOD) and a leucine-rich repeat (LRR) domain. Cryopyrin is expressed in the cytoplasm of non-keratinized epithelial cells, uroepithelial cells, granulocytes, dendritic cells, and both T and B cells. It is also weakly expressed in monocytes (Kummer et al. 2007). Cryopyrin is a member of the CATERPILLER family (Ting et al. 2006) and is also called NALP3 (for NACHT domain-, Leucine-rich-repeat-, and Pyrin domain-containing protein 3) (Tschopp et al. 2003). Cryopyrin bears homologies with the extended NALP family of proteins, the plant cytosolic resistance (R) proteins, which mediate resistance to a variety of fungi, viruses, and bacteria, and the NOD family, a member of which, NOD2/CARD15, is mutated in patients with Crohn's disease and Blau syndrome (Miceli-Richard et al. 2001; Rosé et al. 2006).

The PYD of cryopyrin is involved in cognate interactions with other proteins containing a PYD. The NACHT domain is thought to regulate oligomerization. The LRR domain, which is found in a number of proteins including the Toll-like receptors (TLRs), may mediate interactions with numerous intracellular and extracellular potential pathogens.

Stimulation of cryopyrin leads to the formation of a macromolecular complex called the "NALP3 inflammasome." This inflammasome is formed by homotypic interactions between the PYD domain of cryopyrin and ASC, which in turn interacts via its CARD domain with caspase-1. Cryopyrin also interacts with another adaptor protein CARDINAL, which recruits additional caspase-1. The resultant homo-oligomerization of procaspase-1 is thought to facilitate autocatalytic activation to caspase-1. Active caspase-1 cleaves pro-IL-1β into mature proinflammatory IL-1β (Tschopp et al. 2003).

The demonstration that the NALP3 inflammasome activates IL-1β release in response to gram-negative bacteria in the absence of cell surface TLR4 (Kanneganti et al. 2007) strongly suggests a role for cryopyrin in the intracellular control of infectious agents. Cryopyrin's role as an intracellular sensor of so-called PAMPs has expanded greatly recently. Other activators of the inflammasome include bacterial RNA, imidazoquinoline compounds and the gram-positive bacterial toxins nigericin and maitotoxin (Kanneganti et al. 2006; Mariathasan et al. 2006). Knockout models suggest that cryopyrin plays a central role in the robust inflammatory response to both uric acid and calcium pyrophosphate (CPPD) crystals (Martinon et al. 2006). These findings suggest that both gout (uric acid) and pseudogout (CPPD) are, at least in part, autoinflammatory diseases. The means by which cryopyrin senses PAMPs remains unclear; homologies to the LRR domain present in both TLRs and NOD2/CARD15, however, may suggest a role for this domain in pathogen sensing.

The inflammasome complex alone does not appear to be sufficient for the IL-1β-mediated inflammatory response since knockout experiments implicate SGT1 and HSP90 as being essential for inflammasome activity (Mayor et al. 2007). The precise role of these proteins in modulating the inflammatory response remains uncertain. Cryopyrin may also induce cell death upon stimulation with bacteria or other pathogens independent of ASC and IL-1β (Willingham et al. 2007).

Mutations in cryopyrin lead to constitutive activation, although the molecular mechanism is not clear. Modeling of the cryopyrin structure suggests that the LRR domain self-associates with the NACHT domain, thus preventing activation and interaction with the adaptor protein CARDINAL. Mutations in either the NACHT or LRR domains may prevent self-association, resulting in direct assembly of the inflammasome complex. Most of the described mutations are found in exon 3 of *CIAS1*, which encodes the NACHT domain. Current models of cryopyrin mutants inadequately explain the spectrum of disease seen in CAPS (Aksentijevich et al. 2007; Hentgen et al. 2005), reflecting the limitations of in silico techniques.

Early insights into the role of the inflammasome, supported by the findings that monocytes from patients with CAPS showed increased caspase-1 activation and increased IL-1β release, prompted clinical trials of IL-1 inhibition in patients with CAPS. The use of anakinra, an IL-1 receptor inhibitor, in the treatment of all three syndromes has been met with considerable success, consistent with the key role of IL-1β in CAPS (Hawkins et al. 2003). The use of anakinra in patients with NOMID, the most severe of these conditions, resulted in complete remission in both peripheral and central nervous system (CNS) inflammation in a majority of subjects. Discontinuation of therapy led to a rapid relapse in symptoms, supporting the need for continuous IL-1 blockade in this condition (Goldbach-Mansky et al. 2006). The use of IL-1 blockade represents a significant advance in the treatment of CAPS.

Syndrome of Pyogenic Arthritis, Pyoderma Gangrenosum and Acne

The syndrome of pyogenic arthritis, pyoderma gangrenosum and acne (PAPA) was first described in a large family who attended the Mayo Clinic (Lindor et al. 1997); another family in Texas was later noted to have similar clinical features (Wise et al. 2000). From childhood, patients have episodic destructive arthritis that is sometimes triggered by minor trauma. Arthritis may lead to periosteal proliferation and ankylosis. Skin manifestations usually occur after puberty and range from severe cystic acne on the face, chest, or back to pyoderma gangrenosum, an ulcerating skin lesion that may be triggered by minor trauma.

Studies of both of the originally described families established linkage to chromosome 15q (Wise et al. 2000). Two different missense mutations were identified in the gene encoding PSTPIP1. This protein interacts with pyrin, the protein mutated in FMF, and is largely restricted to the hematopoietic tissues, being prominent in spleen and peripheral blood leukocytes (Li et al. 1998; Shoham et al. 2003).

PSTPIP1 protein contains an N-terminal CIP4 domain, a coiled-coil (CC) domain, and an SH3 domain. The coiled-coil and SH3 domains are important for protein interactions and both are necessary for PSTPIP1's interaction with pyrin's B-box domain (Shoham et al. 2003). PSTPIP1 also interacts via its CC domain with the C-terminal proline-rich homology domain of PTP-PEST (protein tyrosine phosphatase with a proline, glutamate, serine, threonine domain). The mutations

identified in PAPA patients affect the coiled-coil domain of PSTPIP1 and lead to decreased binding of PSTPIP1 to PTP-PEST, which in turn leads to hyperphosphorylation of PSTPIP1. The hyperphosphorylation of PSTPIP1 increases its avidity for pyrin (Shoham et al. 2003; Yu et al. 2007). Cell lines co-transfected with PAPA-associated PSTPIP1 mutants and with pyrin demonstrate elevated production of interleukin-1β (Shoham et al. 2003). While it is agreed that mutant PSTPIP1 binds pyrin with greater avidity, the precise mechanism by which increased IL-1β production results is unclear. It has been proposed that when mutant PSTPIP1 binds more avidly to pyrin, it results in the sequestration of an antiinflammatory pyrin resulting in a net increase in IL-1β production (Shoham et al. 2003). Alternatively, when mutant PSTPIP1 avidly binds pyrin it may permit the unfolding of pyrin to its proinflammatory state, permitting the interaction of PYD with ASC and subsequent pyroptosome-mediated caspase-1 activation (Yu et al. 2007).

As denoted by its alternative name CD2-binding protein 1 (CD2BP1), PSTPIP1 interacts not only with CD2 but also Wiskott–Aldrich syndrome protein (WASp). WASp co-localizes with actin to form the immunologic synapse in natural killer (NK) cells (Orange et al. 2002). Thus, PSTPIP1 may play a role in the adaptive immune system through both CD2 binding and WASp-induced polymerization with actin, with potential effects on the formation of immunologic synapses in antigen recognition (Badour et al. 2003).

Therapeutic strategies have been informed by evidence that LPS-induced IL-1β secretion is markedly increased in cells from PAPA patients compared to controls (Shoham et al. 2003). In contrast, cytokines such as IL-2, IL-5, and IFN-γ were undetectable. Anecdotal reports suggest that anakinra, an IL-1 receptor antagonist, is beneficial (Dierselhuis et al. 2005); however, both the variable natural history and rarity of this condition suggest that definitive clinical trials of targeted therapies will prove difficult.

TNF Receptor-Associated Periodic Syndrome

Clinical descriptions of families of individuals with prolonged fever and localized inflammation were the first hints of an autosomal-dominant recurrent fever syndrome. Early clinical reports highlighted the Celtic ethnicity of affected individuals, resulting in the term “familial Hibernian fever” (McDermott et al. 1997), while smaller family series described a broader North European ancestry and used the term “autosomal-dominant recurrent fever syndrome.” Now known as tumor necrosis factor (TNF) receptor-associated periodic syndrome (TRAPS), patients typically have episodes lasting at least 1 week and sometimes as long as 6–8 weeks. Characteristic manifestations include migratory erythema which may occur on the torso or limbs and spreads distally, with myalgia in the underlying muscle group. Ocular involvement with conjunctivitis or periorbital edema is common. TRAPS is associated with an increased risk of AA amyloidosis in up to 15% of affected individuals.

In 1998, linkage studies placed the susceptibility locus of a subset of these individuals to a region of chromosome 12p13 (McDermott et al. 1998; Mulley et al. 1998). Within a year *TNFRSF1A* (OMIM entry 191190) was identified as the causative gene (McDermott et al. 1999). Dominantly inherited mutations were identified in families of diverse ancestry, and the term TRAPS (OMIM entry 142680) is used to describe all patients with mutations in *TNFRSF1A* irrespective of ancestry.

TNFRSF1A encodes a 55-kDa receptor (TNFR1/p55) for the cytokine TNF. This receptor has four extracellular cysteine-rich domains, a transmembrane domain, and an intracellular death domain. TNFR1 is expressed on a wide range of cell types and can mediate apoptosis and function as a regulator of inflammation. Of the initial six mutations that were described, five were single-nucleotide substitutions resulting in amino acid substitutions in highly conserved extracellular cysteine domains (McDermott et al. 1999). These cysteine residues are required to maintain the stability of the extracellular domain by forming disulfide bonds. A further mutation disrupted a highly conserved intrachain hydrogen bond in the extracellular domain. Mutations in the intracellular or transmembrane domains have not been identified.

Initial studies of affected families suggested that disease-associated mutations are highly penetrant. Not all mutations, however, are associated with such high penetrance or typical disease. Two substitutions occur in over 1% of the Caucasian (R92Q) and African-American (P46L) populations, and one substitution occurs in up to 9% of selected African (P46L) populations. While these substitutions may lead to a proinflammatory phenotype, patients typically do not appear to have typical TRAPS (Tchernitchko et al. 2005).

The pathophysiology of TRAPS remains unclear. In healthy subjects, TNF stimulates TNFR1, which recruits several proteins to form a complex resulting in the activation of nuclear factor-κB (NF-κB), while an alternate pathway leads to apoptosis (Chen and Goeddel 2002). TNFR1 stimulation results in cleavage of the extracellular domains of the receptor following activation. Cleaved soluble TNFR1 (sTNFR1) acts as a decoy receptor for TNF. In patients with TRAPS, low levels of cleaved soluble TNFR1 led to the hypothesis that mutations lead to impaired cleavage of the extracellular domain of TNFR1 by matrix metalloproteinases following stimulation. Thus, the usual regulatory processes in terminating TNF signaling were impaired, with insufficient sTNFR1 to act as a TNF blocker in serum and excess activated TNFR1 remaining on the cell surface. This was supported by early laboratory studies (McDermott et al. 1999); subsequent reports, however, demonstrated variable rates of cleavage across disease-associated mutations, and thus did not fully explain the pathophysiology of this disease. Defective intracellular processing of mutant TNFR1 has also been identified. Following synthesis, mutant TNFR1 is unable to traffic appropriately from the endoplasmic reticulum to the cell surface (Lobito et al. 2006; Todd et al. 2007). It has been postulated that mutant TNFR1 is retained in the endoplasmic reticulum resulting in the proinflammatory unfolded protein response. Thus, inflammation may occur without direct ligand interactions with TNFR1. Alternatively, mutant receptors may be retained within the cell, autoaggregate, and inappropriately activate proinflammatory cascades

(Rebelo et al. 2006). Collectively this is termed the "ligand-independent" hypothesis. Mutant TNFR1 may also result in prolonged survival of inflammatory cells, since neutrophils from patients with TRAPS demonstrate impaired TNF-mediated apoptosis (D'Osualdo et al. 2006).

The initial "defective shedding" hypothesis, whereby the decoy receptor for TNF, cleaved soluble TNFR1, is decreased in patients with TRAPS, led to efforts to restore the balance in favor of inhibition of TNF using etanercept. Etanercept is a p75 TNF receptor fusion protein that binds serum TNF and prevents its engagement with cell surface TNFR1. Use of this agent has been beneficial in terms of clinical and laboratory parameters (Hull et al. 2002). In keeping with the alternative "ligand-independent" hypothesis, in which inflammation results from non-TNF-mediated pathways, the use of anakinra, an IL-1 receptor antagonist, has also been reported to be of benefit (Simon et al. 2004).

Hyperimmunoglobulinemia D with Periodic Fever Syndrome

In 1984 a Dutch group described six patients with periodic fever and elevations in immunoglobulin D (van der Meer et al. 1984). In 1999 two independent Dutch groups identified the gene responsible for Hyperimmunoglobulinemia D with periodic fever syndrome (HIDS), an autosomal-recessive condition. Traditional linkage analysis enabled one group to localize the causative gene (Drenth et al. 1999), while the other group identified elevated mevalonate in the urine of patients with HIDS and decreased enzyme activity in skin fibroblasts (Houten et al. 1999). The causative gene, *MVK*, encodes an enzyme involved in the isoprenoid biosynthesis pathway, and this unanticipated finding suggested a new role for this pathway in regulating inflammatory responses. The isoprenoid metabolism pathway generates a wide variety of important compounds for cell function. Branches of the pathway synthesize over 20,000 compounds including sterols, which includes cholesterol, and the nonsterol isoprene compounds. Two isoprenoid moieties, farnesyl or geranylgeranyl, are added to proteins during posttranslational modification, thus promoting membrane association.

HIDS episodes usually start in infancy, and may be triggered by immunizations. Episodes may occur once or twice per month and typically last 3–7 days. Typical episodes are characterized by initial chills and headache with subsequent fevers and diffuse tender lymphadenopathy. Diarrhea frequently occurs and a number of cutaneous manifestations have been described including painful erythematous macules. Joint symptoms include arthralgia and polyarticular large joint arthritis. Episodes typically occur less frequently in adulthood and are usually less severe (Drenth et al. 1994).

Mutations in *MVK* that result in complete loss of mevalonate kinase (MK) enzymatic activity cause the related condition mevalonic aciduria, a rare metabolic disease with mental retardation, failure to thrive, and early death, in addition to the features seen in HIDS. In contrast, HIDS-associated *MVK* mutations result in

residual MK enzymatic activity, in the range of 1%–8% of normal (Mandey et al. 2006a). Interestingly, in vitro experiments demonstrate that MK enzymatic activity is temperature-sensitive, with decreased activity at higher temperatures (Houten et al. 2002).

Mutations in *MVK* are found throughout the gene, and most HIDS patients are compound heterozygotes for missense mutations. A number of mutations are more strongly associated with either HIDS or MA. Mutations resulting in a base pair change at position 377 (V377I) are most commonly associated with HIDS, and result in modest decreases in enzymatic activity, in contrast to predictions that this mutation would not affect enzymatic activity based on modeling studies (Mandey et al. 2006b). This mutation exhibits a founder effect in the Dutch population, and likely explains the higher prevalence of HIDS in this population. Population-based studies indicate that 0.6% of Dutch people carry the V377I mutation. Given the marked underrepresentation of homozygote V377I patients in HIDS cohorts, it has been suggested that the homozygous state results in either a milder phenotype or none at all (Houten et al. 2003).

The activity of the isoprenoid pathway is tightly controlled. An early rate-limiting step, undertaken by HMG-CoA (3-hydroxy-3-methyl-glutaryl-CoA) reductase, has been extensively studied. The identification of this enzyme, and use of the "statin" group of HMG-CoA reductase inhibitors, has been a major advance in the treatment of hypercholesterolemia. The next step in isoprenoid synthesis is MK, resulting in the phosphorylation of mevalonate to 5-phosphomevalonate. MK deficiency results in an increase in HMG-CoA reductase activity, which may increase mevalonate concentrations.

In HIDS there is no general deficiency in isoprenoid end products, and serum cholesterol levels are only slightly decreased. Mevalonate levels are increased to detectable levels in urine during attacks.

Manipulation of the isoprenoid pathway with statins, in an effort to reduce mevalonate levels, has been considered to have antiinflammatory effects, although contradictory reports have emerged. The use of statins in patients with MA resulted in acute flares in two patients within weeks of commencement of a statin; in contrast, six HIDS patients treated for 6 months did show a decrease in symptoms (Simon et al. 2004).

Peripheral blood mononuclear cells (PBMCs) from patients with HIDS show excess IL-1β production in response to LPS stimulation, a finding that can be reversed by the addition of geranylgeranyl, an isoprenoid deficient in HIDS (Mandey et al. 2006a). In contrast, the addition of mevalonate, which is elevated in HIDS, also reduced IL-1β production, suggesting that it is not central to the proinflammatory phenotype. Apoptosis of activated cells is a key mechanism in the termination of the inflammatory response and, in keeping with the protracted inflammatory response seen in HIDS, defective apoptosis has been observed in PBMCs from patients with HIDS (Bodar et al. 2007).

Therapeutic options in HIDS are limited. As stated earlier, there are conflicting results regarding the usefulness of statins. Manipulation of the isoprenoid biosynthesis pathways to augment the production of nonsterol isoprenoids has been

suggested as a potential therapeutic option based on in vitro studies (Schneiders et al. 2006). Elevations in urinary leukotrienes during HIDS episodes led to the trial of oral leukotriene receptor antagonism, using montelukast, with anecdotal reports suggesting clinical benefit. The demonstration of elevated serum TNF led to a pilot study involving the use of etanercept, the p75 TNF receptor fusion protein, which demonstrated clinical benefit (Takada et al. 2003).

Future Directions

The study of rare autoinflammatory syndromes has informed us of novel proteins involved in the innate immune response. These proteins provide further evidence for the complexity of this ancient host defense system. Many of these recently described proteins are involved in intracellular pathogen sensing, a previously under-recognized role for the innate immune system. Despite the progress made in recent years, there remain a significant number of patients with recurrent fevers in whom the previously described gene defects cannot be found. Efforts are ongoing to identify new genes. Regarding the known genes and their products, the accumulating evidence highlights the difficulties encountered in unraveling their interactions. Further information regarding the roles of these proteins should in time allow clear elucidation of their complex interplay with the cell and host environment.

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Itchy Mice: The Identification of a New Pathway for the Development of Autoimmunity

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Contents

Abstract *Itchy* mice possess a loss-of-function mutation in a HECT-domain-containing ubiquitin ligase (E3), Itch. Homozygous *itchy* mice develop a systemic and progressive autoimmune disease that proves lethal beginning at 6 months of age. Numerous targets of Itch-mediated ubiquitination have been identified, and some of these have defined physiological roles for Itch signaling in T cell anergy and T cell differentiation. Studies of *itchy* mice have also allowed for the identification of a novel pathway involved in autoimmunity: noncanonical Notch signaling. In *itchy* mice carrying an activated *Notch1* transgene, there are increased amounts

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of full-length Notch1, which can complex with $p56^{lck}$ and PI3K to activate a cell survival signal that is mediated by phospho-AKT. This, in turn, leads to a reduction in apoptosis in the thymus and may have consequences in T cell tolerance. A role for noncanonical Notch signaling in autoimmune disease is also supported by numerous mouse knockout studies, and suggests possible new therapeutic approaches for the treatment of autoimmune disease.

Abbreviations HECT: Homologous to E6-AP carboxy terminus; Ub: UbiquitinE1 Ubiquitin-activating enzyme; E2: Ubiquitin-conjugating enzyme; E3: Ubiquitin ligase; RING Really interesting new gene; Ndfip1: Nedd4 family interacting protein 1; ICN Intracellular fragment of Notch; *Su(dx):* Suppressor of *deltex*; FL: Full length

Introduction

Autoimmune disease is a collection of more than 80 discrete clinical entities including systemic lupus erythematosus, type I diabetes, and multiple sclerosis. This group of diseases is estimated to affect upwards of 3% of the United States population and therefore significantly contributes to healthcare costs, morbidity, and mortality (Jacobson et al. 1997). Common to all autoimmune disease is the loss of self vs nonself discrimination in the adaptive immune system. This loss of tolerance ultimately results in the destruction of the body's own tissues by immune effector cells. Most often, autoimmune disease is initiated by the malfunction of a T cell. Normally, T cells play a prominent role in the elimination of invading pathogens. They develop in the thymus through a well-defined program (Fig. 1) that assures a diverse T cell repertoire with a number of safeguards in place to protect against autoreactivity. One such defense is central tolerance, an instructive process occurring in the thymus that identifies and eliminates potentially self-reactive thymocytes through negative selection. Central tolerance requires the presentation of self-antigens by thymic epithelial cells. Since not all self-antigens are expressed and thus displayed by these cells, it is possible for a self-reactive cell to escape into the circulation. Any such escapees are controlled through measures collectively known as peripheral tolerance. Specific mechanisms of peripheral tolerance include the induction of T cell anergy (Schwartz 2003), T cell apoptosis through activation-induced cell death (Zhang et al. 2004), and the generation of suppressive regulatory T cells (Sakaguchi 2004). In autoimmune disease, loss of self-tolerance can result from defects in central tolerance, peripheral tolerance, or both.

Despite the plethora of human patients and mouse models, progress toward the identification of key molecules involved in autoimmunity has been slow. This is likely due to the complex nature of these diseases, in which environmental factors

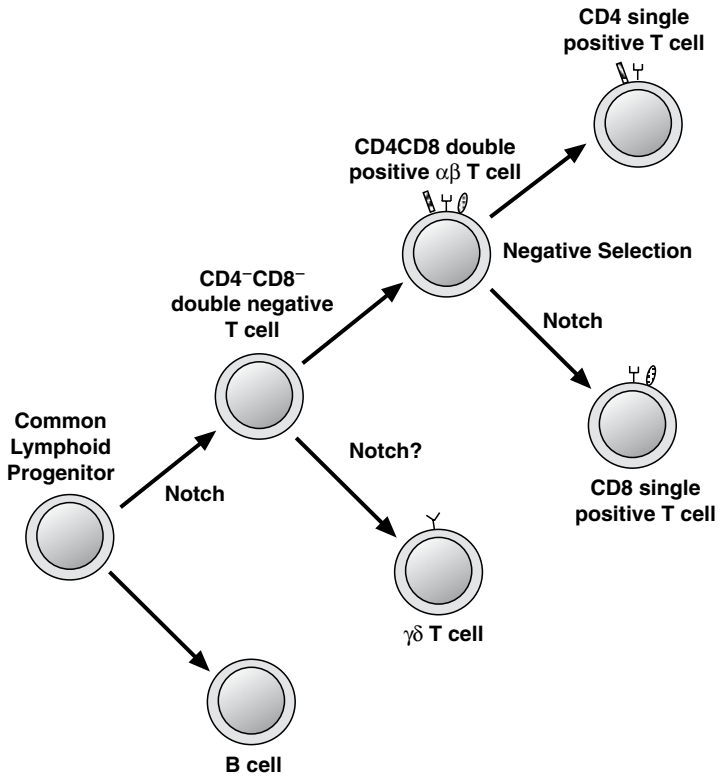

Fig. 1 Notch signaling in T cell development. T cells arise from common lymphoid progenitors that migrate to the thymus under an instructive Notch signal. At that point, they become double-negative cells, because they are doubly negative for the expression of the cell surface markers CD4 and CD8. As these developing thymocytes rearrange their T cell receptors they become either γδ-bearing or αβ-bearing. There are some reports that Notch signaling may affect this decision, although there is not consensus as to whether Notch biases toward αβ or γδ T cells. Cells with an αβ T cell receptor go on to become doubly positive for CD4 (*rectangle*) and CD8 (*oval*). It is at this point that negative and positive selection occurs. There is some preliminary data implicating the involvement of Notch signaling in negative selection (central tolerance). Cells that pass these critical tests become either CD8 single-positive or CD4 single-positive effector cells that enter the peripheral circulation. There is some evidence supporting the involvement of Notch signaling in CD8 vs CD4 lineage commitment

and genetic heterogeneity both contribute. New insight has recently come from spontaneous or induced monogenic mouse models of autoimmune disease. These animal models provide a system where environmental conditions can be precisely regulated and signaling pathways essential in breaching tolerance can be thoroughly characterized in order to translate these findings to human disease. One such model animal system that has helped in our understanding of some of the mechanisms involved in the genesis of autoimmune disease is the *itchy* mouse.

The *Itchy* Mouse

Phenotype of Itchy Mice

On a C57BL/6J background, homozygous a^{18H} mice (also referred to as *itchy* mice or $itch^{-/-}$ mice) are dark agouti in color with black pinna hairs. However, unlike all other alleles of *nonagouti* (*a*), *itchy* mice also develop an autoimmune-like disease characterized histologically by a mixed infiltrate (consisting of lymphocytes, eosinophils, and histiocytes) in nearly every organ system, lymphoproliferation resulting in splenomegaly and lymphadenopathy, and cortical atrophy of the thymus with medullary proliferation (Hustad et al. 1995). Furthermore, these animals produce antinuclear antibodies, and IgG deposits can be detected in the glomeruli as early as 8 weeks of age (Matesic et al. 2006). At about 5 months of age, *itchy* mice develop dermatitis and ulcerations that are especially prevalent on the head and neck region. These mice eventually die between 6 and 9 months of age from asphyxiation, as their lung function becomes compromised from alveolar proteinosis and interstitial inflammation composed of mostly $B220^{+}$ cells. This phenotype can be recapitulated by transplantation of *itchy*-derived bone marrow into a lethally irradiated syngeneic host. However, the mutation is no longer lethal when moved onto a $Rag1^{-/-}$ background where mature lymphocytes are lacking (L.E. Matesic, N.G. Copeland, N.A. Jenkins, unpublished observations). These results suggest that the autoimmune-like disease is cell autonomous to a bone marrow-derived cell, most likely a lymphocyte.

The Molecular Basis of the Itchy Mutation

The molecular defect responsible for the *itchy* phenotype is a small inversion on distal mouse chromosome 2. The breakpoints of this inversion affect the expression of two genes, *Agouti* and *Itch*. Specifically, there is a decrease in the amount of *Agouti* message, the consequence of which is a dark agouti coat color, as well as a complete abrogation of the expression of *Itch*, which presumably accounts for the immune dysfunction (Perry et al. 1998). The *Itch* gene has an open reading frame of 2,562 nucleotides, encoding a protein of 854 amino acids with a molecular weight of approximately 113 kDa. Itch is ubiquitously expressed in all adult tissues as well as throughout development. Sequence alignments of the predicted amino acid sequence demonstrates that Itch contains three important motifs: a C2 domain, four WW domains, and a HECT (homologous to E6-AP carboxy terminus) domain. The C2 domain can be found in a multitude of proteins with diverse biological functions. This motif is thought play a role in membrane targeting or subcellular localization, in some cases responding to increases in levels of intracellular Ca^{2+} (Nalefski and Falke 1996). The WW domain is named for the presence of two conserved tryptophan residues that guide the folding of this protein module. WW domains have been implicated in protein–protein interactions, with high binding affinity for PPLP, PPXY, or

phospho-serine/threonine motifs (Sudol 1996). HECT domains have been shown to have ubiquitin ligase (E3) activity and, as such, serve important roles in regulating protein stability, function, and subcellular localization in diverse cellular processes such as signal transduction, regulation of transcription, DNA repair, cell cycle progression, antigen presentation, and apoptosis (Hershko and Ciechanover 1998).

Ubiquitination

Ubiquitination is a posttranslational modification that directly conjugates a highly conserved 76-amino acid ubiquitin (Ub) molecule to a lysine residue on a target protein. The sequential action of three enzymes mediates this reversible process. First, Ub is activated in an ATP-dependent manner by an Ub-activating enzyme (E1) to form a thioester bond between the active site cysteine in the E1 and the C-terminal glycine residue of Ub. The activated Ub is then transferred to an Ub-conjugating enzyme (E2) to form a similar thioester linkage. This process comes to fruition when the E3 recruits both the E2–Ub complex and the target protein substrate in order to facilitate the transfer of the Ub from the E2 to the target protein. As such, it is the E3 that confers substrate specificity in ubiquitination. Consequently, it is not surprising that E3s are encoded by several hundred genes in the mammalian genome and often contain protein–protein interaction motifs in addition to the E3 catalytic site (Semple 2003). There are two major classes of mammalian E3s: the HECT and RING (really interesting new gene) families, which differ from one another not only in their sequence but also in their mode of action. RING E3s act as a scaffold for the transfer of Ub to the target protein, while HECT E3s have intrinsic enzymatic activity and the Ub is transferred from the E2 to a conserved cysteine residue in the HECT domain before being attached to the target protein (Liu 2007). Itch is a member of the HECT family of E3s.

In addition to the regulation offered by controlling the timing of ubiquitination as well as its reversibility, there are diverse biological outcomes associated with the ubiquitination signature affixed to a substrate. A target protein can be monoubiquitinated at a single lysine residue or serially monoubiquitinated at several different lysine residues via the lysine 63 residue of Ub. Such modifications usually signal for altered protein trafficking (e.g., internalization of membrane receptors). Alternatively, a substrate protein can be polyubiquitinated with a chain of four or more Ub molecules on one or more lysine residues via the lysine 48 residue, which leads to degradation by the 26S proteasome (Wang et al. 2006).

Itch Function in T Cells

Since the molecular lesion responsible for the *itchy* mutation was cloned, much progress has been made in understanding the physiological role of Itch. Numerous targets that are regulated by Itch have been identified. These are summarized in Table 1. A few of these have particular relevance to T cell function and are discussed here in greater detail.

Table 1 Targets of itch binding and ubiquitination

Target	Action of Itch	Function	Reference
LMP2A	PolyUb	EBV infection	Ikeda et al. 2001
ErbB–4	polyUb	Receptor tyrosine kinase	Omerovic et al. 2007
Trpv4 and Trpc4	MonoUb	Ion channels	Wegierski et al. 2006
NF-E2	Acts as transcriptional co-repressor	Heterodimeric transcription factor	Chen et al. 2001
CXCR4	MonoUb	Chemokine receptor	Marchese et al. 2003
Hrs	MonoUb; CXCR4-dependent	Endocytosis	Marchese et al. 2003
p63	PolyUb	Transcription factor involved in epidermal differentiation	Rossi et al. 2006
p73	PolyUb	DNA damage response	Rossi et al. 2005
RNF11	Not determined	RING E3	Kitching et al. 2003
p68	PolyUb	Subunit of Im	Ingham et al. 2005
Smad2	Proteolysis-independent Ub	TGF-β signaling	Bai et al. 2004
HEF1	PolyUb	TGF-β signaling	Feng et al. 2004
JunB	PolyUb	Th2 differentiation	Fang et al. 2002
c-Jun	PolyUb	T cell activation	Fang et al. 2002
Endophilin-A	MonoUb	Clathrin-mediated endocytosis	Angers et al. 2004
Cbl-c	PolyUb	RING E3	Magnifico et al. 2003
Atrophin–1	Not determined	DRPLA gene	Wood et al. 1998
Occludin	PolyUb	Sertoli tight junctions	Traweger et al. 2002
Notch1	MonoUb and polyUb	Various developmental processes	Qiu et al. 2000
Deltex	K29 polyUb	Regulation of Notch signaling	Chastagner et al. 2006
c-FLIP	PolyUb	NF-κB induced anti-apoptotic protein	Chang et al. 2006
Bcl10	PolyUb	Important for activation of NF-κB	Scharschmidt et al. 2004
PLC-γ1	PolyUb	Induced by Ca^{2+}/ calcineurin signaling	Heissmeyer et al. 2004
PKC-θ	PolyUb	Induced by Ca^{2+}/ calcineurin signaling	Heissmeyer et al. 2004
Gli1	PolyUb facilitated by Numb	Transcription factor in hedgehog signaling	Di Marcotullio et al. 2006
FAM/USP9X	Reverses Itch auto-polyUb	Ub protease	Mouchantaf et al. 2006

EBV, Epstein–Barr virus; MonoUB, monoubiquitination; NF-κB, nuclear factor-κB; PolyUB, polyubiquitination; TGF, transforming growth factor; Th, T helper

Itch in T Cell Differentiation

JunB is a transcription factor that plays an important role in T cell differentiation. Subsequent to antigen exposure, naïve CD4 T cells can differentiate into either T helper (Th)1 or Th2 cells, depending on their cytokine profiles and effector functions (Mosmann

and Coffman 1989). JunB is preferentially expressed in Th2 cells and activates the transcription of interleukin (IL)-4 and IL-5 (Li et al. 1999). The increased levels of these cytokines results in an allergic response and antibody class switching to IgG1, IgA, and IgE, as well as the recruitment of eosinophils via IL-5 (Neurath et al. 2002). JunB is polyubiquitinated by Itch, and without proper regulation of this transcription factor, *itchy* mice develop a Th2 bias in T cell differentiation, have increased IgG1 and IgE levels in the serum, and experience eosinophil activation (Fang et al. 2002).

The function of Itch in T cell differentiation is regulated by phosphorylation and interaction with other proteins. The activation of c-Jun NH_2-terminal kinase 1 (JNK1) by MAP/ERK kinase kinase 1 (MEKK1) results in the phosphorylation of Itch on serine or threonine sites (Gao et al. 2004). This induces a structural change in the Itch ligase, so that it adopts a more open and active conformation, which allows for more efficient recruitment and degradation of the JunB substrate (Gallagher et al. 2006). In contrast, Fyn-mediated tyrosine phosphorylation of Itch negatively regulates JunB ubiquitination and turnover (Yang et al. 2006). Finally, Itch has recently been shown to coimmunoprecipitate and colocalize with Nedd4 family interacting protein 1 (Ndfip1) in T cells (Oliver et al. 2006). *Ndfip1*$^{-/-}$ mice have a similar phenotype to *itchy* mice, with severe skin and lung inflammation and a Th2 bias. Furthermore, levels of JunB are increased in *Ndfip1*$^{-/-}$ T cells, suggesting that Ndfip1 is required for efficient ubiquitination of JunB by Itch. Since Ndfip1 is a membrane-bound protein, it may act to recruit Itch to the appropriate subcellular compartment for Itch to exert its effects.

Itch in T Cell Anergy

Itch also plays an important role in T cell anergy, a process that renders a lymphocyte functionally inactive but alive following an encounter with antigen. Itch is upregulated in anergizing conditions and polyubiquitinates phospholipase C (PLC)-γ1 and protein kinase C (PKC)-θ, two key molecules induced by Ca^{2+}/calcineurin signaling; this, in turn, destabilizes the immunological synapse and induces T cell unresponsiveness after T cell receptor engagement in response to restimulation with antigen together with antigen-presenting cells (Heissmeyer et al. 2004). The failure to induce anergy may account for the inability to establish peripheral tolerance and the development of autoimmune disease in *itch*$^{-/-}$ mice. This hypothesis has recently been proved in an in vivo system that measures Th2 tolerance in airway inflammation. In that animal model, the lack of Itch leads to a breach in tolerance of Th2 cells and to the development of allergic responses under experimental conditions that would induce anergy in normal Th2 cells (Venuprasad et al. 2006).

Notch Signaling

Itch has been shown to mono- and polyubiquitinate Notch1 in vitro (Qiu et al. 2000). Notch signaling plays a number of important roles in the immune system, influencing everything from hematopoiesis to T cell lineage commitment to the

function of peripheral T cells (reviewed in Radtke et al. 2004). Notch signaling is also known to mediate critical steps of T cell development (Fig. 1). As such, the mechanism by which Itch may regulate Notch signaling in vivo is of particular interest in the genesis of autoimmune disease.

Canonical Notch Signaling

Notch proteins are evolutionarily conserved transmembrane receptors that play important roles in cellular differentiation, proliferation, and apoptosis (Artavanis-Tsakonas et al. 1999; Miele and Osborne 1999). In mammals, there are four Notch receptors (Notch1–4) and five ligands (Jagged1 and 2; Delta1, 3, and 4) which function through direct cell-to-cell contact since both the ligands and the receptors are integral membrane proteins. The Notch receptors exist at the cell surface as a functional heterodimer, resulting from a furin-like processing event in the *trans* Golgi (Blaumueller et al. 1997). Upon ligand binding, ADAM10 or ADAM17 cleaves the Notch receptor extracellularly, releasing the extracellular domain (Brou et al. 2000). This is followed by another cleavage event mediated by γ-secretase (whose catalytic site is thought to be in presenilin subunits), which generates the intracellular fragment of Notch (ICN). The ICN translocates into the nucleus where it becomes a transcriptional coactivator for recombination signal-binding protein for immunoglobulin κJ region (RBP-Jκ), initiating the transcription of HES (hairy and enhancer of split) and HEY (HES-related with YRPW motif) target genes (Lai 2004). ICN is ubiquitinated in the nucleus by FBXW7 (F-box and WD repeat domain containing 7) and rapidly degraded (Gupta-Rossi et al. 2001; Wu et al. 2001). Studies in *Drosophila* have identified two additional E3s, Suppressor of *deltex* [*Su(dx)*] and DNedd4, which regulate the level of endogenous Notch. Specifically, these C2-WW-HECT E3s ubiquitinate full-length (FL), unactivated Notch in the endosome to target it for proteolysis. In the absence of these E3s, more FL Notch is present in the cell and can either be spuriously activated in the endosome by γ-secretase or recycled back to the plasma membrane, thus effectively lowering the threshold for Notch signaling (Sakata et al. 2004; Wilkin et al. 2004).

Noncanonical Notch Signaling

Through the extensive study of Notch signaling in various model organisms, some exceptions to the rule of canonical Notch signaling have been described. Noncanonical Notch signaling can involve the use of alternative ligands, alternative transcriptional coactivators, or nonnuclear mediators. In the vertebrate nervous system, F3/contactin acts as a Notch ligand to initiate a signal that promotes oligodendrocyte maturation and myelination (Hu et al. 2003). There are a number of other noncanonical ligands that can activate Notch signaling (e.g., NB3, NOV,

MAGP1, and MAGP2), but they have not been shown to have activity in the immune system (Osborne and Minter 2007). In *Drosophila*, Notch can signal through some members of the Wingless pathway (Axelrod et al. 1996; Ramain et al. 2001) instead of through Suppressor of Hairless (the ortholog of RBP-Jκ).

With respect to nonnuclear mechanisms of Notch signaling, there is a growing body of evidence that such pathways do exist under physiological conditions. In neuronal growth cones, Notch signaling has been proposed to directly regulate the actin cytoskeleton via a protein complex containing the tyrosine kinase Abl to regulate axon guidance (Giniger 1998). The cytoplasmic protein Deltex has also been shown to initiate Notch signaling in the late endosome of *Drosophila* (Hori et al. 2004). In Jurkat T cells, FL Notch1 coimmunoprecipitates with $p56^{lck}$ and with phosphatidylinositol 3-kinase (PI3K). This interaction was observed to activate AKT signaling and mediate an antiapoptotic effect (Sade et al. 2004).

Noncanonical Notch Signaling in Autoimmune Disease

The first connection between Notch signaling and autoimmune disease was the description of the combined *presenilin1* and *2* loss-of-function phenotype. Animals that are heterozygous for a knockout allele of *presenilin1* and homozygous for a knockout allele of *presenilin2* develop seborrheic keratosis and an autoimmune disease similar to that seen in *itchy* mice. Specifically, these animals display IgG deposition in the kidneys, produce antinuclear antibodies, and have splenomegaly as well as dermatitis consisting of a mixed inflammatory infiltrate that is predominantly $B220^{+}$ (Tournoy et al. 2004). This was originally interpreted as resulting from a reduction in Notch signaling through the canonical pathway, which caused an excess of B lymphocytes and of CD4 T cells, since canonical Notch signaling is required for T cell lineage commitment and perhaps for progressing from a double positive to a single-positive CD8 T cell (Fig. 1). This autoimmune phenotype, however, could instead result from the increased amount of FL Notch1–4 present in the T cells of these mice, which could then signal through a noncanonical pathway. Since that initial report, there have been a number of studies confirming that ligand-activated Notch signaling in T cells can occur without cleavage of Notch. Specifically, Notch-mediated suppression in human T cells (Kostianovsky et al. 2007), cytokine production by primary CD4 T cells and dendritic cells (Stallwood et al. 2006), and activation and proliferation of peripheral helper T cells (Rutz et al. 2005) all occurred in the presence of γ-secretase inhibitors where there was a complete inhibition of the canonical signaling pathway.

Increased Notch signaling was directly linked to autoimmune disease when it was discovered that some *lck–Notch1* transgenic mice, which overexpress the *Notch1* ICN exclusively in developing T cells, develop a systemic and progressive autoimmune disease (Matesic et al. 2006). Furthermore, this disease is similar to that observed in $itch^{-/-}$ animals, having approximately the same age of onset. *Notch1* transgenic animals with autoimmune disease have splenomegaly and

lymphadenopathy. There is a mixed inflammation in most organ systems with severe kidney involvement, including membranoproliferative glomerulonephropathy and interstitial inflammation that is almost exclusively $CD3^+$. Additionally, the diseased animals display a progressive deposition of IgG complexes in the glomeruli as well the production of antinuclear antibodies.

The similarity of the *itchy* phenotype to that of the Notch transgenics implies that these proteins may function in the same pathway in the genesis of autoimmune disease. This is supported by the fact that Itch can target Notch1 for ubiquitination in vitro (Qiu et al. 2000) and by phylogenetic analysis suggesting that *Itch* is the mouse ortholog of *Drosophila Su(dx)* (Matesic et al. 2006). In *Drosophila*, a class of gain-of-function *Notch* alleles (Ax^{E2}) is enhanced by a loss-of-function *Su(dx)* mutation (Fostier et al. 1998), suggesting that *Su(dx)* is a negative regulator of *Notch* signaling. To determine whether a similar genetic interaction also occurs in mammals, $itch^{-/-}$ mice were bred to the *lck–Notch1* transgenic mice.

All *itchy* mice carrying the *Notch1* transgene are considerably smaller than their littermates and die between 8 and 12 weeks of age. Examination of these animals reveals lymphoproliferation and massive amounts of chronic, active inflammation with eosinophils in almost every organ system examined. As with *lck–Notch1* tg^+ mice, some membranoproliferative glomerulonephropathy is present in the kidneys. Consistent with the autoimmune-like disease aspect of the phenotype, the sera of *itchy* animals carrying the *Notch1* transgene contain antinuclear antibodies, and more IgG deposition can be detected in the glomeruli of 8-week-old $itch^{-/-}$; *lck–Notch1* tg^+ mice (i.e., mice that carry the Notch1 transgene and are homozygous for the itchy mutation) when compared to age- and gender-matched wildtype or single mutant animals. Thus, $itch^{-/-}$; *lck-Notch1* tg^+ animals develop a similar autoimmune-like disease as $itch^{-/-}$ or *lck-Notch1* tg^+ mice but with more severe lesions and a much earlier age of onset. The fact that the mutations in concert yield severe early-onset disease, which was not seen with either mutation alone, supports the hypothesis that these alleles genetically interact. In addition, the combination of these mutations produces novel phenotypes including a perturbation in T cell development, with a reduction in the number of double-positive and an increase in the number of double-negative and single-positive T cells. TUNEL (terminal deoxynucleotidyl transferase biotin dUTP nick end labeling) staining shows reduced apoptosis in the thymi of *itch* animals that carry the *Notch1* transgene (Matesic et al. 2006).

Mechanistically, this reduction in apoptosis can be explained by an increase in noncanonical Notch signaling. Quantitative analysis of transcriptional targets of canonical Notch signaling such as *Hes1* reveals no correlation with the severity of the autoimmune disease. Antibody staining, however, displays increased levels of FL Notch1 in diseased animals, and the scale of the increase correlates with the severity of the autoimmune phenotype. In the *itchy* mice there is increased FL Notch1 due to the lack of ubiquitination and degradation of Notch1 by Itch. This makes more FL Notch1 available for signaling. In contrast, in the transgenic animals there is increased canonical Notch signaling. One of the transcriptional targets of this signaling cascade is *Notch1* itself. This causes an increase in the amount of FL Notch1 at the cell surface, lowering the signaling threshold. Thus, when the

effects of these two mutations are combined, the amount of FL Notch1 increases to an even greater degree, effectively lowering the Notch signaling threshold. This is manifest in the earlier age of onset and greater severity of the autoimmune disease (Matesic et al. 2006).

Increased levels of FL Notch1 can be found specifically in the double-positive thymocyte population prior to the onset of overt pathology. There are also corresponding increases in phospho-AKT in double-positive thymocytes but no change in other signaling pathways including mitogen-activated protein kinase (MAPK), p38, and JNK. Since AKT is known to provide a cell survival signal, it was hypothesized that the increased FL Notch1 complexes with $p56^{lck}$ and PI3K to activate the phosphorylation of AKT, which delivers a survival signal to double-positive thymocytes (Matesic et al. 2006). Normally 95% of double-positive thymocytes will die in the thymus, due to failure to meet the criteria for positive and negative selection (Strasser 1995). In these double mutants, however, there are decreased amounts of apoptosis correlating with the increase in phospho-AKT. It is tempting to speculate that, in these double mutant animals, the noncanonical Notch signal is allowing autoreactive cells to persist, thus providing a breach in central tolerance. However, this remains to be formally demonstrated. A similar noncanonical signaling mechanism has been noted at the point of β selection to promote the survival and glucose uptake/metabolism of pre-T cells via AKT signaling (Ciofani and Zuniga-Pflucker 2005).

Are There Other Aspects of Noncanonical Notch Signaling Involved in Autoimmune Disease?

Studies of *itchy* mice have brought to light many signaling pathways that are altered in this autoimmune disease state. Perhaps one of the most exciting findings is the link between noncanonical AKT-mediated Notch signaling and autoimmunity. It will be interesting to see if other mediators of noncanonical Notch signaling such as Abl and Deltex also play a role in the genesis of autoimmune disease. Recent reports have shown that c-Abl can phosphorylate c-Jun and protect it from Itch-mediated degradation (Gao et al. 2006). What remains to be demonstrated is whether Notch can regulate c-Abl in T cells in a manner analogous to that observed in growth cones. If this is the case, then there should be increased c-Abl activity in T cells derived from the *Notch1* transgenic animals, which would yield the stabilization of c-Jun protein (Fig. 2).

There are also studies linking Itch to Deltex. Specifically, Itch has been shown to ubiquitinate Deltex1 (Dtx1), a RING E3, through an unusual K29 linkage (Chastagner et al. 2006). Deltex, in turn, can catalyze the ubiquitination and degradation of MEKK1 (Liu and Lai 2005). MEKK1 has been shown to phosphorylate Itch and augment its ability to ubiquitinate JunB via JNK1 (Gao et al. 2004). These observations offer the tantalizing possibility that there may be a connection between the Th2 bias and noncanonical Notch signaling (Fig. 3). However, there are some outstanding questions: (1) Do T cells from Notch1 transgenics have a Th2 bias? If

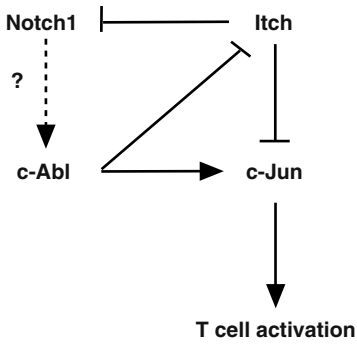

Fig. 2 A hypothetical role for c-Abl-mediated noncanonical Notch signaling in T cell activation. Notch has been shown to signal via a protein complex containing Abl in growth cones. Abl can also phosphorylate c-Jun and protect it from Itch-mediated ubiquitination and degradation during T cell activation. It remains to be determined whether Notch can signal via c-Abl in this cellular context (*dashed line*). (*Arrows* represent activation and *bars* represent inhibitory effects)

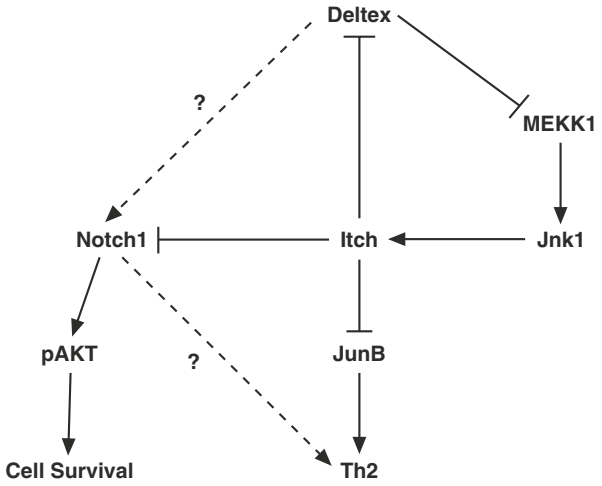

Fig. 3 Deltex could connect noncanonical Notch signaling to T cell differentiation. Deltex is a RING E3 and has been shown to target MEKK1 for ubiquitination and degradation by the 26S proteasome. MEKK1 can activate JNK1, which can phosphorylate Itch and increase its ability to ubiquitinate JunB. Itch can also ubiquitinate Deltex via an unconventional Ub linkage. In this way, Itch and Deltex antagonistically regulate one another. It remains to be determined if the overexpression of Deltex could result in autoimmune disease (*dashed line*). Furthermore, it is not known whether the increased Notch signaling in the Notch1 transgenic leads to a bias in Th2 differentiation (*dashed line*; *arrows* represent activation and *bars* represent inhibitory effects)

so, what is the connection between increased Notch and the Th2 bias, and (2) will overexpression of Dtx1 in developing T cells yield an autoimmune phenotype? The answers will likely be complex since the translation between Deltex function in *Drosophila* and mammals has been confusing at best. Although Deltex has been shown to be a positive regulator of Notch signaling in *Drosophila*, when Deltex1 is overexpressed in hematopoietic stem cells, a phenotype mimicking Notch inactivation

is observed, suggesting that *Dtx1* negatively regulates Notch signaling (Izon et al. 2002). Knockout mice lacking the function of just *Dtx1* (Storck et al. 2005) or both *Dtx1 and Dtx2* (Lehar and Bevan 2006) display normal immune development and normal immune responses. T cells from these animals, however, were not assayed for any bias in Th1 vs Th2 differentiation, so the possibility remains that Deltex could have some physiological function in the immune system.

Conclusions

As our understanding of the *itchy* phenotype continues to grow, one of the remaining challenges we face is the integration of all of these signaling pathways in the explanation of the mutant phenotype. That is, why do *itchy* mice develop autoimmune disease? Is it due to a breach in central tolerance, in peripheral tolerance, or both? Mechanistically, how does this happen? As we arrive at answers to these questions, we will gain a greater understanding of the pathogenesis of autoimmune disease. This will allow for the design of better therapeutics that might be able to benefit a large number of people suffering from a number of diseases characterized by the loss of self-tolerance.

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TIM Gene Family and Their Role in Atopic Diseases

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Contents

Abstract The TIM gene family was discovered seven years ago by positional cloning in a mouse model of asthma and allergy. Three of the family members (TIM-1, TIM-3, and TIM-4) are conserved between mouse and man, and have been shown to critically regulate adaptive immunity. In addition, TIM-1 has been shown to play a major role as a human susceptibility gene for asthma, allergy and autoimmunity. Recently, TIM-4 has been identified as a ligand of phosphatidylserine and to control the uptake of apoptotic cells. These studies together suggest that the TIM gene family evolved to regulate immune responses by managing survival and cell death of hematopoetic cells.

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Introduction

Bronchial asthma is an inflammatory disease of the lungs that has increased dramatically in prevalence over the past two decades in industrialized countries, doubling in prevalence since 1980, so that one in five individuals is now affected. In addition, other atopic diseases, including respiratory allergies (allergic rhinitis) and a skin disease known as atopic dermatitis, have increased greatly in prevalence over the last 20 years. As a result, current healthcare expenditures for the atopic diseases in industrialized countries are enormous. The large increase in prevalence is thought to be due to the dramatic changes in the environment of industrialized countries that have occurred over the past 20 years, but the specific environmental changes that are responsible for driving the increased development of allergic inflammation and asthma are not yet clear. Possible environmental changes that have affected the prevalence of asthma, allergy, and atopy include reductions in the frequency of infections (e.g., measles, mumps, rubella, tuberculosis, hepatitis A virus, and others) (Bach 2002; Matricardi et al. 1997, 2002) due to improved public health measures, increased use of vaccines and antibiotics (Umetsu et al. 2002), smaller family size (Strachan 1989), increased exposure to indoor allergens (Platts-Mills et al. 1996), and changes in diet (Devereux 2006), to name a few.

Allergy and asthma are complex genetic traits caused by environmental factors in genetically susceptibility individuals. It is estimated that a dozen or so susceptibility genes affect the development of asthma. Because each of the atopic diseases occurs in the same families and each has similar pathogenic mechanisms (affecting body surfaces that interact with the environment and involving eosinophilic inflammation), many of the susceptibility genes for asthma, allergic rhinitis, and atopic dermatitis are shared, although disease-specific genes (lung-, skin- or nasal mucosa-specific) may be involved as well. However, the identification of specific asthma, allergic rhinitis, atopic dermatitis, or atopy susceptibility genes has been difficult, because each susceptibility gene exerts only a small effect in the overall disease pathogenesis, and because each susceptibility gene interacts both with other genes on other chromosomes and with the environment in nonadditive ways. Furthermore, each susceptibility gene segregates independently, complicating the identification of these susceptibility genes. Thus, although multiple genome-wide scans have linked numerous chromosomal regions to asthma and allergy, only a few specific susceptibility genes within these chromosomal regions have been identified with any degree of certainty (Cookson and Moffatt 2000).

Several years ago our laboratory set out to identify an atopy susceptibility gene that might regulate the development of asthma and allergy, and which might provide important insight into the regulation of allergic inflammatory responses. Because of the genetic complexity of asthma and allergy, however, we first sought to simplify the problem by reducing the number of interacting atopy susceptibility genes. We therefore developed a unique mouse model of asthma through the use of congenic mice, generated by genetically moving discrete chromosomal segments from one mouse strain, DBA/2 (asthma resistant), into another strain, BALB/c (asthma susceptible), by repeated backcrossing (Ruscetti et al. 1985). In our mouse

model, the BALB/c strain produces high levels of interleukin (IL)-4 and develops severe airway hyperreactivity (AHR), a cardinal feature of asthma, on exposure to allergen. In contrast, the DBA/2 mice develop low IL-4 responses and have normal airway reactivity when exposed to the same allergen. The congenic strains we developed had discrete chromosomal segments from the DBA/2 on the BALB/c background, and thus converted a complex genetic trait (asthma and allergy) into a single locus trait, thereby eliminating interference from other atopy susceptibility genes on other chromosomes. These mice were then screened for the development of AHR and IL-4 on exposure to allergen. One congenic strain, called C.D2/Es-3/Hba, stood out in that it exhibited the DBA/2 phenotype of resistance to AHR and produced low levels of IL-4. Moreover, the DBA/2 chromosome segment that the C.D2/Es-3/Hba strain inherited from the DBA/2 chromosome 11 was syntenic to human chromosome 5q23–35, a region that has been repeatedly linked to asthma and allergy in humans (Marsh et al. 1994). Using the congenic C.D2/Es-3/Hba strain along with BALB/c mice and traditional positional cloning techniques (powerful for identification of single gene but not multigenic traits), we identified a novel atopy susceptibility gene locus called *Tapr* (T cell and airway phenotype regulatory). Within the *Tapr* locus, we then positionally cloned the TIM (T cell, Ig domain, and mucin domain) gene family (McIntire et al. 2001).

TIM Gene Family

The TIM family of genes consists of eight members (*Tims 1–8*) on mouse chromosome 11B1.1, and three members (*TIM-1, -3*, and *-4*) on human chromosome 5q33.2 (McIntire et al. 2001). All of the mouse and human TIM genes encode a type 1 membrane protein, consisting of an N-terminal Cys-rich IgV-like domain, a mucin-like domain, a transmembrane domain, and an intracellular tail. The intracellular tails of TIM-1, TIM-2, and TIM-3, but not TIM-4, contain predicted tyrosine phosphorylation motifs, suggesting that these TIMs are involved in transmembrane signaling. Whereas TIM-3 has only three predicted glycosylation sites, human TIM-1 has 60, which are primarily *O*-linked glycosylation motifs located within the mucin-like domain. The N-terminal Cys-rich regions of the TIM homologs have a sequence identity of about 40%, whereas sequence identity between the mouse and human orthologs is around 60% (Santiago et al. 2007a). The structural similarities between all the TIMs suggest that they arose from an ancestral gene by successive gene duplication events.

Human TIM-1 as an Atopy Susceptibility Gene

TIM-1 (HUGO designation HAVCR1) is highly polymorphic in monkeys and humans as it is in mice [with single nucleotide polymorphisms (SNPs) as well as insertion/deletion variants occurring primarily in the mucin-like domain in both

mice and humans], suggesting that human TIM-1 might serve as a susceptibility gene on human chromosome 5q23–35, a chromosomal region that has been repeatedly linked with asthma. Association analysis of the insertion/deletion variants of TIM-1 in human subjects with asthma and allergy demonstrated that allelic variation of TIM-1 contributes to the risk of atopy, but that this association depended upon past exposure to the hepatitis A virus (HAV). Specifically, the 157insMTTTVP and 195delT were associated with protection from atopy, but this protection was only observed in individuals who were seropositive to HAV (McIntire et al. 2003). The relationship to HAV is important, particularly since TIM-1 was previously discovered as the cellular receptor for HAV (HAVcr-1) in African green monkeys (Kaplan et al. 1996) and in humans (Feigelstock et al. 1998). Moreover, previous epidemiological studies in several distinct populations indicated that the prevalence of allergy and asthma was significantly lower in HAV seropositive individuals compared to that in HAV seronegative individuals (Matricardi et al. 1997, 2002).

The association analyses of TIM-1 and atopy, among the first to demonstrate a link between environmental factors (HAV infection) with an important susceptibility gene (TIM-1), provides a molecular mechanism for the hygiene hypothesis. The hygiene hypothesis attempts to explain the dramatic increase in the prevalence of atopic diseases that has occurred over the past 20 years, and states that infections, which have decreased substantially in industrialized countries over the past two decades, stimulate the immune system in such a way as to protect against asthma and allergy. The specific infectious agents that might be responsible for protection against allergy in the hygiene hypothesis are unclear, although infection with HAV has been associated with a reduced risk for developing atopy (Matricardi et al. 1997, 2002). Initially, the protection against atopy associated with HAV infection was assumed to be due to poor hygiene, because infection with HAV was thought to be a marker of poor hygiene, as the virus is transmitted through fecal–oral routes. However, the discovery of TIM-1 as an atopy susceptibility gene and of TIM-1 as the receptor for HAV suggests HAV has direct and long-lasting effects on T cells and on the immune system. As such, childhood infection with HAV, which occurred in nearly all children two decades ago, protects children from the development of asthma and allergy. In the past, childhood infection with HAV was for the most part mild and clinically inapparent, but recognized by the presence of antibodies to HAV, which approached 100% prevalence in Western countries prior to 1970 (Bach 2002). In contrast, the prevalence of infection with HAV today is less than 5% in young children in the United States, and this great reduction in the prevalence of infection may contribute to the increase in the prevalence of atopic diseases. Of note, currently in the United States, infection with HAV occurs primarily during food-borne epidemics or in daycare settings (Haaheim et al. 2002). Since protection against the development of atopy is also associated with early entrance into daycare (Ball et al. 2000), it is possible that in daycare settings HAV may be the important microbe that protects against atopy, although other infectious agents may also contribute to protection.

The association between polymorphic variants of TIM-1 and protection against atopic diseases has been reproduced in a number of studies, including one in African-American asthmatics (Gao et al. 2005), and others in children with atopic

dermatitis in Arizona (Graves et al. 2005) and in Australia (Page et al. 2006), in Koreans with asthma and atopic dermatitis (Chae et al. 2003), but not in Japanese children with asthma (Noguchi et al. 2003). The lack of association of TIM-1 in Japanese children with asthma may be due to a reduced incidence of HAV infection in Japan, which is now close to zero in young Japanese children. However, the precise immunological mechanisms by which HAV infection alters TIM-1 and the immune system to protect against atopy are not yet clear. The immunology of TIM-1 is only beginning to be understood (see below), and the results so far indicate that TIM-1 potently regulates immune responses through novel mechanisms.

The powerful effects of TIM-1 on the immune system and in atopy may reflect the high degree of polymorphisms that occur in TIM-1, primarily in exon 4. In exon 4 of TIM-1, nonsynonymous nucleotide substitutions occur much more frequently than synonymous substitutions, similar to patterns observed in major histocompatibility complex loci (McIntire et al. 2001; Nakajima et al. 2005). Together with the fact that the sequence variability in TIM-1 occurs in humans, chimps, and gorillas, these results suggest that the gene sequence variability of TIM-1 is driven by evolutionary natural selection, presumably due to pressures from infection with HAV. Further investigation of the differential functions of the TIM-1 polymorphic variants will provide important insight into immune regulation and potentially into the understanding of host defense against HAV.

Human TIM-1 in Autoimmune Disease

TIM-1 has been associated not only with atopic diseases, but also with several autoimmune diseases, suggesting that TIM-1 regulates the immune system more globally. For example, rheumatoid arthritis was associated with polymorphisms in exon 4 (5509_5511delCAA) of TIM-1 (Chae et al. 2004a), while in patients with rheumatoid arthritis, C-reactive protein or rheumatoid factor levels were associated with polymorphisms in the promoter region of TIM-1 (Chae et al. 2005). How TIM-1 regulates autoimmune disease is not known, nor is it known whether HAV infection is associated with protection from autoimmunity. However, TIM-1 mRNA is expressed in the cerebrospinal fluid mononuclear cells of patients with multiple sclerosis (MS), primarily in patients in remission rather than in patients in relapse, suggesting that TIM-1 regulates the development of MS, possibly as a beneficial element, perhaps associated with tolerance (Khademi et al. 2004).

Role of TIM-3 as a Susceptibility Gene

Both human and mouse TIM-3 are polymorphic with several SNPs present in the coding regions of the IgV regions. These polymorphisms have not been associated with atopic disease (Page et al. 2006), although a promoter polymorphism

may be associated with allergic rhinitis (Chae et al. 2004b). SNPs in the coding regions of the IgV regions of TIM-3, however, have been associated with rheumatoid arthritis (Chae et al. 2004b). The immunological mechanisms by which TIM-3 functions to regulate Th1 biased immune responses and autoimmunity will be discussed below.

TIMs as Costimulatory Molecules on T Cells

TIM-1 is a type 1 cell surface molecule expressed on $CD4^+$ but not on $CD8^+$ T cells, and initial studies indicated that TIM-1 is preferentially expressed on $CD4^+$ Th2 cells (McIntire et al. 2001). In contrast, TIM-3 is preferentially expressed on Th1 cells (Monney et al. 2002). Naïve $CD4^+$ T cells do not express either TIM-1 or TIM-3, but upon activation with specific antigen and dendritic cells or with anti-CD3 and CD28 monoclonal antibody (mAb), $CD4^+$ T cells express TIM-1. On further differentiation, TIM-1 expression is maintained on Th2 cells, but not on Th1 cells (Umetsu et al. 2005), although TIM-3 expression increases on differentiating Th1 cells (Monney et al. 2002).

Cross-linking of TIM-1 on T cells with an agonist mAb provides a very potent costimulatory signal to $CD4^+$ T cells that increases T cell proliferation and cytokine production [IL-4, interferon (IFN)-γ, and IL-10] (Umetsu et al. 2005). The costimulatory effect is seen only in the presence of T cell receptor (TCR) signaling, and could not be observed with monomeric Fab fragments of the anti-TIM-1 mAb. In vivo administration of the agonist anti-TIM-1 mAb along with antigen also greatly increased antigen-specific T cell proliferation and cytokine production, indicating that the agonistic anti-TIM-1 mAb provided a potent adjuvant effect. The adjuvant effect of anti-TIM-1 mAb potently blocked the development of respiratory tolerance (Umetsu et al. 2005), consistent with the idea that TIM-1 costimulation potently activates T cells. Normally, respiratory exposure to antigen induces T cell unresponsiveness, and is associated with the development of antigen-specific regulatory T cells expressing FoxP3 (Akbari et al. 2002; Stock et al. 2004), but treatment with an agonist anti-TIM-1 mAb prevented this tolerance induction. It is possible that distinct regions of TIM-1 interact with different receptors or molecules, and mAbs recognizing these distinct regions may have different effects on the immune response, or that the affinity of an antibody for TIM-1 on different cell types may affect the type of response that is induced. Thus, for example, anti-TIM-1 mAbs recognizing exon 4 of the mucin/stalk domain greatly exacerbated airway inflammation and Th2 cytokine production, but another mAb blocked inflammation in a mouse model of asthma (Sizing et al. 2007). However, the precise events that regulate the various outcomes of TIM-1 signaling by distinct mAbs are not yet known.

The molecular signal transduction mechanisms by which TIM-1 costimulates T cell activation are also not fully known. It is known, however, that overexpression of TIM-1 in T cells results in an increase in production of IL-4 but not IFN-γ (de Souza et al. 2005). Furthermore, transfection of D10 cells with TIM-1 results

in increased transcription from the IL-4 promoter and activation of NFAT/AP1 elements (de Souza et al. 2005), suggesting that TIM-1 preferentially enhances Th2 cytokine production, which is consistent with the preferential expression of TIM-1 on Th2 cells (McIntire et al. 2001). Activation of T cells appears to result in the phosphorylation of a conserved tyrosine in the cytoplasmic tail (Y276) of TIM-1 (de Souza et al. 2005). In studies of TIM-1 utilizing overexpression of TIM-1 on Jurkat T cells, which normally do not express TIM-1 proteins, investigators have found that TIM-1 colocalizes on the T cell surface with CD3 (Binne et al. 2007). TIM-1 coimmunoprecipitates with the TCR complex upon TCR cross-linking and T cell activation, and TCR signaling increases upon TIM-1 cross-linking. Furthermore, TIM-1 cross-linking caused rapid tyrosine phosphorylation of TIM-1, as well as phosphorylation of Zap70 and ITK.

TIM-1 Ligands

Several approaches have been taken to determine the natural ligands of TIM-1, and these approaches have identified TIM-1 itself, TIM-4, and IgAλ as molecules that can bind to TIM-1. The structure of TIM-1 includes a glycosylated mucin domain, which imparts a degree of promiscuity to the TIM-1 molecule. This may explain the identification of multiple ligands, and has made it difficult to determine which if any of the already identified ligands is the primary ligand of TIM-1. Staining with TIM-1-Ig fusion proteins (consisting of the TIM-1 IgV domain with or without the mucin domain coupled to the Fc portion of IgG) as well as with a TIM-1 tetramer demonstrated that TIM-1 binds to $CD11c^+$ splenocytes and more weakly to $B220^+$ B cells and $CD11b^+$ splenocytes (Meyers et al. 2005; Wilker et al. 2007). This binding was dependent on divalent cations for high-affinity binding as the addition of EGTA significantly reduced binding (Wilker et al. 2007). TIM-1-Ig and TIM-1 tetramer bound to CHO cells transfected with TIM-4 (Meyers et al. 2005), and this interaction could be specifically inhibited by anti-TIM-1 mAb, indicating that TIM-4 was a ligand of TIM-1. In addition, TIM-1-Ig and TIM-1 tetramers bound to cells expressing TIM-1, and this binding was dependent on the presence of the glycosylated mucin stalk, although the mucin stalk alone was insufficient for TIM binding, indicating that homotypic TIM-1-TIM-1 binding also occurred.

Administration of TIM-4-Ig in vivo along with antigen induced high levels of splenocyte proliferation and cytokine production. The interpretation was that TIM-4-Ig bound to TIM-1 on T cells resulting in T cell activation (Meyers et al. 2005). TIM-4 is expressed on $CD11b^+$ and $CD11c^+$ cells, including macrophages and dendritic cells (DCs), particularly on lymphoid $CD8\alpha^+$ DCs or on splenic stromal cells, but not on T cells (Shakhov et al. 2004). Administration of a TIM-1-Ig fusion protein, however, produced similar results, which was initially difficult to understand. Nevertheless, it is likely that TIM-1 may bind to itself, which is suggested by clumping of TIM-1 transfectants (Umetsu et al. 2005) and by the crystal structure of TIM-1, which was solved in 2007 (Santiago et al. 2007a).

IgAλ was also recently described as a putative ligand of human TIM-1 (Tami et al. 2007). This ligand was identified using an expression cloning strategy based on binding of a human TIM-1-Fc fusion protein to cells transfected with a human lymph node cDNA library. The interaction of TIM-1 with IgAλ was blocked by mAbs against IgA, Igλ, and human TIM-1. Since IgA did not inhibit HAV infection of African green monkey kidney cells, it is likely that the IgA and virus binding sites on TIM-1 are distinct. The precise physiological implications of this finding are not yet clear, though it is possible that the IgA-TIM-1 interaction has a synergistic effect in host defense against HAV.

TIM-1 Crystal Structure

Crystal structure analysis of TIM-1 confirmed the homotypic TIM-1–TIM-1 interactions, which are conserved in mice and humans, suggesting that this interaction is an important immunoregulatory mechanism. The TIM family members share a common structural organization with other Ig superfamily members. The IgV domain of TIM-1 has two antiparallel β-sheets, bridged by the first and last of six Cys residues in the IgV domain, similar to the structure of Ig superfamily members. The remaining four Cys residues link two loops, forming a cleft in the IgV domain. One loop connects two β-strands (the FG loop) and another loop connects two other β-strands (the CC′ loop). The six conserved Cys residues in all of the TIM molecules appear to provide a distinctive structural feature of TIM IgV domains. The major differences between the IgV domains of TIM-1 and TIM-2, which display high sequence identity (66%), occur mainly in this cleft region, suggesting that the cleft provides important functionality to the TIMs. Importantly, the HAV appears to bind to human TIM-1 at this cleft region, with Ser37 in the CC′ loop possibly the critical virus-binding residue (Santiago et al. 2007a).

The TIM-1 IgV domains crystallized in asymmetric pairs, such that each TIM-1 domain was related by a rotation angle of about 180°, with their C-terminal ends extending in opposite directions. This suggested that two TIM-1 molecules on two different cells might interact through two Thr17 residues at TIM-1 molecular surfaces opposite the clefts. This idea is supported by the observation that TIM-1 transfected cells tend to aggregate in clumps (Umetsu et al. 2005), and that TIM-1 molecules on transfected cells cluster at intercellular junctions (Santiago et al. 2007a). In addition, soluble TIM-1-Ig fusion proteins bind to cells transfected with TIM-1 (Umetsu et al. 2005) and to plastic surfaces coated with TIM-1–Ig. The homophilic binding in BIAcore assays was about 0.6 μM, and required contributions from the mucin domains and divalent cations (Santiago et al. 2007a). It is therefore possible that the homophilic binding of TIM-1 at intercellular junctions could facilitate phosphorylation of TIM-1, resulting in T cell activation. Since TIM-1 is also overexpressed after ischemic kidney injury (Han et al. 2002), and in renal carcinoma (Vila et al. 2004), the homophilic interactions of TIM-1 could also mediate cell adhesion interactions for renal cell regeneration and tumor development. Importantly, most of the TIM-1

molecules in transfected cells accumulated in intracellular vesicles, but trafficked to the cell surface after treatment with ionomycin or PMA (Santiago et al. 2007a). This may explain the observation of the presence of TIM-1 mRNA in lymphocytes that express minimal levels of TIM-1 cell surface protein (Mesri et al. 2006).

TIM-4, a Receptor for Phosphatidylserine

Although TIM-4 can bind to TIM-1, TIM-4 appears to have a critical role in other settings, notably in facilitating the uptake of apoptotic cells by macrophages and dendritic cells. Macrophages in the peritoneum and subsets of macrophages and DCs in the spleen express TIM-4, which has been shown recently by two independent groups to be an important and specific receptor for phosphatidylserine, a membrane phospholipid expressed by apoptotic cells (Kobayashi et al. 2007; Miyanishi et al. 2007). The specificity of the binding was confirmed by crystallographic studies of TIM-4 with phosphatidylserine binding in the cleft of the IgV domain (Santiago et al. 2007b). Moreover, cells expressing TIM-4 avidly phagocytized apoptotic cells (expressing PS), and this process was specifically blocked by anti-TIM-4 mAb (Kobayashi et al 2007). These studies suggest that TIM-4, by controlling the uptake of apoptotic cells, may regulate the development of tolerance and autoimmunity.

TIM-2, an Inhibitory Costimulatory Molecule

Murine TIM-2, which has no counterpart in humans, has sequence similarities to TIM-1, although their crystal structures indicate that the two molecules are quite distinct (see below). TIM-2 is expressed by B cells and by epithelial cells in bile ducts and renal tubules (Chen et al. 2005), as well as by activated Th2 cells (detected by mRNA analysis) (Chakravarti et al. 2005). A number of reports have suggested several distinct ligands for TIM-2. These ligands include Sema4A, which is expressed on activated macrophages, B cells, and DCs (Kumanogoh et al. 2002), and H-ferritin, which could act as an immune regulator by inhibiting T cell proliferation or impairing B cell maturation (Chen et al. 2005). Sema4A plays an important role in T cell activation, and Sema4A-deficient mice exhibit defective Th1 responses (Kumanogoh et al. 2005). In addition, blockade of TIM-2 signaling by administration of a TIM-2–Ig fusion protein results in enhanced Th2 responses (IL-4 and IL-10) and inhibition of IFN-γ production (Chakravarti et al. 2005). Furthermore, administration of the TIM-2–Ig fusion protein during the induction phase inhibits the severity of experimental autoimmune encephalomyelitis. TIM-2-deficient mice develop increased Th2 responses (Rennert et al. 2006), suggesting that TIM-2 activation provides an inhibitory signal to T cells which would normally produce Th2 cytokines. Thus, T cells from TIM-2-deficient mice immunized with antigen proliferate more vigorously and produce increased quantities of Th2 as well as Th1

cytokines, and develop increased airway inflammation in an asthma model. On the other hand, overexpression of TIM-2 in human T cell lines (Jurkat cells) results in a reduction in NFAT and AP-1 transcriptional activity (Knickelbein et al. 2006). Together, these studies indicate that TIM-2, which is preferentially expressed on Th2 cells, signals to inhibit the development of Th2 responses.

The crystal structure of TIM-2 has also been solved and indicates that TIM-2 IgV molecules readily form homodimers. The IgV domain of TIM-2, like that of TIM-1, has six Cys residues, which stabilizes two β-sheets and a cleft formed by the FG loop and the CC′ loop (Santiago et al. 2007a). In contrast to the homodimers of TIM-1, the angle between the two TIM-2 IgV domains was 60°, suggesting that dimerization of molecules occurs in a *cis* manner on the same cell surface, rather than between TIM-2 molecules on two different cells, as appears to occur with TIM-1 IgV molecules. IgV dimerization creates an extended glycan-free surface at the top, which allows accessibility to ligands. It is possible that the ligands of TIM-2 may differ depending on whether monomeric versus dimeric TIM-2 is available.

TIM-3, Another Inhibitory Costimulatory Molecule

Mouse TIM-3 (HUGO designation HAVCR-2) was independently identified using an expression cloning strategy of Th1 cells, and is expressed by Th1 cells after two to three rounds of polarizing stimulation in vitro (Monney et al. 2002; Sanchez-Fueyo et al. 2003). Mouse TIM-3 encodes a 281-amino acid protein, while human TIM-3 encodes a 302-amino acid protein that shares 63% homology with mouse TIM-3 (McIntire et al. 2001; Monney et al. 2002). TIM-3 is preferentially expressed on Th1 cells and $CD8^+$ T cells, as well as macrophages, DCs, and natural killer (NK) cells (Khademi et al. 2004). TIM-3 appears to provide a negative signal to T cells, and thus, blockade of TIM-3 signaling with a blocking anti-TIM-3 mAb in mice developing experimental autoimmune encephalopathy greatly worsens disease, increases IFN-γ production, and is associated with an increase in the activation status of macrophages (Monney et al. 2002). Similarly, administration of a TIM-3–Ig fusion protein, which blocks TIM-3 activation signals, resulted in greatly increased Th1 cell development with increased IFN-γ and IL-2 production, and blockade of peripheral tolerance (Sabatos et al. 2003). Furthermore, blockade of TIM-3 signaling accelerated diabetes in NOD mice, and prevented acquisition of transplantation tolerance induced by costimulation blockade (Sanchez-Fueyo et al. 2003). The proinflammatory effects of TIM-3 blockade were mediated in part by dampening of the antigen-specific immunosuppressive function of $CD4^+$ $CD25^+$ regulatory T cell populations. On the other hand, enhancing TIM-3 signaling appears to cause rapid Th1 cell death, inhibits Th1-mediated allo-immune responses, and enhances transplantation tolerance. Thus, TIM-3 signaling downregulates Th1-dependent immune responses, and facilitates the development of tolerance.

Th1 cell clones generated from the cerebrospinal fluid of patients with MS produced increased amounts of IFN-γ but expressed lower levels of TIM-3 and T-bet,

suggesting that the failure to upregulate expression of TIM-3 might represent an intrinsic defect that contributes to the pathogenesis of MS (Koguchi et al. 2006). In addition, blockade of TIM-3 signaling during coxsackievirus infection resulted in increased myocarditis and reduced regulatory T cell activity (Frisancho-Kiss et al. 2006). Furthermore, in a model of asthma induced by the pulmonary transfer of ovalbumin (OVA)-specific Th2 cells, TIM-3 blockade significantly enhanced IFN-γ production, decreased eosinophils and Th2 cells in the lung, and greatly reduced AHR, presumably because Th1 responses can inhibit allergen-induced AHR (Kearley et al. 2007). However, since Th1 responses can also play a proinflammatory role in asthma, the precise role of TIM-3 or of TIM-3 blockade in the regulation or treatment of asthma is unclear.

TIM-3 Ligands

Using TIM-3-Ig and an expression cloning strategy, galectin-9 was identified as a ligand of TIM-3 (Zhu et al. 2005). Galectins are mammalian protein lectins recognizing conserved carbohydrates (Liu and Rabinovich 2005). Galectin-9 has been known to induce T cell apoptosis, and it appears that binding to TIM-3 mediates this process, although galectin-9 inhibits apoptosis in eosinophils, presumably through a non-TIM-3-mediated pathway (Hsu et al. 2006). Galectin-9 is expressed on endothelial cells, fibroblasts, and astrocytes, and it attaches to TIM-3 on T cells resulting in rapid Th1 but not Th2 cell clumping and cell death. This suggests that induction of galectin-9 by IFN-γ helps to resolve Th1 inflammatory responses.

Analysis of the crystal structure of the IgV domain of TIM-3 demonstrates that TIM-3 has a structure very similar to TIM-1 and TIM-2, in having six Cys residues in the IgV domain, resulting in a cleft formed by two β-strands, the FG loop and the CC′ loop, which are stabilized by two of the disulfide bonds (Cao et al. 2007). This cleft is critical for the binding of TIM-3 to a ligand(s) other than galectin-9, as binding of TIM-3 to cell lines [and the presumed ligand(s)] was abolished by site-directed mutagenesis of residues located in proximity to the cleft. These results suggest that there may be at least two independent TIM-3 ligands: galectin-9, which does not bind to the cleft region, and another ligand(s) that binds to the cleft region. As with TIM-1 and TIM-2, ligands may bind specifically to the conserved FG-CC′ cleft of TIM-3 or the cleft may contribute to local structural or dynamic changes associated with the recognition of ligand epitopes on the target molecules. The TIM-3 ligands may include specific carbohydrate moieties, as suggested by a recent report indicating that TIM ligands might be promiscuous (Wilker et al. 2007). Finally, polymorphisms in the sequence of mouse TIM-3 all occur in the IgV domain, and may affect ligand binding. Since these polymorphisms occur distal to the FG-CC′ cleft of TIM-3, the polymorphisms are unlikely to directly affect ligand recognition associated with the FG-CC′ cleft, but they might affect the binding to galectin-9, or could modulate the interactions between the IgV and mucin domains, thus altering the overall presentation of the ligand-binding surfaces.

Summary and Conclusions

The TIM gene family, which includes eight murine and three human members, was identified using a genetic approach and a unique congenic mouse model of allergic asthma. This model converted a complex genetic trait (allergic asthma) into a monogenic problem, allowing the positional cloning of the TIM gene family in 2001, and using AHR and Th2 cytokine production as readouts. This approach proceeded without knowledge or assumptions of the previously unknown TIM genes, which turn out to code for distinctive proteins with previously unsuspected structures and function. TIM-1 in particular, but TIM-3 as well, are polymorphic and both are associated with development of atopy and autoimmunity, and play important roles in regulating T cell function. Importantly, TIM-1 is the receptor for the hepatitis A virus, infection with which had previously been shown to protect against the development of allergy and asthma. Since improved hygiene and public health measures over the past two decades have considerably reduced the prevalence of HAV infection, TIM-1 provides a molecular explanation for the hygiene hypothesis.

The mechanisms by which the TIM molecules function in the immune system are now the focus of a large number of investigations. TIM-1 and TIM-2 are preferentially expressed on Th2 cells, while TIM-3 is preferentially expressed on Th1 cells, consistent with the idea that the TIMs play important roles in the immunoregulation and biology of T cells. TIM-1 costimulatory signaling causes enhanced T cell activation, whereas TIM-2 and TIM-3 costimulation generates inhibitory signals in T cells. The precise mechanisms by which TIM-1 affects the development of atopy or how HAV affects T cell signaling, however, are still unknown. The answers to these questions will generate important new information about the TIM molecules and their ligands in immune regulation. Although several TIM ligands have been identified already, knowledge of the crystal structure of mouse TIM-1, TIM-2, and TIM-3 has helped us to understand the specific function of known ligands, and explain how different ligands might each bind to the TIMs. We believe that future studies of the TIMs will lead to a much improved understanding of the regulation of atopy and autoimmunity, and lead to novel and effective immunotherapies for these diseases.

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Index

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