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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