Genetic dissection of the host response to *Salmonella* Typhimurium infection in *Toll-like receptor 4* transgenic mice and recombinant congenic strains.

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ABSTRACT

Different individuals react differently to infection with similar pathogens and weakly pathogenic organisms can cause life-threatening infections in some, while highly virulent microbes may go undetected in others. The basis of these differences lies within the genetic makeup of each individual, which determine their response to infection. Unraveling the genetic determinants of susceptibility to infection brings a much clearer understanding of the pathogenesis of diseases and paves the way to potential prophylactic and therapeutic interventions urgently needed in the context of increasing antimicrobial resistance, globalization of infectious diseases, and emerging or re-emerging pathogens.

Salmonella spp are highly successful pathogens that have co-evolved with countless host species. Even today, they continue to threaten public health throughout the world. Their zoonotic nature, their propensity to establish long-term carrier states and the emergence of antimicrobial resistant, highly virulent strains greatly complicate the fight against this pathogen. As for other infectious diseases, the host response to *Salmonella* is genetically controlled. In order to genetically dissect this response, a mouse model was developed and allowed identification of a few genes having a strong impact on the outcome of *Salmonella* infection. The mouse response to *Salmonella* is, however, complex and several additional genetic variants influencing the response to infection remain to be identified.

Here, we present a series of experiments, which contribute to our understanding of the host response to acute *Salmonella* Typhimurium infection in mice. First, we investigated the impact of Tlr4 expression during *Salmonella* infection by comparing host responses in mice carrying 1, 2 and 3 copies of *Tlr4* on the same genetic background. We show for the first time, in this narrow range of *Tlr4* expression, an incremental protective effect against *Salmonella* due to improved control of bacterial growth and increased expression of important downstream immune genes. Second, using a set of reciprocal A/J and C57BL/6J recombinant congenic strains, we identified five novels QTL influencing the outcome of *Salmonella* Typhimurium infection in mice. Finally, we present evidence for the genetic basis for one of the newly identified QTL and describe a role for anemia and iron balance in the mouse response to *Salmonella*.

RÉSUMÉ

Différents individus répondent différemment à des pathogènes similaires et alors que certains sont gravement malades suite à une infection par un organisme peu virulent, d'autres seront complètement résistants à une infection potentiellement grave. Ces différences ont leur fondement dans le bagage génétique de chacun. L'identification des gènes responsables de la susceptibilité aux pathogènes est essentielle à une meilleure compréhension de la pathogénie des infections et peut ouvrir la voie à des interventions prophylactiques ou thérapeutiques nécessaires dans la lutte contre la résistance aux antimicrobiens, contre la globalisation des maladies infectieuses et contre la menace de pathogènes émergeants ou ré-émergeants.

Les *Salmonellae* sont des pathogènes accomplis ayant co-évolué avec de nombreux hôtes. Encore aujourd'hui, ils sont une menace pour la santé des populations. Leur potentiel zoonotique, leur capacité à établir des états de porteurs chroniques et la récente émergence de souches résistantes aux antimicrobiens et très virulentes compliquent grandement la lutte contre ce pathogène. Comme c'est le cas pour toutes maladies infectieuses, la réponse de l'hôte à l'infection à *Salmonella* est contrôlée génétiquement. Afin d'étudier cette réponse, un modèle d'infection chez la souris a été développé et a permis l'identification de quelques gènes importants dans l'infection à *Salmonella*. Cependant, comme cette réponse est complexe, il est certain que plusieurs variants génétiques additionnels importants dans l'infection à *Salmonella* existent et restent à découvrir.

Nous exposons ici les résultats d'une série d'expériences visant à approfondir notre compréhension des facteurs génétiques impliqués dans la réponse de la souris à l'infection à *Salmonella* Typhimurium. D'abord, nous présentons des données indiquant que le niveau d'expression de *Tlr4* chez des souris possédant 1, 2 ou 3 copies du gène influence la survie suite à l'infection à *Salmonella* à cause d'un meilleur contrôle de la prolifération bactérienne et d'une régulation fine de gènes effecteurs. Ensuite, nous présentons les résultats d'expériences effectuées chez des souris recombinantes congéniques qui ont permis d'identifier cinq nouveaux QTL influençant la résistance aux *Salmonellae*. Finalement, nous démontrons la nature moléculaire d'un des nouveaux

QTL identifiés et démontrons un rôle pour l'anémie et la surcharge en fer dans la réponse de l'hôte à *Salmonella* Typhimiurium.

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TABLE OF CONTENT

ABBREVIATIONS

129S6: 129S6/SvEvTac

BIR: blood invasiveness ratio

CBC: complete blood count

CGD: chronic granulomatous disease

FACS: fluorescent antibody cell sorting

IFN: interferon

IL: interleukin

iNOS: inducible nitric oxide synthase

IRF: interferon regulatory factor

Ity: immunity to Typhimurium

LBP: lipoprotein binding protein

LOD: logarithm of the odds ratio

LPS: lipopolysaccharide

M6PR: mannose 6 phosphate receptor

MDR: multidrug resistant

MHC: major histocompatibility complex

MyD88: myeloid differentiation factor 88

NADPH: nicotinamide dinucleotide phosphate

NF-κB: nuclear factor κB

NO: nitric oxide

NRAMP1: natural resistance associated macrophage protein 1

PAMP: pathogen associated molecular pattern

Pklr: liver and red blood cell specific pyruvate kinase

PK: pyruvate kinase

PBS: phosphate buffered saline

PCR: polymerase chain reaction

PRR: pattern recognition receptors

qPCR: quantitative real-time PCR

QTG: quantitative trait gene

QTL: quantitative trait loci

RBC: red blood cell

RES: reticuloendothelial system

RNI: reactive nitrogen intermediate

ROI: reactive oxygen intermediate

SCV: *Salmonella*-containing vacuole

Sifs: *Salmonella*-induced filaments

SNP: single nucleotide polymorphisms

SPI: *Salmonella* pathogenicity island

TIR: Toll-Interleukin-1 receptor domain

TIRAP: TIR domain-containing adaptor protein

TLR: Toll-like receptor

TNF: tumor necrosis factor

TRAM: TRIF-related adaptor molecule

TRIF: TIR domain-containing adaptor protein inducing interferon-β

TTSS: type III secretion system

Xid: X-linked immunodeficiency

CONTRIBUTION OF AUTHORS

Parts of the Sections 2 and 3 of Chapter I were previously published in a review paper written by the author and published in Genes and Immunity: "Roy MF, Malo D. Genetic regulation of host responses to Salmonella infection in mice. *Genes Immun* 2002; **3**: 381-393." This paper is partially reproduced here with the permission of the publisher. The published version of this paper is reproduced in Appendix 1. The author, with guidance and advice from Dr Danielle Malo, mostly wrote this review paper.

Chapter II contains a manuscript previously published in Genes and Immunity and reproduced with the permission from the publisher: "Roy MF, Lariviere L, Wilkinson R, Tam M, Stevenson MM, Malo D. Incremental expression of Tlr4 correlates with mouse resistance to Salmonella infection and fine regulation of relevant immune genes. *Genes Immun* 2006; **7**: 372-383." The author, under the supervision of Dr Danielle Malo, performed this study. Line Larivière and Rosalie Wilkinson provided technical help for the infection of the mice, and tissue handling and processing. Mifong Tam provided invaluable technical help for the FACS analysis and the CBC. Mary Stevenson provided advice regarding the planning and interpretation of the FACS analysis experiments. The histopathology was performed at CTBR Bio-Research Inc (Senneville, Québec). All other work was performed by the author including: breeding and maintenance of the mice colonies; generation of the B10.Cg-*Nramp1/Tlr4*; genotyping; infectious dose preparation; assisting during infections; monitoring of mice during infections; harvesting and processing of the tissues for CFU determination, RNA analysis, and FACS analysis; RNA extraction; cRNA target synthesis, array hybridization, image acquisition and analyses; cDNA synthesis; quantitative PCR; data compilation and analyses, including statistical analyses; and preparation of the manuscript.

Chapter III consists of a manuscript accepted for publication in Genes and Immunity: "Roy MF, Riendeau N, Loredo-Osti JC, Malo D. Complexity in the host response to *Salmonella* Typhimurium infection in AcB and BcA recombinant congenic strains. (Accepted for publication)". The author, under the supervision of Dr Danielle Malo, essentially did the work presented in this manuscript. Noémie Riendeau performed the DNA extractions and the genotyping necessary to close the gaps in the genome scans.

Dr Chon Loredo-Osti performed the one locus mapping for the survival phenotype using a parametric survival regression at the markers and assessed the significance via 10 000 bootstrapped resamples. Line Laroche performed the breeding of the F2s, and Line Laroche and Rosalie Wilkinson infected the mice. Line Laroche, Line Larivière and Rosalie Wilkinson also provided technical assistance for the harvest and processing of tissues for the bacterial load experiments. Their contribution is acknowledged at the end of the manuscript. The author's contribution included preparing the infectious dose; assisting during infections; monitoring the mice during infections; harvesting and processing the organs for bacterial load experiments; compiling and analyzing the results including statistical analysis; identifying the polymorphic markers, organizing the genotyping with KBioscience; dosing and adjusting the concentration of the DNAs; preparing the DNA plates for SNP genotyping; analyzing/verifying the genotyping data; preparation/verification of the genetic maps; performing one locus interval mapping in R/qtl under various models; assessing the genome-wide significance level through permutations; and preparation of the manuscript.

Chapter IV consists of a manuscript in preparation that will be submitted for publication shortly: "Roy MF, Riendeau N, Bédard C, Hélie P, Canonne-Hergaux F, Gros P, Malo, D. Pyruvate kinase deficiency confers susceptibility to *Salmonella* Typhimurium infection in mice. (Manuscript in preparation)." The author, under the supervision of Dr Danielle Malo, did the studies reported in this manuscript. Noémie Riendeau performed part of the *Pklr* genotyping and all of the genotyping associated with the fine mapping (including the selection, and testing of the markers, PCR amplification, agarose gel verification of the amplified products and preparation of the samples for sequencing). Dr Christian Bédard supervised the CBC analysis, reviewed the CBC results and provided useful advice regarding the planning and interpretations of the hematological studies. Dr Pierre Hélie performed the bone marrow histology. Dr François Canonne-Hergaux provided advice and technical assistance for the iron studies. Dr Canonne-Hergaux also performed the Perls staining of the spleen and liver. Dr Philippe Gros was one of the instigators of the project and he provided some of the AcB61 mice. Dr Gros was also primarily involved in the identification of the *Pklr* mutation as a malaria resistance gene and he provided useful advice for this project.

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CHAPTER 1: INTRODUCTION

Infectious diseases have accounted for most of the mortality in human history, accounting for approximately 60% of all deaths in England in the mid 19th century.[1] With the improvements in hygiene, the development of vaccines and the discoveries of effective treatments against infectious agents, this figure has now decreased to approximately 25%.[2] Despite these improvements, infectious diseases remain, more now than ever, a threat to the world health with much of the burden falling on the population of the developing countries and especially on infants less than 5 years old (WHO report on infectious disease 1999; www.who.int/infectious-diseases-report). With the globalization of exchanges and the circulation of people between countries from all over the world, emerging or re-emerging infections can now rapidly spread to numerous and remote human populations.[2] Moreover, the never-ending battle against antimicrobial resistance puts a toll on our capacity to successfully treat microbial infections that were once easily overcome with antimicrobials. If we want to continue to have success in our battle against invading microorganisms we must extend our understanding of the pathogenesis of infectious diseases in the context of *in vivo* host-pathogen interactions. In this regard, the genetic dissection of the complex host response to infection will undoubtedly bring a higher level of understanding of the pathogenesis of infectious diseases and pave the way to novel prophylactic or therapeutic interventions.

Section 1. *Salmonella* spp: clinical manifestations and disease burden

1.1 The genus *Salmonella*

The genus *Salmonella*, member of the family of Enterobacteriaceae, comprises two species, *Salmonella enterica* and *Salmonella bongori*.[3] More than 2500 serovars are recognized[4] and most belong to the subspecies I of *Salmonella enterica* designated *Salmonella enterica* subsp. *enterica*. This subspecies encompasses most of the clinically important serovars infecting human or domestic animals and will be the focus of this short overview.

Salmonella spp are Gram-negative, facultative-intracellular bacilli that are ubiquitous in nature and have the capacity to infect a wide variety of different host including domesticated and wild mammals, reptiles, birds and insects. The clinically important *Salmonella* serovars are often classified as host-adapted or not. For instance, *Salmonella enterica* subsp *enterica* serovar Typhi and Paratyphi (thereafter *Salmonella* Typhi and Paratyphi) are only capable of infecting humans and higher primates and are therefore considered host-adapted to humans. By the same principle, the serovars Gallinarum, Abortusovis and Abortusequi cause disease only in poultry, sheep and horses respectively, and are considered host-adapted to these species. Other serovars, however, such as Dublin and Choleraesuis, are also considered to be host-adapted (in cattle and swine), although they can at times cause a highly invasive illness in humans. Host-adaptation of *Salmonellae* should therefore not be seen as an indication of the virulence of a serovar in a given species, as it has been classically implied, but more as an indication of its ability to circulate, maintain itself and cause disease in a particular population.[5]

The vast majority of the *Salmonella* serovars, however, are considered non host-adapted since they have the ability to cross species barriers, infect successively various hosts belonging to distant species, and do not appear to be maintained predominantly in a specific population. Examples of such serovars are numerous and include Typhimurium, Enteritidis, Newport and Heidelberg. These *Salmonella* serovars are considered zoonotic organisms and most human cases can be traced to food animals, pets or wildlife either through direct contact or contamination of the food chain and environment.[6-11]

The clinical manifestations associated with *Salmonella* spp are numerous and differ according to the serovar and the host species involved. In the following paragraphs I will discuss the epidemiology, the clinical features and the main clinical syndromes associated with *Salmonella* infections in human and relevant domestic animals.

1.2 Enteric (typhoid and paratyphoid) fever

Enteric fever is a systemic disease of human caused by the host-adapted *Salmonella* serovars Typhi (typhoid fever) and Paratyphi (paratyphoid fever).[12-15] This disease is transmitted from human to human through the feco-oral route and is a problem mainly in areas where overcrowding, poor sanitation and lack of access to clean water

prevail. While the disease was endemic in most parts of the world up to the 20th century, the improvement in hygiene and sanitation in industrialized countries has led to a dramatic decrease in its incidence from more than 35 000 cases annually in the USA in 1920 to less than 500 cases annually nowadays.[13] The disease remains, however, a threat to public health in developing countries where people do not have access to clean water and where sanitation infrastructures are lacking. The global burden of enteric fever is estimated to ~21.7 million cases and ~200 000 deaths annually for typhoid fever, and ~5.4 million cases annually for paratyphoid fever.[16] The highest incidence rates (>100/100 000 cases/year) are found in south-central Asia and south-east Asia. Medium incidence rates (10-100/100 000 cases/year) are found in the rest of Asia, Africa, Latin America and the Caribbean, and Oceania (except Australia and New Zeland) while low incidence rates (< 10/100 000 cases/year) are reported from Europe, North America, and the rest of the developed world (Figure 1).

The pathogenesis of typhoid fever has been well described.[12-15,17] Following ingestion, the bacteria must endure the acidity of the stomach before reaching the small intestine. In this regard, conditions associated with decreased acidity of the stomach or perturbations of the endogenous microbiota are associated with increased risk of infection. Once in the small intestine, *Salmonella* attaches to the epithelium and invades the M-cells of the Peyer's patches. The ability of *Salmonella* to survive inside mononuclear cells in the deeper layers of the gut wall is key to its virulence and allows dissemination (primary bacteremia) to the spleen, liver, lymph nodes, gallbladder, and bone marrow. During the incubation period (5 to 21 days) the bacterium replicates within these sites and the appearance of clinical signs correlates with the onset of secondary bacteremia at a time when the bacterial loads in the reticuloendothelial system (RES) reach a certain threshold. The most common clinical signs include fever, generalized malaise, headache, gastrointestinal symptoms, relative bradychardia, splenomegaly and leukopenia. Secondary bacteremia may, in rare instances, lead to the seeding of additional organs, resulting in extra-intestinal complications such as meningitis, myocarditis, arteritis, pneumonia, osteomyelitis or septic arthritis.[17] Additionally, intestinal bleeding secondary to the necrosis of the lymphoid tissue of the ileocecum (Peyer's patches) can occur. Patients with clinical symptoms of more than two weeks

duration are at increased risk for such complications. Chronic carrier state occurs in 2 to 5% of the cases with shedding of the organism for more than one year, thereby contributing to disease dissemination and persistence.[18]

Without treatment, enteric fever is a severe, debilitating and often fatal disease.[19] With appropriate and timely treatment, however, the average case fatality rate is now estimated to approximately 1%.[16] While disease-associated deaths can usually be prevented by antimicrobial therapy, there is increasing concern that the rise in antimicrobial resistant isolates[20] may pave the way to a dramatic increase in case fatality rates.

Chloramphenicol was introduced for the treatment of typhoid fever in 1948 resulting in greatly decreased morbidity and mortality associated with this disease.[19] While resistance to chloramphenicol was reported only two years later, it is not until the 1970s that outbreaks of chloramphenicol resistant *Salmonella* Typhi were reported from several locations throughout the world.[12,14,15] Thereafter, additional antimicrobials such as ampicillin, sulfonamides and trimethoprime were used successfully in the treatment of typhoid fever patients but resistance to these antimicrobial was soon reported. By the early 1990s, multidrug resistance (MDR), defined as resistance to all first line antimicrobials (chloramphenicol, ampicillin and sulfonamides/trimethoprime) had become common in several parts of the world. The introduction of fluoroquinolones in 1990s was a major advance in the treatment of enteric fever, however, resistance to and treatment failure with ciprofloxacin are now increasingly reported and complicates the treatment of resistant infections.[21-23] Finally, third generation cephalosporins remain an alternative for the treatment of MDR/fluoroquinolone resistant isolates although resistance to cetriaxone as been reported as well.[24]

The emergence of highly resistant Typhi isolates greatly complicates the fight against this pathogen because of increased duration and decreased efficacy of treatment as well as increased shedding of the organisms by the infected host, resulting in a higher potential for dissemination. The higher cost of treatment associated with more expensive drugs is especially of concern in the developing world where people are most at risk but lack sufficient resources to cope with the high treatment costs. These observations emphasize the need for finding alternative strategies to the current antimicrobial treatment

in the fight against *Salmonella* Typhi and Paratyphi. The study of the genetic determinants of the host response to infection *in vivo* is one way of gaining a better understanding of the pathogenesis of the disease, which may give insights to the development of effective prophylactic measures. However, it is obvious that the most efficient measures in our battle against typhoid fever would be the improvement of hygiene and sanitation in endemic areas as well as the vaccination of school-age children.[15]

1.3 Salmonellosis

Non-typhoidal *Salmonellae*, such as *Salmonella enterica* serovars Enteritidis or Typhimurium, are associated with a more localized gastrointestinal illness in humans known as salmonellosis. Infection occurs through the feco-oral route and is usually associated with contamination of the food chain[25] or contact with infected domestic animals.[10] Salmonellosis continues to be one of the most common food-born diseases, accounting for a third of all bacterial isolates reported through the United-States FoodNet,[26] and it is widely distributed throughout the world.[27,28] In the United-States, it is estimated that 1.4 million cases of salmonellosis occur each year, associated with 168 000 physician office visits, 15 000 hospitalizations and 400 deaths.[25,29] Non-typhoidal *Salmonellae* are the pathogens most often associated with food-related deaths, accounting for 38% of all deaths reported through FoodNet from 1996-1999.[26] The highest incidence rates of salmonellosis are found in infants aged less than 6 years, while the highest death rates are found in older people (aged ≥ 60 years), immunocompromised patients or individuals having received antimicrobials prior to infection.[26] The economical consequences of non-typhoid *Salmonellae* have been estimated to 3 billion US dollars annually for the United-States alone. (WHO, Drug-resistant Salmonella, 2005. www.who.int/mediacentre/factsheets/fs139).

Clinical salmonellosis is associated with acute onset of fever, abdominal cramping, diarrhea, nausea and vomiting, occurring within 48 hours of consumption of contaminated food or water.[12] The illness is usually self-limiting and most people recover without treatment within 3 to 7 days. In some cases, particularly in the very young or the elderly, dehydration from diarrheal fluid losses can become severe and life threatening,

requiring medical attention. Bacteremia may develop in a small percentage of immunocompetent patients although in higher proportion in patient populations with compromised immunity or in cases of infection with some specific serovars. Blood dissemination of *Salmonellae* may result in various complications such as endocarditis, aortitis, pneumonia, osteomyelitis or septic arthritis and most of the fatal *Salmonella* infections are associated with invasive illness.[26] While treatment of infectious diarrheal illnesses with antimicrobials is not recommended for the general population, patients at risk for extra intestinal dissemination may benefit from appropriate antimicrobial therapy. For patients with bloodstream dissemination, effective treatment may be life saving.[30,31] While the acute death rates associated with non-typhoid *Salmonella* infection are relatively low (0.6-1.2% of cases[26,32]), follow-up studies have demonstrated long term consequences to acute salmonellosis with one year relative mortality being 2.85 (95% CI: 2.56 to 3.17) in *Salmonella* cases compared to the general population.[32]

As it is the case for most bacterial pathogens, the development of resistance to antimicrobial agents among non-typhoidal *Salmonella* isolates is common and worrisome. Of most concern, is the spread of MDR isolates such as MDR *Salmonella* Typhimurium definitive phage type 104 (DT104),[27] which commonly shows resistance to ampicillin, chloramphenicol, streptomycin, sulfonamides and tetracycline. After appearing for the first time in the United-Kingdom in the early 1980s, *Salmonella* Typhimurium DT104 has now spread to most parts of the world, and is commonly found in poultry, pigs, cows, sheep, and human. In addition to the 5 above-mentioned antimicrobials, *Salmonella* Typhimurium DT104 is capable to acquiring resistance to other drugs such as quinolones, trimethoprim and cephalosporins,[27,33,34] potentially leading to a complete lack of effective treatment for infected patients. Even more concerning are the recent reports of increased virulence associated with MDR *Salmonella* isolates leading to excess blood stream infections, excess hospitalization and excess death rates. Indeed, when looking at outbreaks of *Salmonella* infection in the United-States between 1984 and 2002, it was found that significantly more people were hospitalized among those infected with resistant *Salmonella* isolates compared to pansusceptible isolates.[35] Additionally, multivariate analysis showed that patients infected with non-typhoidal *Salmonella* serovars resistant to ≥ 1 antimicrobial had excess bloodstream infections, and excess

hospitalizations with bloodstream infection compared to patients infected with pansusceptible isolates.[36] Finally, a Danish study, looking at *Salmonella* Typhimurium infections, found that while infection with pansusceptible isolates increased the two year death rates in patients compared to the general population by a factor of 2.3, patients infected with MDR isolates were 4.8 times more likely to die and patients infected with quinolone resistant isolates were 10.3 times more likely to die, even after adjusting for differences in comorbidity.[37]

The reality of antimicrobial resistance for non-typhi *Salmonella* stresses again the importance of improving our understanding of the host-pathogen interaction *in vivo* with the hope of finding alternatives to antimicrobial treatment.

1.4 Invasive non-typhoidal *Salmonella* infections

Although most infections attributed to nontyphoidal *Salmonellae* are limited to the gastrointestinal tract, systemic dissemination may occur, resulting in potentially serious consequences for affected patients. Based on the FoodNet data from 1996 to 1999, it is estimated that more than 2500 cases of invasive *Salmonella* infection occur annually in the United-States, resulting in ~1800 hospitalization and 150 deaths.[38] Invasive *Salmonella* infections are associated with higher rates of hospitalization (71% versus 17%) and higher mortality rates (5.4% versus 0.2%) when compared to enteric infections.[38] The propensity of *Salmonellae* to cause invasive illnesses appears to be serovar specific. The invasiveness of each serovar is measured by its blood invasiveness ratio (BIR), the number of blood isolates per 100 blood plus stool isolates.[28] The highest BIR are found, as expected, in the human specific serovars Typhi (BIR = 70/100 blood plus stool isolates) and Paratyphi (60/100) compared to BIR usually much lower, 8/100, for non-host specific serovars such as Enteritidis or Typhimurium. Interestingly, the pig and cow adapted serovars, Choleraesuis and Dublin, are capable of causing disease in humans with BIR ranging from 40 to 70/100. Although the number of cases caused by these serovars in human remains low, their propensity to cause invasive illness makes them more dangerous for humans, especially for people with comorbidity. For instance, infection with serovars Dublin is associated not only with a much increased risk of bloodstream infection compared to serovar Typhimurium (OR 77.7, 95% CI: 25.5-

237.2)[36] but also with a dramatic increase in relative mortality within one year of infection (15.55; 95%CI-6.57-36.80) compared to the general population, a figure much higher than what is reported for serovar Typhimurium infections (3.01; 95%CI: 2.43-3.74).[32]

1.5 Infection in domestic animals

Salmonella spp infections are common in domestic animals and are, by themselves, associated with a wide array of detrimental consequences including economical losses for producers, losses of valuable breeding stock, and emotional burden for owners of affected animals. Indirectly, the infection of domestic animals is at the source of most cases of human salmonellosis through contamination of the food chain.[25] The increasing antimicrobial resistance noted in non-typhoidal *Salmonellae* has been linked to the use of antimicrobial in food animal either for therapeutic purpose or as growth promoter agents.[39] *Salmonella* infections in domestic animals should therefore be taken seriously and all possible measures should be taken to minimize their impact and prevent the spread of antimicrobial resistance. In the following paragraphs, I will briefly describe the various clinical syndromes and consequences of *Salmonella* infection in poultry, swine, cattle and horses.

1.5.1 *Salmonella* infection in poultry.

Salmonella infection in poultry can be grouped into two categories: 1) pullorum disease and fowl typhoid caused by the bird specific *Salmonella enterica* serovars Pullorum and Gallinarum and 2) paratyphoid caused by a wide variety of non-host specific serovars such as Enteritidis, Typhimurium and Arizonae.[40]

Pullorum disease is an acute systemic disease of young birds while fowl typhoid is an acute or chronic systemic disease found both in young and older birds. Both diseases used to cause significant losses in commercial flocks but extensive eradication programs have successfully freed most commercial operations in the United-States, Canada, Australia, Japan and Western Europe from these diseases. Pullorum disease and fowl typhoid may still be found in other parts of the world or in backyard flocks. Chickens are the natural host for *Salmonella* Gallinarum and Pullorum although outbreaks have been reported in other birds. Infection of humans or other mammals are rare. Transmission

within bird flocks occurs both horizontally and vertically. Young birds hatching from contaminated eggs or infected soon after hatching will suffer significant morbidity and mortality. Typical clinical signs include somnolence, weakness, decreased appetite, poor growth, labored breathing and swollen joints. Mortality rates usually peak around the second or third week of life and rates up to 100% have been reported. In older birds, *Salmonella* Pullorum rarely causes clinical signs but *Salmonella* Gallinarum may cause acute outbreaks in chickens characterized by sudden drop in food consumption, ruffled feathers, pale and shrunken combs, drop in egg production, decreased fertility and diminished hatchability. Death may occur within 5 to 10 days.

Paratyphoid in chickens is of significantly more public health concern because it is caused by the non-specific, zoonotic serovars of *Salmonella enterica*.[40] Contaminated poultry meat and eggs are among the most often incriminated source of *Salmonella* outbreaks in humans. The economical consequences for poultry producers are enormous and associated with growth depression and mortality in young birds, negative publicity resulting from poultry-associated *Salmonella* outbreaks, and the cost associated with risk reduction such as biosecurity practices, cleaning and disinfection programs, rodent control, vaccination and testing. Transmission of non-host specific serovars within bird flocks can also occur horizontally and vertically. Clinical signs are usually found only in very young birds and include progressive somnolence, drooping wings, ruffled feathers, anorexia, emaciation, profuse watery diarrhea and lameness. Mortality in young birds may be as high as 50%. In older birds, however, significant intestinal and even systemic colonization may occur without significant clinical signs except for some mild transient diarrhea. The lack of clinical signs in birds that are shedding *Salmonellae* in their feces or potentially laying contaminated eggs significantly complicate the control of this pathogen.

Eradication of *Salmonella* Gallinarum from most commercial flocks in North America and Europe in the 1970s may have paved the way to the subsequent outbreak of food born *Salmonella* Enteritidis in humans.[5] Because both serovars belong to the same D1 serogroup, it is believed that eradication of *Salmonella* Gallinarum in commercial flocks lead to decreased immunity against the O9-antigen common to both Gallinarum and Enteritidis, allowing the latter to colonize the now naive flocks. Since Gallinarum

cannot infect human, the public health consequences of colonization of poultry by Enteritidis were greater. As mentioned previously, *Salmonella* Gallinarum infection in birds is usually associated with obvious clinical signs while *Salmonella* Enteritidis infection appears completely silent, complicating the detection of infected animals.[7] Control of *Salmonella* Gallinarum through effective vaccination instead of systematic killing of infected birds might have prevented the public health consequence of the *Salmonella* Enteritidis outbreak.

1.5.2 *Salmonella* infection in swine.

Salmonella spp are associated with two distinct problems in the pig industry. First, infections with the swine specific serovar Choleraesuis or with the non-specific serovar Typhimurium are associated with clinical disease in pigs. Second, infection with other *Salmonella enterica* serovars is rarely associated with disease in swine but contaminates the carcasses and pork products, with potential for public health consequences.[41] Although much of the contamination of pork products occurs through cross contamination at abattoirs, the source of infection remains pigs that are infected at the time they leave the farm. It is believed that the stress of shipping and food deprivation during transportation increases the shedding of *Salmonella* by inapparent carriers and leads to contamination of the truck and environment of the slaughterhouse with subsequent infection of additional individuals.

Salmonella Choleraesuis, a host-adapted serovar found almost exclusively in swine, is the most common cause of *Salmonella* outbreaks on pig farms. The disease is found most often in intensively reared weaned pigs less than 5 months old. Transmission occurs from pig to pig or environment to pig either through the feco-oral route or even through nose-to-nose contact since *Salmonella* Choleraesuis also resides in the pharyngeal tonsils. The clinical signs of *Salmonella* Choleraesuis infection are those of a systemic disease, because of the highly invasive nature of this serovar. Anorexia, lethargy and fever are accompanied by a moist, shallow cough, slight expiratory dyspnea, icterus, reluctance to move and cyanosis of extremities. Diarrhea usually does not develop before day 3 or 4 of the disease. During an outbreak, the case fatality rate is usually quite high while the morbidity is usually less than 10%. Stressful situations are

often associated with the onset of an outbreak. Surviving pigs may remain carrier and continue to shed *Salmonella* in their feces for several weeks.

The second most common serovar causing clinical disease in pigs is *Salmonella* Typhimurium, which causes a more localized enterocolitis. Disease also occurs most often in weaned pigs, up to four months of age. Affected pigs present with a watery, yellow diarrhea that spreads rapidly to involve most pigs in a pen within a few days. Diseased animals are usually febrile, depressed, inappetent and dehydrated but most make a complete clinical recovery. A proportion of the affected pigs remains carrier and shedder for five months post-infection.

The control of *Salmonella* infection and prevalence on pig farms is complicated by the frequent chronic carriers that do not exhibit clinical signs and the numerous potential sources of contamination (food, rodents, insects, chronic carriers, etc) although for *Salmonella* Choleraesuis, the only source of contamination appears to be carrier pigs. Detection of carriers through fecal culture is not sensitive enough to detect chronic carriers because of the intermittent nature of the shedding while serology allows detection of previously infected pigs but does not necessarily indicate the carrier or shedder state of the animal. Sound management practices, however, are likely to decrease the incidence of *Salmonella* infection in pigs. These include filling of grower and finishing rooms with single source, single age pigs, respecting proper animal density, providing adequate environment such as a dry, comfortable pen, regulated ambient temperature and adequate ventilation.

1.5.3 *Salmonella* infection in cattle.

Salmonella infection in cattle is caused by the host-adapted serovar Dublin or by non-host specific serovars such as Typhimurium, Newport or Montevideo.[42-44] As it is the case for other food animals, *Salmonella* infections in cows pose a significant public health threat through the contamination of meat and milk. Of major concerns are the outbreaks of MDR *Salmonella* Typhimurium DT104 in dairy cattle and humans in the UK and USA.[45,46]

Salmonella Dublin infection in cattle is associated with long-term carriage and intermittent shedding, which may persist for the life of the animal. Shedding occurs

through the feces or through the milk and contributes to the persistence of infection within a particular herd. Stressors, such as parturition, high ambient temperature or food and water deprivation will increase the chances of shedding of *Salmonella*. Infection in calves is associated with systemic disease with fever, depression and respiratory symptoms. Diarrhea is often not a predominant clinical sign, a fact that complicates the diagnosis. Adults may present with diarrhea, abortion or mastitis. Infection with non-host adapted serovars, such as Typhimurium, is associated with sporadic cases or outbreaks. Because these serovars are not associated with long-term carriage as seen with *Salmonella* Dublin, the disease does not tend to persist within a herd but reappears periodically from accidental introduction of infected animals (cows, rodents and birds) or feedstuffs. Septicemia occurs in neonatal calves and the disease may be fatal with 24 to 48 hours without treatment. Disease in adult cattle and older calves occurs most commonly at times of stress and the clinical signs are typical of acute, febrile enterocolitis with severe dehydration. Mortality rates may be quite high in the absence of appropriate supportive treatment and abortion is common in pregnant female.

1.5.4 *Salmonella* infection in horses.

Equine facilities often face problems associated with *Salmonella*, especially large breeding farms where chronic carriers (the mares) and availability of susceptible host (neonatal foals) contribute to disease dissemination and persistence.[47] *Salmonella* spp have the capacity to survive for long periods of time in the environment, especially when protected in organic matter or dust, thereby allowing persistence until availability of a suitable host for replication, and re-contamination of the environment.

Four clinical syndromes have been reported in horses and reproduced experimentally: 1) subclinical infection with latent or active carriage; 2) depression, fever, anorexia and neutropenia without colic or diarrhea; 3) fulminant enterocolitis with diarrhea; and 4) septicemia with or without diarrhea.[48] Any stressful event or any change in gastrointestinal homeostasis may transform a latent carrier into an active shedder or a full blown clinical case.

Acute septicemia is often seen in neonatal foals, which present in the first few days of life with lethargy, anorexia, weakness and fever. Septicemia in neonates is

usually fatal without appropriate treatment and often necessitates intensive supportive care. Older foals and adult horses with acute *Salmonella* infection most often present with acute, severe enterocolitis with profuse diarrhea and severe dehydration. Increased permeability of the colonic mucosa due to intestinal inflammation allows lipopolysaccharides (LPS) and other bacterial products to reach the systemic circulation leading to development of systemic inflammatory response syndrome, with potential complications such as severe sepsis, multiple organ dysfunction syndrome, septic shock and death.[49]

Large animal hospitals experience relatively frequent outbreaks of salmonellosis and in this regard, equine hospitals appear particularly at risk with outbreaks reported from several University teaching hospitals.[50-53] The vulnerability of equine hospitals may be explained by several factors. First of all, *Salmonella* spp are capable of establishing silent carrier state in horses, even in those that have no history of salmonellosis, and it is estimated that 0.8% of the resident horses in equine facilities shed *Salmonella* in their feces based on single fecal cultures.[54] This figure is most likely higher in hospitalized horses.[55,56] Second, *Salmonella* spp can survive in the hospital environment for long periods of time[53,57] even after cleaning and disinfection.[58] Third, hospitalized horses appear as a population vulnerable to infection or reactivation most likely because of stress (shipping, hospitalization, pre-existing disease, anesthesia, surgery), and change in microbiota (change in food and water, fasting, antimicrobial drug or anti-acid medication).[59] Finally, a horse infected with *Salmonella* and presenting clinical signs can rapidly contaminate its environment leading to the rapid spread of the bacteria to adjacent hospitalized horses or even to the attending personnel if strict isolation and control procedures are not rapidly taken. As seen in most species, the appearance of MDR isolates from horses[53,60] will complicate the treatment of infection and pose a public health threat given the zoonotic nature of the pathogen.

The description of the clinical diseases and the problematic associated with *Salmonella* infection in both humans and domestic animals found in this section emphasizes the need to continue our efforts to understand more fully the pathogenesis of *Salmonella* infection, control its spread and protect the usefulness of current antimicrobials.

Section 2. The mouse model of typhoid fever

Because of difficulties associated with experimental studies in humans or large animal species, the laboratory mouse has become the preferred animal model for the study of numerous human disorders, including infectious diseases.[61] Although, as with any model system, the use of the mouse for the study of human infectious disease is fraught with limitations,[62] the usefulness of this animal model far outweigh its shortcomings. The first advantage of the mouse model resides in the similarities between mice and men: they both are mammals that use innate and adaptive immunity in the face of invading organisms, they are often susceptible to the same or similar pathogens and they share about 99% of their genes.[63] The second advantage in using the mouse resides in the ease one has to work with these animals: laboratory mice are usually docile and easy to handle, they have a very short generation time, they are prolific breeders within the laboratory setting and their small size limit the cost associated with the care of the colonies.[64] Finally, the resources associated with the laboratory mouse are almost unlimited and include, among others, the availability of hundreds of fully inbred strains, recombinant inbred strains, or recombinant congenic strains; the facility to manipulate the mouse genome to create congenic lines, knockout or transgenic strains; and the availability of complete genome sequences, and numerous microsatellites and single nucleotide polymorphism (SNP) markers.

For these reasons, the study of the host response to infection and the understanding of the host-pathogen interactions *in vivo* have been greatly facilitated by the use of appropriate mouse models.[61,65] In particular, the mouse model of typhoid fever has been invaluable in understanding the pathogenesis of *Salmonella* infection, identifying the bacterial virulence genes important during infection and dissecting the genetic components of the host response to this invading pathogen. In the following paragraphs, I will briefly describe the pathogenesis of *Salmonella* infection and review some aspects of *Salmonella* pathogenicity, as understood from the study of the mouse model.

2.1 Pathogenesis of *Salmonella* Typhimurium infection in mice

In 1892, Loeffler described an epidemic in mice that closely resembled human typhoid fever. Since the growth characteristics of the organism isolated from the affected mice were similar to what was seen with isolates from human typhoid patients (known at the time as *Bacillus typhi*), the organism was named *Bacillus typhimurium* (now *Salmonella* Typhimurium).[66] *Salmonella* Typhimurium appears to be a natural pathogen of mice, based on its frequent isolation from this natural reservoir.[67] Because it induces a systemic disease in mice similar to human typhoid fever, and also because *Salmonella* Typhi does not cause disease in species other than human or higher primates, the mouse model of *Salmonella* Typhimurium is now widely accepted as a good model for the study of the pathogenesis of typhoid fever.

Oral inoculation of mice with nontyphoidal *Salmonellae* rapidly results in the localization of the bacteria within the Peyer's patches of the distal ileum and cecum.[68] *Salmonella* spp appear to enter the Peyer's patches through invasion of M-cells, a type of specialized epithelial cells overlying these intestinal lymphoid follicles.[69] The destruction of the M-cells following *Salmonella* invasion exposes the basement membrane and allows the bacteria to penetrate deeper into the lamina propria. Additionally, the inflammatory reaction associated with invasion of *Salmonella* promotes the infiltration of neutrophils and macrophages, contributing to tissue inflammation, erosion of the intestinal mucosa and penetration of *Salmonella* into deeper tissues.[70] *Salmonella* may also passively cross the intestinal barrier following phagocytosis by CD-18 positive cells which contribute to extra intestinal dissemination by migration to systemic sites.[71] Two days post-infection, the mesenteric lymph nodes draining the ileum and cecum also harbor bacteria and systemic dissemination to the spleen and liver rapidly follows.[68] In susceptible mice, rapid replication within these target organs occurs until lethal levels are reached. The proliferation of *Salmonella* within the spleen and liver activates the host innate immune system, leading to the infiltration of neutrophils (early in infection) and mononuclear phagocytes, and development of hepatosplenomegaly.[72] The lesions within the spleen and liver appear as acute foci of necrosis that gradually mature to organized granulomas. During infection, *Salmonella* resides within the macrophages of the spleen and liver where it is capable of surviving and replicating.[72,73]

Systemic infection with a sublethal inoculum of *Salmonella* Typhimurium in mice is characterized by four distinct phases of infection[74,75] (Figure 2). The first phase involves rapid clearance of the organisms from the blood stream (within 2 hours), followed by localization of the inoculum within macrophages and polymorphonuclear cells of the spleen and liver where a proportion of the *Salmonellae* will survive and start replicating.[72,73] The second phase of infection takes place over the following week, with an exponential growth of the organisms within the RES of the spleen and liver. The macrophages exert an important regulatory function during this phase since administration of silica (a macrophage poison) results in a major increase in bacterial load and a substantial decrease in the LD_{50} by a factor of 100 times.[76] The host innate immune system, through the recognition of *Salmonella* or *Salmonella*-conserved motifs such as LPS, plays a major role at this stage. Recognition of pathogen-associated molecular patterns (PAMPs) by the host cell pattern recognition receptors (PRRs)[77] triggers an innate immune inflammatory response characterized by the production of several cytokines including tumor necrosis factor-α (TNFα) and interferon-γ (IFNγ) and by an abundant mononuclear infiltration of the spleen and liver aimed at the elimination of the pathogen. The activation of the innate immune system to stop bacterial growth results in the establishment of a plateau (third) phase. The final (fourth phase) resolution of infection is clearly a function of activation of the acquired immune system and depends on T and B cell activation.[78-81]

In resistant mice, infection with *Salmonella* Typhimurium results in a subclinical infection with chronic carriage and intermittent shedding.[82] At 60 days post-infection, *Salmonella* can be isolated from the liver, spleen, cecum, mesenteric lymph nodes, Peyer's patches and the gall bladder with intermittent shedding in the feces. Gradually, the infection is cleared from most of the sites but persists within the mesenteric lymph nodes of several mice and, in fewer mice also within the spleen and liver, up to one year following infection. The lesions associated with this chronic *Salmonella* infection consist of foci of necrosis, microgranulomas or accumulation of polymorphonuclear cells (at day 60), progressing to rare inflammatory foci consisting primarily of macrophages with minimal central necrosis. Similarly to what is seen with human typhoid fever, bacteria persist despite high levels of anti-*Salmonella* antibodies. Throughout infection, and as

seen in acute infections, the *Salmonellae* were localized intracellularly within macrophages. Interestingly, in mice infected for 260 days with *Salmonella* Typhimurium, clinical signs of systemic infection can be induced by injection of neutralizing antibodies against IFN-γ, confirming the essential role of this cytokine in the control of *Salmonella* infection (see section 3.3.3).

Mice are usually resistant to intestinal colonization and pathology following oral *Salmonella* Typhimurium infection, a phenomenon possibly attributed to their endogenous microbiota. Instead, as previously discussed, oral infection of mice with *Salmonella* results in the rapid dissemination of the pathogen to systemic sites without significant intestinal lesions. However, disruption of the resident microbiota with antimicrobial administration such as streptomycin, allows intestinal colonization by *Salmonella* Typhimurium in mice. This observation has been exploited for the study of the pathogenesis of *Salmonella* enterocolitis using the mouse as a model.[70,83] Mice pre-treated with streptomycin and infected orally with *Salmonella* Typhimurium excrete much higher levels of *Salmonella* in their feces compared to water pre-treated mice. Moreover, histopathological examination reveals that streptomycin pre-treated mice develop significant colitis and typhlitis by 24 hours post-infection while no lesions are present in control mice. The lesions are more pronounced in the cecum and consist of edema of the submucosa and lamina propria, crypt elongation, disruption of the crypt architecture, reduced number of goblet cells, epithelial erosion and ulceration, pronounced polymorphonuclear cells infiltration and transmigration into the intestinal lumen. Intestinal inflammation is, however, soon followed by systemic colonization of the spleen and liver as seen in non-streptomycin treated mice.

2.2 *Salmonella* virulence mechanisms identified in the mouse model of typhoid fever

2.2.1 Role of the *Salmonella* pathogenicity islands.

The ability of *Salmonella* to invade cells, survive and replicate within macrophages, and evade the immune system to establish long term carriage with intermittent shedding are key to its success as a pathogen. Acquisition, by the member of the genus *Salmonella*, of virulence genes found on a virulence plasmid or grouped into genomic regions named *Salmonella* pathogenicity islands (SPI) appear to have

contributed to the success of this pathogen.[84] Two of the SPI, SPI-1 and SPI-2, encode a specialized molecular syringe, called type III secretion system (TTSS), that allows the delivery of effector molecules to the host cells.[85,86] The TTSS effectors permit a fine manipulation of the host cell to the benefit of invading *Salmonellae*. SPI-1 and SPI-2 TTSS work at different times during the invasion and infection processes, delivering different effectors to the host cell. It is usually believed that SPI-1 TTSS is important for intestinal cell invasion in mice[87] while SPI-2 TTSS is required for intracellular replication and systemic disease.[88] This view is certainly overly simplified, however, since there appears to be an overlap of the SPI-1 and SPI-2 functions for both intestinal disease[70] and intracellular replication.[89]

During natural infection, *Salmonella* gains entry into the host by invading M-cells.[69] *Salmonella* invasion is a process actively controlled by the pathogen through various effector proteins that are injected into the host cell by the SPI-1 TTSS. Through the injection of various bacterial effectors (SipA and SipC, SopE, SopE2 and SopB), *Salmonella* induces remodeling of the host cytoskeleton, resulting in disappearance of apical microvilli, localized membrane ruffling that surrounds and eventually engulfs the bacteria (Figure 3).[90] SPI-1 mutants are attenuated when given orally to mice because they lack the ability to invade the intestinal mucosa. However, if injected intraperitonelly, SPI-1 mutants show full virulence.[91] SPI-1 TTSS is also essential for development of typhlocolitis in the streptomycin pre-treatment model[83] although SPI-2 TTSS also contributes to the severity of the lesions.[70]

Once inside the cell, *Salmonella* uses SPI-2 encoded TTSS to deliver a second set of effector proteins into the host cell cytosol. These effectors are essential for replication within the splenic and hepatic macrophages and for virulence in mice.[73,88] Following invasion, *Salmonella* resides in a membrane-bound compartment known as the *Salmonella* containing vacuole (SCV). The early stages of maturation of the SCV resemble those seen with true phagosomal maturation with acquisition of markers of early and late phagosomal stages such as Rab5, Rab7, LAMP along with the recruitment of vacuolar ATPase resulting in acidification of the SCV (Figure 4).[92] However, the maturation process appears to be altered by the *Salmonella* and fusion with pre-lysosomes and lysosomes fails to occur[93] as indicated by the complete absence of mannose-6-

phophate receptor (M6PR)[94,95] from the SCV. Both SPI-1 and SPI-2 TTSS secreted effectors appear to play a role in preventing the merging of the SCV with lysosomes.[89,96] Throughout this process, the SCV also avoids the delivery of nicotinamide dinucleotide phosphate (NADPH) oxidase[97] or inducible nitric oxyde synthase (iNOS),[98] thereby minimizing the exposure of *Salmonella* to harmful reactive oxygen and reactive nitrogen intermediates (ROI and RNI). The maturation of the SCV also differs from normal phagolysosome maturation in that it soon undergoes extensive fusion with endosomal compartments, resulting in the formation of long tubular extensions of the SCV called the *Salmonella*-induced filaments (Sifs).[99,100] This process is dependant on a SPI-2 TTSS effector, SifA, that is translocated from the SCV into the cytosol.[101,102] Although the exact function of the Sifs remains unknown, their presence indicate successful establishment of late-stage infection and the start of bacterial replication. SifA appears essential for maintaining the SCV since SifA mutant *Salmonellae* are found in the cytosol and not in a SCV.[73]

2.2.2 *Salmonella*-induced apoptosis.

Salmonella may be able to escape the phagocyte where it resides to infect adjacent cells through the induction of cell death. *Salmonella* is capable of inducing cell death through two distinct mechanisms: rapid cell death, dependent on SPI-1 TTSS and delayed cell death, dependent on SPI-2 TTSS.[103] The ability of *Salmonella* to cause inflammation within the intestinal mucosa and thereby invade deeper layers of the gut wall was linked to its ability to activate caspase-1 in a particular mouse strain.[104] Following invasion of M cells, *Salmonella* activates caspase-1 through translocation of the SPI-1 TTSS effector SipB into the host cell cytoplasm.[105] Caspase-1 is not only a pro-apoptotic cystein protease but it also directly cleaves the proinflammatory cytokines interleukin (IL)-1β and IL-18 into their bioactive forms, thereby promoting inflammation within the intestinal mucosa. A study done with caspase-1 knockout mice on a B10.RIII genetic background showed that these mice are more resistant to oral challenge with *Salmonella* while they are as susceptible as their wild-type counterpart to intraperitoneal infection, suggesting a role for caspase-1 in tissue colonization and systemic dissemination. This view has, however, recently been challenged since additional studies

done with caspase-1 knockout mice of a different genetic background have showed increased susceptibility to oral *Salmonella* challenge, an effect attributed mainly to a deficiency in IL-18 activation.[106,107] The biological relevance of cell death during *Salmonella* infection certainly continues to be unclear and it still remains to be shown whether *Salmonella*-induced cell death is a bacterial manipulation of the host or a host adaptation to the pathogen. However, the results of these recent studies emphasize the crucial role of caspase-1 dependent activation of IL-1β and IL-18 in the host response to acute *Salmonella* infection.

2.2.3 *Salmonella*-induced immunosuppression.

The propensity of *Salmonella* to persist within its host may be related to its ability to manipulate the host immune response. Several lines of evidence indicate that *Salmonella* has an immunosuppressive effect. Mice challenged with an attenuated strain of *Salmonella* exhibit a marked depression in their primary antibody response to sheep erythrocytes, an effect that persists for several weeks.[108] This immunosuppressive effect appears to be dependant on the production of NO by macrophages.[109] *In vitro* experiments also identified a strong suppression of MHC class II-dependant presentation of ovalbumin by murine dendritic cells infected with virulent *Salmonella* Typhimurium.[110] This effect was dependant on NO production by the dendritic cells and transcription of SPI-2 genes. This mechanism may be relevant to the pathogenicity of *Salmonella* infection *in vivo*, since the pathogen was frequently found within dendritic cells of the subepithelial dome of the Peyer's patches or of the mesenteric lymph nodes, and that vaccination with SPI-2 mutant conferred significantly more protection to challenge with a virulent strain compared to vaccination with a PurD mutant (attenuated to the same level than an SPI-2 mutant but expressing a functional SPI-2 TTSS).

Section 3. Genetic determinants of the host response to *Salmonella*

Several lines of evidence suggest that the genetic makeup of the host greatly contributes to susceptibility or resistance to infectious diseases. In 1988, a seminal study published by Sorenson *et al*[111] showed that the risk of dying from infectious disease was much increased for an adoptee if its biological parent had died of infectious disease

before age 50. The contribution of the host genetic background to the risk of infection and disease severity has also been evidenced in humans by studies documenting racial differences in disease susceptibility, a higher concordance in monozygotic versus dizygotic twins and, more recently, using large-scale family-based genome scans and association studies.[112-115] It is clear from these genetic analyses that the molecular mechanisms of resistance and susceptibility to infectious diseases are extremely complex and multifactorial with microbial virulence determinants and geographical environment factors modifying the expression of specific host susceptibility loci.

In humans, the risk and outcome of *Salmonella* infection are also influenced by genetic factors. For instance, patients with mutations causing sickle cell anemia,[116] chronic granulomatous disease (CGD)[117] or defects within cytokine signaling pathways[118,119] are at increased risk for *Salmonella* infection. Additionally, MHC class II and III genes have been associated with susceptibility to typhoid fever in a population of Vietnam.[120] While these examples illustrate that specific gene mutations or variants within the MHC have an impact on the host response to *Salmonella*, it is very likely that several additional genetic variants influence the outcome of *Salmonella* infection and remain to be identified. Because of inherent difficulties associated with genetic analysis in a human population, the mouse model of typhoid fever is used to identify genes that are important in the host response to *Salmonella* infection. These genes may then be good candidates to be tested for their impact on human *Salmonella* infections.

It was recognized over 30 years ago that the susceptibility of inbred mice to infection with virulent *Salmonella* Typhimurium varied from strain to strain and that these differences were genetically controlled.[121,122] In general, classical inbred strains of mice can be classified into three distinct categories in regard to their susceptibility to *Salmonella*.[122,123] 129S6/SvEvTac (129S6) mice are extremely resistant to infection with *Salmonella* Typhimurium compared to A/J mice that present an intermediate susceptibility phenotype, showing increased survival time with decreasing infectious dose but without surviving the infection (Figure 5). Other strains such as C57BL/6J, BALB/cJ and C3H/HeJ are extremely susceptible to infection and all succumb within the first week independently of the inoculum size. Differential susceptibility to *Salmonella* is also recognized among the wild-derived mice with CAST/Ei being very resistant in

comparison to MOLF/Ei or SPRET/Ei mice.[123] The development of genomic technologies (large scale cloning and sequencing, gene targeting, etc.) and mouse genome databases (http://www.informatics.jax.org) in the late 1980s and early 1990s combined with classical genetics contributed to the successful identification of several *Salmonella* resistance genes in laboratory mice. More recently, the development of novel models of infection together with quantitative trait mapping has identified additional host susceptibility loci.[123-125] This section will highlight key studies that led to the discovery of major *Salmonella* resistance genes in mice using different approaches including the generation of congenic mouse strains, positional cloning of spontaneous mouse mutations associated with susceptibility to *Salmonella* infection, targeted disruption of candidate genes and quantitative trait loci (QTL) mapping.

3.1 *Salmonella* resistance loci identified using congenic mice: genes of the major histocompatibility complex (H2).

The mouse histocompatibility complex (H2 complex) on MMU17 is a large genomic region encoding dense clusters of immune loci defining more than 120 genes.[126] The mouse H2 was first identified during the course of transplantation and serological studies, and was later shown to influence the outcome of several immune diseases including resistance to infection with *Salmonella* Typhimurium.[78,127] Using C57BL/10 congenic lines (all *Ity*^{s}-see below for a description of *Ity*), Hormaeche and Harrington[78] showed that mice carrying $H2^b$ and $H2^d$ haplotypes were more susceptible ($LD_{50} < 10^3$ CFUs) to a strain of *Salmonella* Typhimurium of intermediate virulence than those carrying $H2^a$, $H2^k$ and $H2^f$ haplotypes ($LD_{50} \geq 10^4$ CFUs). F1 hybrids between $H2^b$ (susceptible) and $H2^f$ (resistant) showed an intermediate phenotype suggesting a codominant mode of inheritance. Susceptibility of $H2^b$ mice was apparent 4 weeks following the infectious challenge and reached a maximum at seven to eight weeks post inoculation. The bacterial load was 10 to 100-fold higher in the spleen and liver after infection of susceptible $H2^b$ congenic strains compared to resistant $H2^f$. Using congenic mouse strains carrying recombinant H2 haplotypes, *Salmonella* susceptibility was mapped to the MHC class II I-Eα subregion. The major role of MHC class II-dependent

immune mechanisms in the elimination of *Salmonella* Typhimurium during the late course of infection was later highlighted using mice lacking MHC class II molecules (i.e. lacking H2-I-Aβ chain) and mature CD4+ TCRαβ cells.[128,129]

In another series of experiments using *Salmonella* Typhimurium C5TS (a temperature-sensitive mutant), three categories of susceptibility were defined by comparing late bacterial clearance among H2 congenic mice on a C57BL/10 genetic background.[130] The lowest rate of bacterial clearance was observed in $H2^b$, intermediate clearance in $H2^d$, $H2^f$, $H2^k$, $H2^p$, $H2^r$, $H2^s$ and $H2^v$, and high clearance in $H2^j$, $H2^q$, and $H2^u$. The impact of the H2 haplotype on bacterial clearance was influenced by different genetic backgrounds. Further analysis using H2 recombinant congenic mice on a C57BL/10 genetic background suggested that at least two additional regions of the H2 complex, H2-D and H2-K are involved in determining the late clearance phenotype.[127] A role for class-I restricted T cells in the immune response to *Salmonella* Typhimurium infection was later shown using mice lacking β2-microglobulin (β2m).[131] β2m is a 12 kDa protein known to associate with class I (H2-K, H2-D, H2-Q and H2-T) molecules that promotes activation of CD8+ T cells that specifically recognized cells infected with *Salmonella*.

In humans, recent studies support the contribution of the MHC to the host immune response to infection with *Salmonella*. Class II MHC was shown to be associated with susceptibility to *Salmonella* Typhi in Vietnam where typhoid fever is endemic[120] and MHC class Ib molecules were associated with the development of autoimmune reactive arthritis following *Salmonella* infection.[132,133] The inherent complexity and polymorphism of the MHC complex and the linkage disequilibrium between loci will continue to pose a significant challenge to the identification of specific *Salmonella* susceptibility genes located in this area of the genome.

3.2 *Salmonella* resistance genes identified by positional cloning

3.2.1 Nramp1.

The first description of *Ity* (Immunity to Typhimurium) in the mouse appeared in 1976.[121] In this early paper, the authors showed that eight strains of inbred mice fell into two sharply defined groups with respect to resistance to infection. Four strains (CBA,

A/J, C3H/He and DBA/2) were resistant ($LD_{50} > 10^5$) to *Salmonella* Typhimurium C5 and the other four strains (BALB/c, C57BL, B10.D2 and DBA/1) were susceptible (LD_{50}, < 10). These strain differences in disease resistance were shown to behave as a simple Mendelian trait with a dominant mode of inheritance. Three years later, *Ity* was located to mouse chromosome 1 using distinguishable phenotypes as chromosome markers.[134] Around the same time, two other groups identified host resistance loci for two unrelated pathogens, *Mycobacterium bovis* (*Bcg*) and *Leishmania donovani* (*Lsh*).[135,136] These two loci were also located on mouse chromosome 1 to the same genomic subregion than *Ity*.[137,138] At that time, it was not clear if *Bcg*, *Ity* and *Lsh* were either the same or very closely related genes. The identification of a unique gene underlying *Ity/Bcg/Lsh* came almost 20 years after its first description.[139,140]

Two allelic forms were recognized for *Ity*: a resistance allele, Ity^r and a susceptibility allele, Ity^s.[134] The resistance allele is dominant and influences the rate of bacterial growth during the exponential phase of multiplication in the RES and the LD_{50} after lethal challenge with high doses of *Salmonella* Typhimurium.[121] *Ity* is expressed by macrophages and Ity^r confers an increased bactericidal capacity to this cell type in comparison to Ity^s.[141] Studies involving experimental mouse model of infection with *Mycobacteria* and *L. donovani* showed that Bcg^r and Lsh^r behaved similarly, being expressed in macrophages and conferring resistance to bacterial multiplication of the intracellular parasites during the early phase of infection.[135,142,143] A positional cloning approach was undertaken to identify the gene underlying the *Bcg* phenotype.[144,145] *Bcg* was mapped by segregation analysis to the proximal portion of mouse chromosome 1 closely linked to the villin (*Vil*) gene.[146] High resolution linkage and physical maps were generated and allowed to narrow the chromosomal segment encompassing the *Bcg* locus to a size amenable to positional cloning.[147,148] Subsequently, using exon amplification, a candidate gene for the *Bcg* locus was found and named natural resistance-associated macrophage protein-1 gene or *Nramp1*.[140] *Nramp1* was recently renamed *Slc11a1* because of its membership to a family of solute carriers.[149] Predicted protein sequence analysis of *Nramp1* between Ity^r and Ity^s strains revealed a single mutation resulting in glycine to aspartic acid substitution at position 169.[150] This mutation results in complete lack of function of Nramp1 in susceptible mice.[151] The identity of *Nramp1*, *Bcg*, *Ity* and

Lsh was confirmed by targeted disruption of *Nramp1* in mice and phenotypic comparison among different genotypes of mice during experimental infections with *Salmonella* Typhimurium, *M. bovis* and *L. donovani.*[139] Additionally, susceptible mice were rendered resistant to BCG and *Salmonella* Typhimurium by transfer of the resistance allele, further confirming the identity of *Nramp1* with the phenotypic resistance to *Salmonella* Typhimurium.[152]

The identification of *Nramp1* and its function opened a whole new field in the area of host resistance to intracellular pathogens. *Nramp1* encodes a highly hydrophobic 56 kDa protein, which possesses 12 transmembrane (TM) domains and a glycosylated extracytoplasmic loop. It is expressed in the membrane fraction of macrophages and neutrophils as a phosphoglycoprotein of 90 to 100 kDa.[149] During phagocytosis, Nramp1 is recruited to the membrane of the phagosome and remains associated with this structure during its maturation to a phagolysosome.[153] Its effect on *Salmonella* replication within the macrophage may be related to its capacity to counteract *Salmonella*'s ability to become secluded from the endocytic pathway. Cuellar-Mata *et al* [154] have indeed shown that Nramp1 has an impact on SCV maturation. SCV formed in *Nramp1*$^{-/-}$ macrophages fail to acquire M6PR and become inaccessible to extracellular markers. In contrast, Nramp1 wild-type macrophages do acquire M6PR and remain accessible to extracellular dextran. Since M6PR is known to regulate the delivery of a subset of lysosomal enzymes from the trans-golgi network to the pre-lysosomal compartment, it is conceivable that Nramp1 wild type macrophages may have increased bactericidal activity.

These findings support the hypothesis that Nramp1 controls the replication of intracellular parasites by altering the intravacuolar environment of the phagosome. In fact, it was shown that Nramp1 functions as a pH-dependant cation transporter.[155,156] Divalent cations, like manganese and iron, are likely important for the survival of pathogens and removal of these from the phagosome probably results in enhanced bacteriostatic or bactericidal activity and increased resistance to intracellular pathogens. Nramp orthologues have been identified in *Salmonella* (MntH) and other bacteria. They are also proton-dependent manganese transporters and appear to function as an import system for the acquisition of divalent metals from the extracellular environment.[157] *Salmonella* MntH and mammalian Nramp1 proteins might influence the outcome of

bacterial infection through competition for the same essential substrates within the microenvironment of the phagosome.[149]

Because of the critical role of Nramp1 in the mouse model of typhoid fever, the human homologue of mouse *Nramp1* was investigated in resistance to typhoid fever in humans. Despite the fact that *NRAMP1* contributes clearly to the risk and the progression of mycobacterial infections in humans,[114,158-160] no allelic association was identified between *NRAMP1* and typhoid fever susceptibility in humans in southern Vietnam.[161] However, these studies do not preclude a role for *NRAMP1* in susceptibility to human *Salmonella* infections because of the known effect of the ethnic genetic background on the expression of disease susceptibility. The role of allelic variation at *NRAMP1* was also examined in a chicken model of *Salmonella* infection.[162] Using crosses between resistant (W1) and susceptible (C) chickens, *NRAMP1* was shown to account for 18% of the early differential resistance to infection.

3.2.2 Tlr4.

Toll like receptor 4 (Tlr4; first described as the *Lps* locus) is another critical gene that regulates innate resistance to infection with *Salmonella* Typhimurium and the host response to LPS in mice. Bacterial LPS is a major constituent of the outer membrane of Gram-negative bacteria and is essential to the structure and survival of these bacteria.[163] Through evolution, the immune system of eukaryotes has learned to recognize LPS as an indicator of Gram-negative bacterial infection. Indeed, very small amounts of LPS are able to initiate a robust inflammatory response in the host. LPS molecules are PAMPs that are naturally recognized through receptors of the innate immune system known as PRRs.[164] The Toll-like receptor (TLR) family is a good example of PRRs and among them, Tlr4 was identified as an important component of the signal transduction initiated by LPS.

The discovery of Tlr4 was rendered possible because of earlier identification of LPS hyporesponsiveness of C3H/HeJ mice.[165] C3H/HeJ mice can withstand 20 to 38 times the LD_{50} for other inbred strains when challenged intravenously with LPS and show an altered intraperitoneal inflammatory response with a reduced ratio of neutrophils to mononuclear cells following local injection of LPS.[165,166] However, if challenged with

Gram-negative bacteria such as *Salmonella* Typhimurium, C3H/HeJ mice present a markedly increased susceptibility to this pathogen. The LD_{50} for LPS responsive mice to *Salmonella* Typhimurium infection is $\geq 2 \times 10^3$, while LPS hyporesponsive strains succumb to infection with less than 2 organisms.[167] LPS hyporesponsiveness was also recognized in other strains including C57BL/10ScCr,[168] its progenitor C57BL/10ScN[169] and more recently in C57BL/6.KB2-mnd.[170] The failure of (C3H/HeJ x C57BL/10ScCr)F_1 and (C3H/HeJ x C57BL/6.KB2-mnd)F1 to respond to LPS suggest that these three strains carry mutations at the same gene.[170,171] Segregation analysis in backcrosses between responsive and hyporesponsive strains revealed that this phenotype was inherited as a simple Mendelian trait.[172,173] The locus was named *Lps*, and two alleles were defined: Lps^n and Lps^d for normal and defective response to LPS respectively.[174] The mode of inheritance varied with the phenotype studied and the strain combination used: heterozygote mice issued from C3H/HeJ and C57BL/6J present an intermediary LPS-response consistent with a codominant mode of inheritance[173] whereas crosses made between C57BL/10ScCr and Lps^n mice show a fully dominant wild-type allele.[171] These observations may be explained by monoallelic expression of Tlr4 and the nature of the *Lps* allele in the studied strains (see below).[175] The response to *Salmonella* infection was inherited as a single dominant trait in all crosses performed between Lps^d and Lps^n mice.[167,176]

Early linkage analysis studies revealed that *Lps* cosegregated with the major urinary protein locus (*Mup-1*) and the polysyndactyly (*Ps*) mutation indicating that *Lps* is located on mouse chromosome 4.[173,174] High resolution genetic, physical and transcriptional maps of the area were thereafter generated[176,177] and led to the identification of *Tlr4* as a candidate for *Lps*.[177,178] Three different *Tlr4* mutant alleles were identified: C3H/HeJ mice present a single missense mutation resulting in a proline for histidine substitution at codon 712 within the signaling domain;[178,179] in C57BL/10ScCr mice, there was no *Tlr4* transcripts detected[178,179] as a consequence of a 75 kb chromosomal deletion encompassing the whole *Tlr4* gene;[180] the mutation identified in C57BL/6.KB2-*mnd Tlr4* consists in a complete deletion of exon II. This mutation leads to a frameshift resulting in the appearance of a stop codon just downstream of the exon junction. The putative Tlr4 mutant protein is equivalent to the first 31 N-terminal

residues of its wild-type counterpart (835 residues).[170] Confirmation of the role of *Tlr4* in LPS hyporesponsiveness was obtained through examination of mice that had been rendered deficient for *Tlr4*.[181]

The TLR family[164,182,183] is composed of type 1 TM receptors characterized by an extracellular leucine-rich repeat domain and an intracellular domain similar to the intracellular domain of the IL-1 receptor called the TIR (Toll/IL-1 receptor) domain. The first identified member of this family, *Drosophila Toll*, functions in a pathway that controls the dorso-ventral axis formation of the fly.[184] In adults, mutations in *Drosophila Toll* lead to increased susceptibility to fungal infection because of failure to induce the antifungal peptide Drosomysin thereby linking this gene to innate immunity. In mammals, 11 TLRs have been identified and most of them have been shown to be essential for defense against different pathogens by sensing specific PAMPs. TLR4 appears to interact directly with LPS with the cooperation of LBP (LPS-binding protein) and co-receptors CD14 and MD-2.[185-187] Ultimately, LPS sensing by TLR4 leads to the activation of various transcription factors through the activation of two known signaling pathways: 1) a MyD88-dependant signaling pathway, dependant on the adaptors MyD88 and TIRAP, leading to a rapid activation of NFκB and transcription of inflammatory cytokines, and 2) a TRIF/TRAM-dependant, MyD88-independent pathway leading to delayed activation of NFκB, specific activation of IRF3, and expression of IFN-β and interferon responsive genes. Activation of TLR4 by LPS leads to the induction of various host defense genes including pro-inflammatory cytokines such as IL-1, IL-6, IL-8 and IL-12, chemokines, costimulatory molecules (CD80 and CD86), MHC class II and iNOS by antigen presenting cells.[188-190] Induction of CD80/CD86 and IL-12 by TLRs contribute to the initiation of adaptive immunity and the induction of TH1 effector responses.[189]

In humans, the role of TLR4 polymorphisms was originally investigated in the response of airways to inhaled LPS. Two co-segregating missense mutations (Asp299Gly and Thr399Ile) were identified and found to be associated with a blunted response to inhaled LPS.[191] These hypomorphic alleles have since been associated with protection or susceptibility to various human diseases including Gram-negative infection,[192] septic shock due to gram-negative organisms,[193] severe malaria,[194] brucellosis,[195] Crohn's disease,[196] severe sepsis following burn injury,[197] atherosclerosis,[198] Legionnaire's

disease[199] and late-onset Alzheimer's disease.[200] TLR4 has also been associated with resistance to *Salmonella* Typhimurium in chickens following linkage analysis in 274 progeny derived from the *Salmonella*-resistant W1 and the *Salmonella*-susceptible C inbred lines of chickens.[201]

3.2.3 Btk.

The role of B lymphocytes in immunity to *Salmonella* Typhimurium was studied initially in CBA/N inbred mice, a strain that has impaired humoral immunity because of a peripheral defect in B-cell activation and function.[202] This defective phenotype was mapped to chromosome X and named *xid* for x-linked immunodeficiency. The B cell defect of CBA/N mice is characterized by an impaired maturation of B cells, diminished immunoglobulin production (CBA/N mice have low serum IgM and IgG_3 levels) and compromised T-independent immune response. Because of their immunodeficiency, CBA/N mice present a late susceptibility to infection with pathogens such as *Salmonella* Typhimurium.[81] Susceptibility of *xid* mice to *Salmonella* Typhimurium is recessive; hemizygous male and homozygous female present the susceptibility phenotype while heterozygous females are resistant[81] due to preferential inactivation of the X chromosome carrying the defective *xid* allele.[203] Passive transfer of immune serum restores resistance in affected males, an effect attributed to the presence of specific anti-*Salmonella* antibodies.[204] These results clearly indicated a role for circulating antibodies in resistance to *Salmonella* Typhimurium during the late (>10days) phase of infection.

The *xid* mutation was localized to a region of mouse chromosome X showing conserved homology with a region of the human genome carrying the gene involved in X-linked agammaglobulinaemia (XLA)[205] a disease that resembles the phenotype expressed in *xid* mice.[206] Male patients with XLA have a severe B cell immunodeficiency characterized by reduced numbers of mature circulating B cells, diminished serum Ig levels and disrupted secondary lymphoid architecture. Intestinal infections with *Salmonella* spp have been described in XLA patients however bacterial infections involving the respiratory tract caused by *Streptococcus pneumoniae, Haemophilus influenzae, Staphylococcus aureus,* and *Pseudomonas* spp are the most frequent infections.

In 1993, mutations in the gene encoding Bruton's tyrosine kinase (btk) were reported to cause *XLA* in humans[207,208] and *xid* in mice.[205,209] btk belongs to the Tec family of non-receptor protein-tyrosine kinases, known to be highly expressed in hematopoietic cells.[210] *btk* is expressed at all stage of B cell development from pro to mature B cells, but is down regulated in plasma B cells. Its expression occurs also in erythroid precursors, myeloid cells, mast cells and megakaryocytes but not in T or NK cells. btk participates in intracellular signal transduction in a number of cell activation pathways such as those mediated by the B cell antigen receptor (BCR) and the toll-like receptor RP105.[211] *btk* encodes a tyrosine kinase that possesses pleckstrin-homology (PH) and Tec-homology (TH) domains in addition to src homology (SH) domains (SH1, SH2 and SH3).[212] btk, as a major component of the BCR signalosome, plays a critical role in the regulation of pre-B and mature BCR signaling.[213-215] Recruitment of btk to the cellular membrane and its subsequent activation triggers the mobilization of intracellular calcium and the activation of PKC resulting in the degradation of the NF-κB inhibitory protein I-κBα and the translocation of NF-κB to the nucleus.[216-218] In humans, more than 175 different mutations involving all domains of the BTK gene have been identified in XLA patients.[206] In *xid* mice, a missense mutation at a conserved arginine residue (R28C) within the PH domain of btk impairs its ability to translocate to the plasma membrane and trigger signaling cascades that regulates B cell survival and growth,[205,209,215] consequently affecting resistance to infection with *Salmonella*.[81]

3.3 *Salmonella* resistance loci identified using gene-deficient mice

The use of gene targeting has been very successful for investigating the role of several *Salmonella* resistance genes in mice. Candidate genes have been selected based on the biological understanding of the disease phenotype or based on the *in vitro* response to the pathogen. In the following paragraphs, I will discuss selected critical genes which role in mouse resistance to *Salmonella* infection was uncovered by engineering knockout mice.

3.3.1 Lbp and Cd14.

Lbp and Cd14, like Tlr4, are known to bind LPS and have been involved in innate defense against *Salmonella* Typhimurium. Lbp is an acute phase protein found in the serum that accelerates the binding of LPS to Cd14 and initiates signals through membrane-bound Tlr4 in monocytes and myeloid cells.[219] Work with Lbp-deficient mice has shown that Lbp is essential for the induction of a rapid inflammatory response and for survival following intraperitoneal infection with *Salmonella* Typhimurium.[220] The critical function of Lbp in resistance to infection was confirmed by the rescue of the susceptible $Lbp^{-/-}$ mice with recombinant mouse Lbp supplementation.[221]

Cd14 is a glycosylphosphatidylinositol-anchored molecule that is expressed on monocytes and neutrophils and acts as a high affinity receptor for LPS. Cd14-deficient mice were found to be extremely resistant to the effect of LPS, with 100% survival and almost no detectable clinical signs following challenge with 10 times the LD_{100} for control mice. This increased resistance correlated with markedly decreased expression of cytokines such as TNFα and IL-6.[222] Interestingly, Cd14-deficient mice were also more resistant to Gram-negative (*E. coli*) bacterial challenge with decreased level of bacteremia, suggesting a role for Cd14 in bacterial dissemination.[223] However, in the case of the Gram-negative intracellular pathogen *Salmonella* Typhimurium, Cd14 appears as an important resistance gene.[224]

3.3.2 NADPH oxidase and iNOS.

Following phagocytosis of virulent *Salmonella* Typhimurium, two major enzyme systems come into play to inactivate the pathogen within the phagosome: the phagocytic NADPH oxidase and iNOS. NADPH oxidase and iNOS participate to the generation of ROI and RNI respectively.[225,226] The importance of NADPH oxidase in resistance to *Salmonella* infection was originally uncovered in humans suffering from CGD, a group of inherited disorders characterized by recurrent infections and chronic inflammation. The disease results from mutations in any one of four subunits of the NADPH-oxidase. The majority of patients with CGD present X-linked deficiencies of $gp91^{phox}$ (renamed CYBB); most autosomal recessive CGD are associated with mutations within $p47^{phox}$ (*NCF1*) or $p67^{phox}$ (*NCF2*); a rare autosomal recessive form is associated with mutations

within *p22^{phox}* (CYBA).[227] Mice deficient in gp91^{phox} were engineered as a model for CGD and found to be extremely susceptible to infection with *Salmonella* Typhimurium.[228,229] Early death of gp91^{phox} deficient mice after *Salmonella* infection was associated with spleen and liver bacterial load exceeding by at least 3 log the wild type controls.[228]

Studies with iNOS-deficient mice support a dual role for NO during virulent *Salmonella* infection *in vivo*. Enhanced production of NO provides increased host defense against pathogens but also contributes to inflammation, tissue damage and even endotoxic shock.[228,230,231] Additionally, NO is essential for survival following infection with an attenuated strain of *Salmonella* but at the same time, it mediates *Salmonella*-induced immunosuppression.[109] Although *Nos2* (the gene encoding for iNOS) knockout mice are able to control the early replication of *Salmonella* in the RES organs, they are unable to suppress bacterial growth later during infection and eventually die.[228] This observation contrasts with the gp91^{phox} deficient mice which are extremely susceptible to *Salmonella* Typhimurium early during the course of infection even with a very low inoculum.[228]

It is clear from these studies that intracellular killing of *Salmonella* Typhimurium is dependent on both ROI and RNI systems, however these reactive intermediates appear to act at different stages of infection (NADPH oxidase being more critical early after infection and iNOS later during infection). Double mutant mice (*gp91$^{phox-/-}$/Nos2$^{-/-}$*) exhibited spontaneous infections caused by organisms of the normal flora, resulting in formation of large internal abscesses.[229] This phenotype was not exhibited by mice deficient in only one of the enzyme activity, indicating that in spontaneous infections with indigenous flora, gp91^{phox} and Nos2 can compensate for each other and that no other pathway could compensate for their simultaneous absence.

3.3.3 Cytokines: TNFα, IFNγ and IL-12.

The release of proinflammatory cytokines from activated T cells, NK cells and macrophages is pivotal in controlling the primary immune response to *Salmonella*. TNFα[232] is a pleiotropic pro-inflammatory cytokine produced mainly by macrophages but also by activated NK cells and TH1 lymphocytes. It is encoded by the *Tnf* gene and exerts

its effects through two types of receptors: TNFRp55 (encoded by *Tnfrsf1a* for TNF receptor superfamily 1a gene) and TNFRp75 (*Tnfrsf1b*). This cytokine plays a key role in host defense against pathogens through several mechanisms including activation of neutrophils and platelets, enhancement of killing activity of macrophages and NK cells, and activation of the immune system. Mice carrying a targeted disruption of *Tnfrsf1a* were found to be more susceptible to challenge with virulent *Salmonella* Typhimurium and to attenuated *purE*, *aroA* and *sseB* mutants.[233,234] The early susceptibility of *Tnfrsf1a*$^{-/-}$ mice to *Salmonella* was attributed to a defect in the early bactericidal capacity of *Tnfrsf1a*$^{-/-}$ macrophages.[234] Although this early phase of bacterial killing within the macrophages is associated with activation of the NADPH oxidase system, comparable levels of superoxide were detected within the infected macrophages of normal and knockout mice. Further analysis, using fluorescence microscopy and transmission electron microscopy, showed that TNFRp55 is necessary for targeting of NADPH phagocyte oxidase-harboring vesicles to SCVs.[234]

IFNγ is produced by activated T cells and NK cells following IL-12 stimulation and plays a key role in TH1 responses.[118] IFNγ is responsible for activating macrophages and influences also antibody class switching. The growth of attenuated *Salmonella* Typhimurium *aroA*$^{-}$ is contained in wild type mice, however IFNγ-deficient mice succumb to infection due to unrestricted bacterial proliferation.[129,235] These experiments point out the central role that IFNγ plays in mice against bacterial strains of poor virulence. The anti-*Salmonella* effect of IFNγ appears to be in part due to stimulation of the NO production by activated macrophages, which would have a repressive effect on the transcription of SPI-2 genes. Inhibition of SPI-2 transcription would facilitate the maturation of the SCV by allowing fusion with late endosomes and lysosomes.[236] Deficiencies in the IFNγ axis are not only associated with higher susceptibility to infection with *Salmonella* in mice but also with increased susceptibility to other intracellular pathogens.[61]

IL-12 is an heterodimeric cytokine composed of two subunits, IL-12p35 (encoded by *Il12a*) and IL-12p40 (encoded by *Il12b*), linked by two disulfide bonds.[118,119] IL-12 is produced and secreted mainly by antigen presenting cells (dendritic cells and macrophages). The IL-12 receptor (composed of two subunits IL12Rβ1 and IL12Rβ2) is

found predominantly on T and NK cells. The principal known function of IL-12 is the induction of IFNγ and consequently the development of TH1 responses. Administration of monoclonal antibodies directed against IL-12 exacerbate the mild disease usually caused by *Salmonella* Typhimurium *aroA*$^-$ in BALB/c mice, eventually leading to death.[237] The administration of anti IL-12 antibodies resulted in decreased local and systemic IFNγ concentration, lower tissue iNOS activity and increased serum IL-10 levels in infected mice. The role of IL-12 in resistance to *Salmonella* infection was also studied in mice carrying targeted disruptions of genes encoding for either subunits of IL-12, *Il12a* or *Il12b*.[238] In a model of systemic infection with *Salmonella* Enteritidis, *Il12a*$^{-/-}$ and *Il12b*$^{-/-}$ mice were more susceptible to infection than wild type mice. However, the increased susceptibility was more pronounced in *Il12b*$^{-/-}$ mice. Lack of *Il12b* resulted in a TH2 response, which was inadequate for immunity against the intracellular pathogen *Salmonella* Enteritidis.

Pathogenic mutations in genes coding for the IFNγR1, IFNγR2, IL-12p40 and IL-12Rβ1 were reported in the syndrome of Mendelian susceptibility to mycobacterial disease.[118,119] These pediatric patients were first identified because of their susceptibility to poorly virulent mycobacterial species such as BCG. The patients rarely develop other infectious diseases with the exception of *Salmonella* infections that are found in almost half of the cases. The clinical manifestations of the disease are heterogenous and range from abdominal abscesses and adenitis to severe sepsis. Several *Salmonella* serotypes have been identified and include *Salmonella* Paratyphi[239,240] or nontyphoid *Salmonella* serotypes such as *Salmonella* Typhimurium or Enteritidis.[239-243]

3.4 Host resistance loci identified using QTL analysis

Other loci influencing resistance or susceptibility to *Salmonella* infection in mice have been identified using QTL analyses.[123-125] The wild-derived inbred mice, MOLF/Ei was initially found to be extremely susceptible to *Salmonella* Typhimurium with survival time comparable to the survival time of C57BL/6J (*Nramp1*^{s}) despite having a wild type allele at *Nramp1*. Subsequently, linkage analysis using 252 (C57BL/6J x MOLF/Ei) F2 mice allowed the mapping of two new QTL, which significantly affect survival time following lethal infection with *Salmonella* Typhimurium.[123] A *Salmonella*-resistant

phenotype (*Ity2*) was linked to a region on mouse chromosome 11 (LOD score of 7.0 at *D11Mit5)* and contributed 10% to the variance. Several candidate genes were detected in the surrounding region including granulocyte/macrophage colony-stimulating factor (*Csfgm*), interleukin 3 (*Il3*), inducible nitric oxide synthase (*Nos2*) and myeloperoxydase (*Mpo*). The candidacy of *Nos2* was evaluated by measuring *Nos2* mRNA levels and nitrite production in MOLF/Ei mice during infection. MOLF/Ei mice showed a decreased capacity to induce *Nos2* mRNA and to produce NO.[244] As mentioned earlier, studies with *Nos2*-deficient mice support a dual role for NO during virulent *Salmonella* infection *in vivo*. Enhanced production of NO provides increased host defense against pathogens but also contributes to inflammation, tissue damage and even endotoxic shock.[245] Although *Nos2* knockout mice are able to control the early replication of *Salmonella* in the RES organs, they were unable to suppress bacterial growth and eventually die from infection.[228] In this model, low *Nos2* mRNA levels correlate with a reduced NO production and a decreased inflammatory response, a finding that may explain the protective effect of *Ity2* on chromosome 11.

A second QTL, *Ity3*, was located on mouse chromosome 1, approximately 25 cM distal to *Nramp1* (LOD score of 4.8 at *D1Mit100*). *Ity3* was inherited recessively with the MOLF/Ei allele conferring susceptibility to infection. The effect of this locus was identified only after adjustment for the effects of *Nramp1*, and contributed to 7% of the variance in survival time. The chromosomal region harboring *Ity3* is also rich in candidate genes, including *Tlr5* and the neutrophil cytosolic factor 2 gene, *Ncf2*. *Tlr5* was first considered a great candidate based on its biological function as a signaling PRR for flagellin, on its sequence analysis revealing a unique haplotype in MOLF/Ei and on its very low expression levels in the liver of MOLF/Ei compared to other inbred strains, including C57BL/6J.[246] However, further analyses did not support the candidacy of *Tlr5* as the gene underlying *Ity3* since, when removed from its original genetic background, the MOLF/Ei *Tlr5* allele was found to be expressed at higher level than the C57BL/6J allele.[247] Moreover, during *in vivo* flagellin stimulation, MOLF/Ei mice were found to produce very little IL-6 or CXCL-1 while C57BL/6J mice congenic for *Ity3*, including the MOLF/Ei *Tlr5* allele, showed higher levels of both IL-6 and CXCL-1 compared to

MOLF/Ei or C57BL/6J. These results suggest that the MOLF/Ei genetic background influences the activity of *Tlr5 in vivo* and that *Tlr5* is not the gene underlying *Ity3*.

The candidacy of *Ncf2* was then evaluated and is now supported by sequence analysis and functional data in B6.MOLF-*Ity/Ity3* congenic mice.[248] *Ncf2* encodes the protein $p67^{phox}$, one of the five subunits of the phagocyte NADPH oxidase. In MOLF/Ei, the *Ncf2* allele was found to carry a nonconservative amino acid change (R394Q) within a crucial functional domain of the protein. Additionally, B6.MOLF-*Ity/Ity3* congenic mice, which are more susceptible to *Salmonella* compared to control mice heterozygous at *Ity3*, showed a trend for decreased expression of *Ncf2* in their spleen. Furthermore, the MOLF/Ei *Ncf2* allele showed decreased PMA and *Salmonella*-induced superoxide production compared to the wild type allele *ex vivo*. Finally, in humans, a mutation at the same amino acid results in CGD and increased risk of *Salmonella* infection. These findings indicate that *Ncf2* remains a very good candidate for *Ity3*.

In another study, a chronic model of infection with *Salmonella* Enteritidis was used to identify loci implicated in the late bacterial clearance of the organism from the spleen.[124] Two strains, C57BL/6J ($Nramp^s$, $H2^b$) and 129S6 ($Nramp1^r$, $H2^b$) were characterized according to their ability to clear *Salmonella* Enteritidis from the spleen. 129S6 mice were found to have markedly impaired ability to clear the parasite when compared to C57BL/6J. Pairwise epistatic QTL mapping performed on a dense set of markers in 300 (129S6 x C57BL/6J) F2 mice allow identification of several QTL having individual or interacting effect on the bacterial clearance.[124,125] The detected loci were successively numbered as *Salmonella* Enteritidis susceptibility (*Ses*) loci. A sex effect was detected in this model with the females showing increased bacterial clearance compared to males and forcing the separate analysis of the male and female data. The final model retained for the female mice included an individual effect of *Ses3* (*D15Mit29*) and two interactions involving *Ses1* (*D1Mcg5*) and *Ses4* (*DXMit48*), and *Ses1* and *Ses5* (*D7Mit267*). In males, the model included also one QTL with individual effect, *Ses1.1* (*D1Mit123*) and three significant interactions involving *Ses1* and *Ses6* (*D9Mit218*), *Ses7* (*D2Mit197*) and *Ses8* (*D4Mit2*), and *Ses9* (*D3Mit356*) and *Ses10* (*D13Mit36*). For all loci, the C57BL/6J allele was associated with increased bacterial clearance and behaved recessively except for *Ses7*, which was dominant. For both the males and the females, the

proposed model explained 47% of the phenotypic variance. The effect of *Ses1* and *Ses1.1* on the bacterial clearance was confirmed in congenic mice.

Ses1 maps to the genomic region of *Nramp1*, which segregates in this cross. Because of the known effect of *Nramp1* on the mouse response to *Salmonella*, the candidacy of *Nramp1* as the gene underlying *Ses1* was investigated in 129S6 and 129S6 *Nramp1*$^{-/-}$ mice.[124] Following infection with *Salmonella* Enteritidis, mice carrying a functional *Nramp1* gene had higher CFUs in their spleen compared to the *Nramp1*$^{-/-}$ mice, indicating that, in this model of chronic infection, having a functional *Nramp1* allele has a detrimental impact on bacterial clearance. Further studies indicated that functional polymorphisms at *Nramp1* influence the T helper differentiation during chronic infection with *Salmonella* Enteritidis in mice with increased bacterial clearance linked to a TH2 response (seen in mice carrying a non-functional *Nramp1*) and delayed bacterial clearance linked to the development of a TH1 response (as seen in mice carrying a wild-type *Nramp1*).[249]

These studies illustrate the genetic complexity associated with the host response to *Salmonella* infection in mice and emphasize the need of accounting for sex effect and epistasis in genetic mapping studies. The future of genetic mapping of complex trait certainly lies within the ability we will have to compute and model multiple genetic and environmental interactions.

Section 4. Iron balance and anemia of inflammation in infectious diseases

The systemic activation of the host innate and adaptive immune system during infection has several consequences on normal body homeostasis. Among others, the various cytokines and acute phase proteins produced during systemic inflammation have a strong impact on the host erythropoietic response and iron balance. Various observations made throughout my PhD work have motivated the investigation of the role of anemia and iron balance during *Salmonella* infection in mice. In the following paragraphs, I will briefly introduce two related topics, the pathogenesis of anemia of inflammation and the role of iron during infectious diseases.

4.1 Anemia of inflammation

Sustained immune activation may result in the development of anemia, a clinical entity referred to as anemia of inflammation.[250] Several clinical conditions have been associated with anemia of inflammation, including infections, cancer and autoimmune diseases. Anemia of inflammation is usually mild to moderate, normochromic, normocytic and non-regenerative. Additional laboratory findings include low serum iron, low to normal transferrin, decreased transferrin saturation, normal to high serum ferritin and increased circulating cytokines. The mechanisms underlying this phenomenon are numerous and complex and include changes in iron homeostasis, decreased red blood cell (RBC) life span, decreased erythropoietin production and decreased proliferation of erythroid progenitors, which are all direct consequences of the immune system activation and cytokine productions (Figure 6).

Anemia of inflammation develops because of the incapacity of the erythropoiesis response to increase slightly in reaction to a small decrease in RBC life span.[251] The decreased survival of RBC is believed to result from the activation of macrophages which prematurely remove aging RBC from the blood stream. Whether some bacterial products or host-derived factors also contribute to the decreased life span of RBC is still open to debate. The destruction of RBC in anemia of inflammation is, however, only mildly accelerated, and in a normal setting should be easily compensated for. A relative resistance to the effect of erythropoietin and changes in iron homeostasis appear to prevent such increases in the production of erythrocytes. In fact, the changes in iron homeostasis appear central to the development of anemia during infection. The cytokines produced by the host increase the sequestration of iron within macrophages and decrease its absorption from the intestine, thereby greatly diminishing the availability of iron for RBC synthesis. The acute phase protein, hepcidin, appears to play a central role in iron regulation during inflammation and in the development of anemia of inflammation. Produced by the hepatocytes under the influence of cytokines such as IL-6,[252] hepcidin decreases the export of iron from macrophages, hepatocytes, duodenal enterocytes and placental syncytiotrophoblasts. Iron is usually exported from these cells when needed through the iron export channel, ferroportin. Under the influence of hepcidin, ferroportin is internalized and degraded, thereby sequestering the iron within these cells.[253]

Therefore, despite seemingly adequate iron stores, iron is unavailable for erythropoiesis and anemia ensues. In the case of long standing anemia of inflammation, true iron deficiency may develop since absorption from the duodenum is also inhibited.

Although the pathways regulating hepcidin gene expression are still incompletely understood, various signals have been identified. First, raised body iron enhances hepcidin expression in a mechanism that is independent of IL-6.[252,254] Up regulation of hepcidin expression during iron overload would result in decreased iron release from macrophages and decreased duodenal absorption, thereby counteracting the iron overload. Alternatively, hypoxia and anemia lead to down regulation of hepcidin expression,[255] which would allow increased export of iron from the RES and enhanced duodenal absorption, allowing increased erythropoiesis to compensate for anemia and hypoxia. Finally, cytokines produced during inflammation and most notably IL-6, also lead to up regulation of hepcidin expression by the hepatocytes.[252,254,255] In this case, the increased sequestration of iron within the macrophage would decrease the pool of iron available for RBC synthesis and contribute to anemia of inflammation. This phenomenon may be an adaptation of the host to deprive invading microorganisms of iron and thereby limit their growth. The unwanted but inevitable consequence of this sequestration is the development of anemia.

4.2 The role of iron in infection

Iron is an essential nutrient to both the host and invading microorganisms. While mammalians have evolved several mechanisms to sequester, regulate and conserve iron, bacteria have at the same time acquired means of getting enough iron from the ecologic niches they occupy. During infection, both the host and the invading pathogen will compete for this precious nutrient. On one hand, almost all bacteria necessitate a minimal amount of iron for growth, and such quantity is not normally freely available in the host where iron is bound to various proteins such as transferrin and lactoferrin. On the other hand, the right balance of iron is needed by the host for adequate innate and adaptive immune responses and both iron overload and iron depletion have been shown to impair the immune response.[256] It is, therefore, not surprising that the iron status of the host has been linked to susceptibility to various pathogens.

The host response to *Salmonella* infection appears also to be dependent on the iron status of the host. In mice, iron overload induced by repeated injections of human blood or iron results in decreased resistance to *Salmonella* infection.[257,258] On the other hand, mice kept on an iron-deficient diet were found to be more resistant to *Salmonella* compared to mice fed a regular diet or mice fed with an iron-deficient diet but injected with iron 10 to 14 days prior to infection.[259] Interestingly, acute iron depletion through the administration of deferoxamine, an iron chelator capable of binding both intra and extracellular iron, increased the susceptibility of mice to *Salmonella*.[257,260] This effect was noted in both *Nramp1* resistant and *Nramp1* susceptible strains and appeared related to an impaired capacity of macrophages to restrict the growth of *Salmonella* because of impaired NADPH-dependent respiratory burst.[260] These results indicate that a fine balance of iron is needed for optimal host defense against *Salmonella* and that both iron overload and iron depletion can exacerbate the growth of this intracellular pathogen.

FIGURES

Fig. 2. **Geographical distribution of typhoid fever**

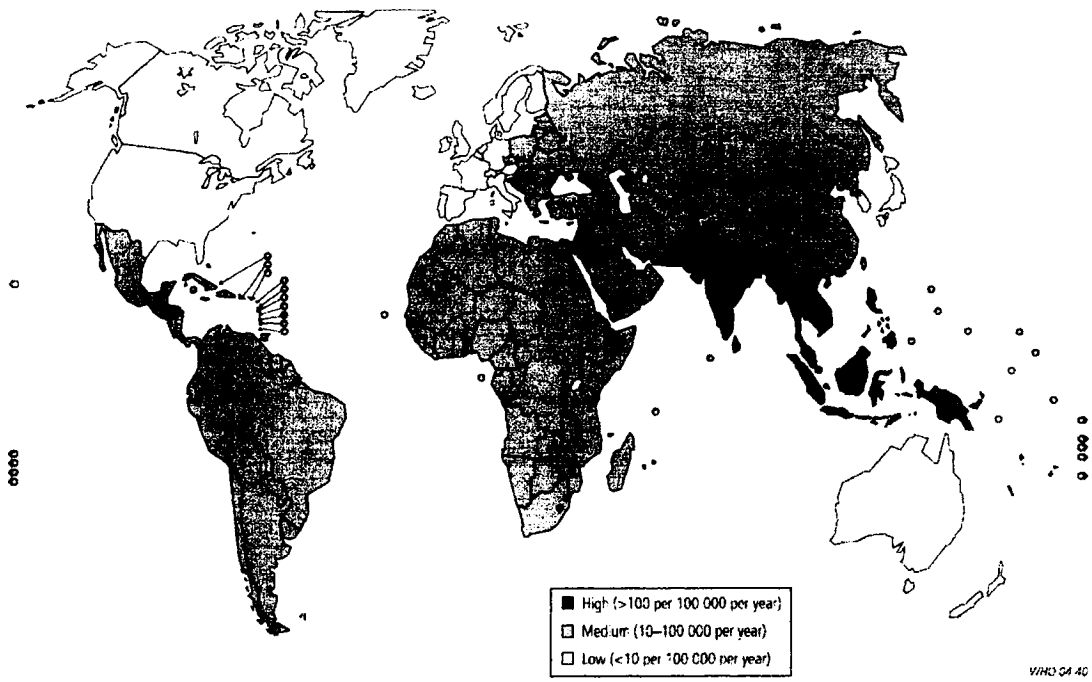

Figure 1: Geographical distribution of typhoid fever. Geographical distribution of estimated typhoid fever incidence rates showing areas of high, medium and low incidence. From Crump JA, Luby SP, Mintz ED. The global burden of typhoid fever. *Bull World Health Organ* 2004; **82**: 346-353.

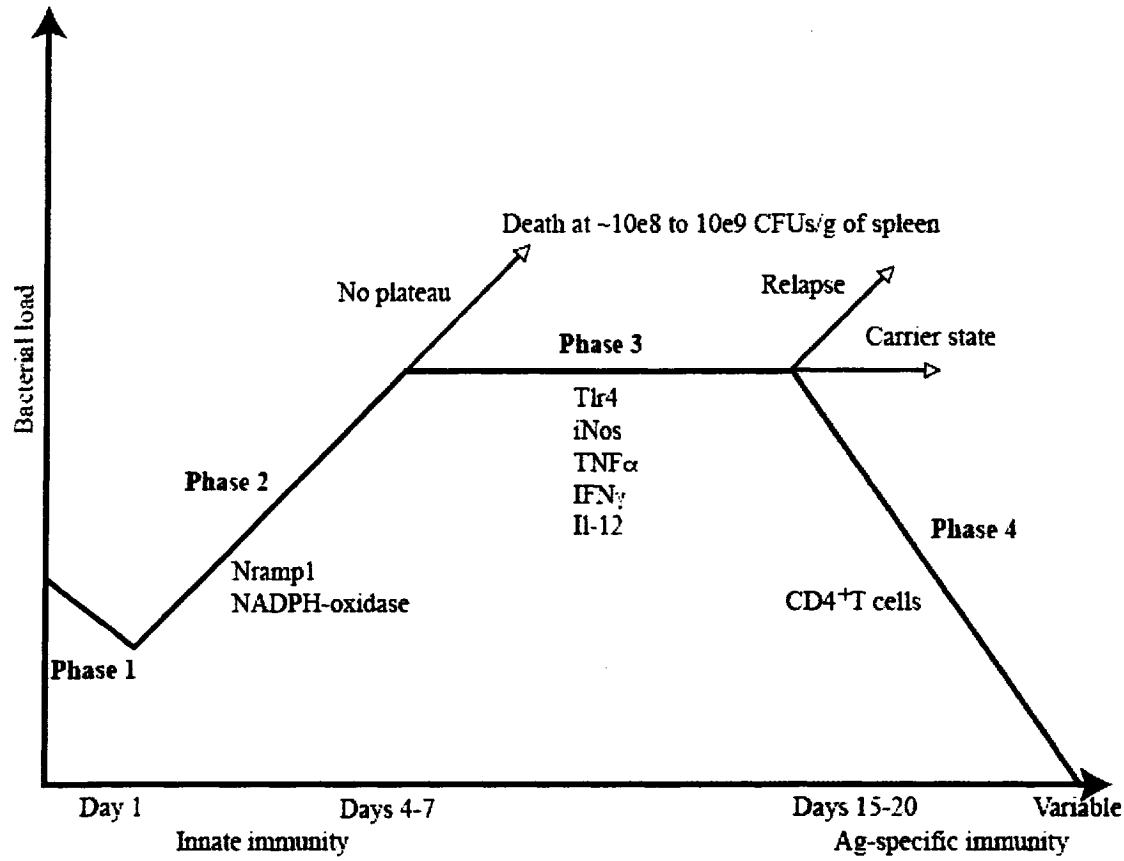

Figure 2: Systemic infection with a sublethal inoculum of *Salmonella* Typhimurium in mice is characterized by four distinct phases of infection. The first phase involves rapid clearance of the organisms from the blood followed by localization of the inoculum within macrophages and polymorphonuclear cells of the spleen and liver. The second phase of infection takes place over the following week, with an exponential growth of the organisms within the RES of the spleen and liver. The activation of the innate immune system to stop bacterial growth results in the establishment of a plateau (third) phase. The final (fourth phase) resolution of infection is clearly a function of activation of the acquired immune system and depends on T and B cells activation. Adapted from Mastroeni P. Immunity to systemic Salmonella infections. *Curr Mol Med* 2002; **2**: 393-406.

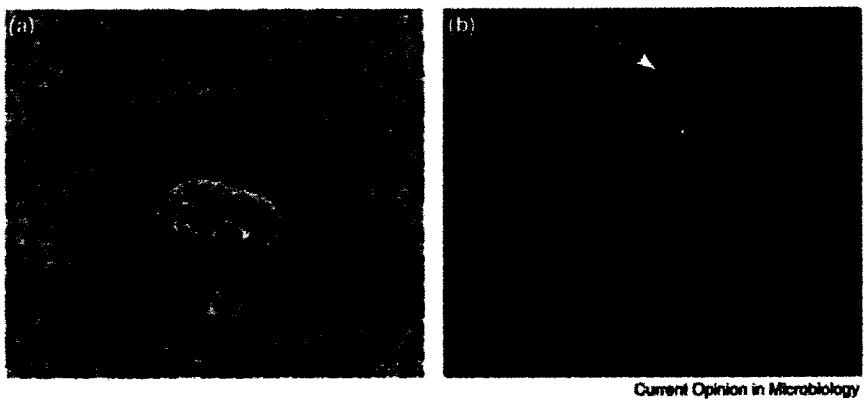

Figure 3: Interaction of *Salmonella* with epithelial cells. a) Electron micrograph of *Salmonella* Typhimurium infected MDCK cells. *Salmonella* induces remodeling of the host cytoskeleton, resulting in disappearance of apical microvilli and localized membrane ruffling that surrounds and eventually engulfs of the bacteria. *Salmonella* is colored in green, and the apical surface is colored in red. b) Immunofluorescence image showing actin cytoskelettal rearrangements (arrow) induced by *Salmonella* infection of Henle 407 cells. Actin stained in red, *Salmonella* stained in green and DNA stained in blue. Patel JC, Galan JE. Manipulation of the host actin cytoskeleton by Salmonella-all in the name of entry. *Curr Opin Microbiol* 2005; **8**: 10-15.

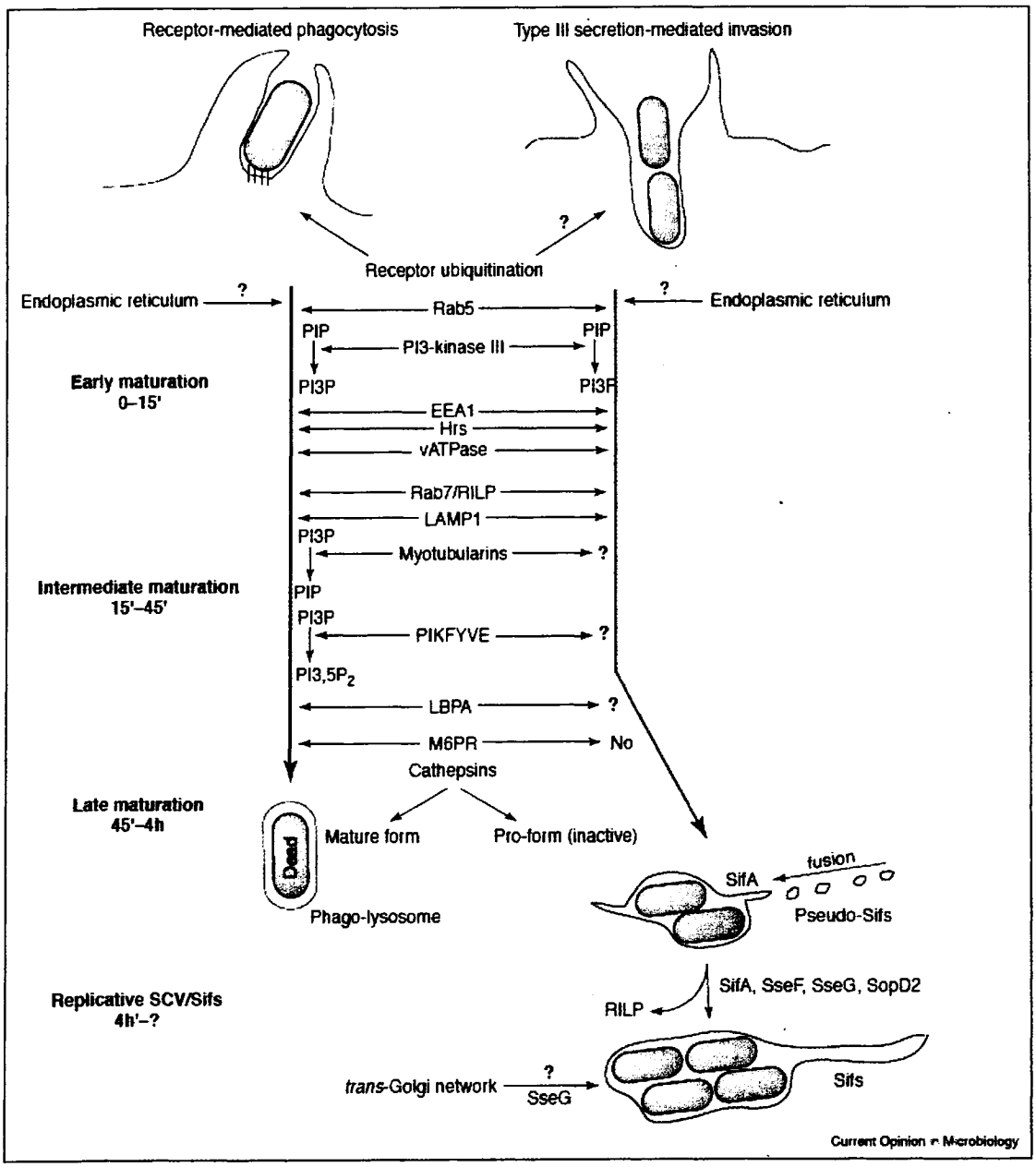

Figure 4: ***Salmonella* redirects phagosomal maturation.** The receptor-mediated phagocytosis resulting in the formation of a mature phago-lysosomes is shown on the left. The *Salmonella* TTSS-mediated invasion is shown on the right. The SCV resembles the conventional phagosome at early and intermediate stages of maturation. However, at later stages, the SCV is redirected away from the phagosomal maturation pathway and does not fuse with lysosomes. SPI-2 encoded TTSS mediates the delivery of SifA to the cell cytoplasm resulting in the formation of Sifs. Brumell JH, Grinstein S. Salmonella redirects phagosomal maturation. *Curr Opin Microbiol* 2004; **7**: 78-84.

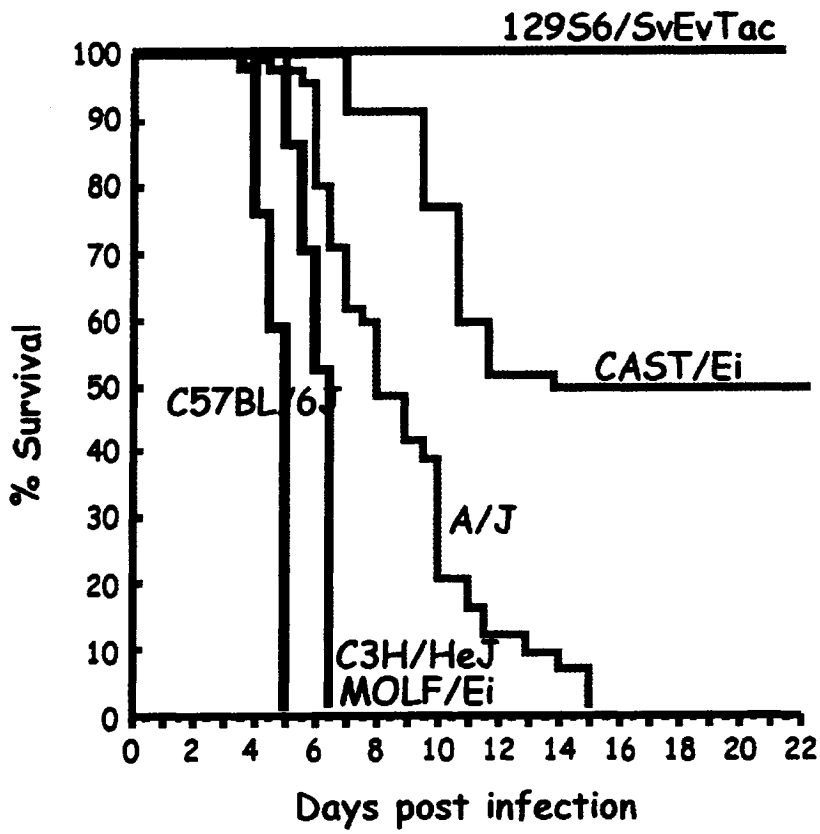

Figure 5: Survival curves following intravenous challenge with 10^4 CFUs of *Salmonella* Typhimurium strain Keller. 129S6/SvEvTac are the most resistant mice followed by CAST/Ei and A/J. C57BL/6J, MOLF/Ei and C3H/HeJ are extremely susceptible to *Salmonella* Typhimurium with no individual surviving beyond 7 days post infection. From Roy MF, Malo D. Genetic regulation of host responses to Salmonella infection in mice. *Genes Immun* 2002; **3**: 381-393.

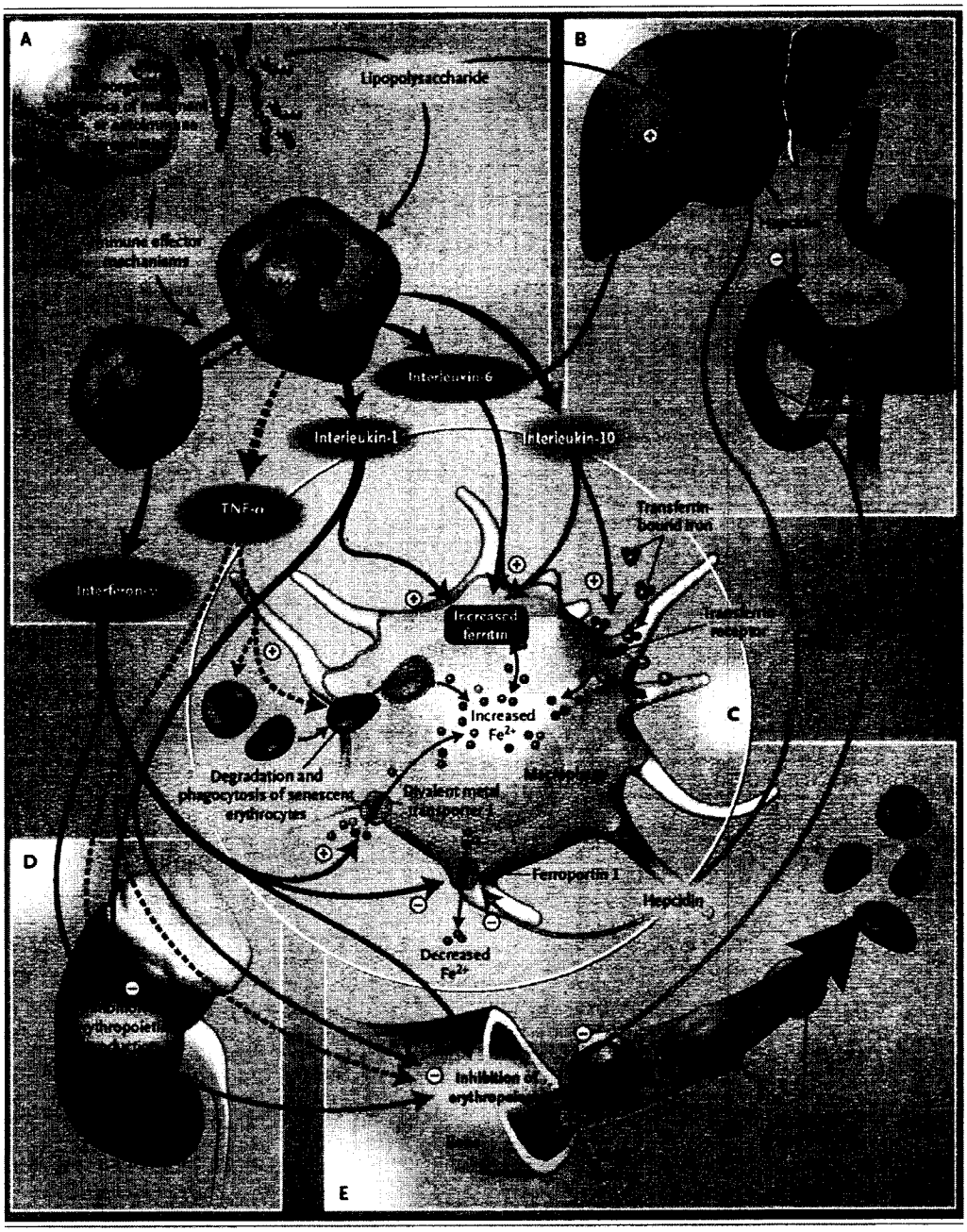

Figure 6: Mechanisms underlying the development of anemia of inflammation. Recognition of invading microorganisms by the host innate immune system leads to the secretion of various cytokines, which impacts on the host erythropoiesis response through several mechanisms. "+" stimulates. "-" inhibits. From Weiss G, Goodnough LT. Anemia of chronic disease. *N Engl J Med* 2005; **352**: 1011-1023.

CHAPTER II: Incremental expression of Tlr4 correlates with mouse resistance to *Salmonella* infection and fine regulation of relevant immune genes.

PROLOGUE

Mice infected either orally or parenterally with *Salmonella* Typhimurium develop a systemic disease similar to what is seen in human typhoid fever, with localization of the bacteria in the spleen and the liver. The response of mice to acute *Salmonella* Typhimurium infection is genetically controlled. Using a positional cloning approach, it was shown that a functional *Tlr4* gene is essential for mouse resistance to *Salmonella* infection. Compared to wild type mice, mice carrying a non-functional *Tlr4* allele show decreased survival and increased bacterial load following infection with *Salmonella.* In order to investigate the impact of the level of expression of *Tlr4* on the mouse response to *Salmonella,* four lines of *Tlr4* transgenic mice were generated. These mice carried 1, 3, 6 and >30 copies of the transgene on a C57BL10/ScNCr background. In the context of a wild type allele at *Nramp1*, the mouse survival following *Salmonella* infection was influenced by the number of copies of the transgene, with a plateau effect starting at three copies.

In this chapter, we report studies aimed at understanding how increasing the level of expression of *Tlr4* translates into improved host resistance to *Salmonella.* Given the plateau effect noted at three copies of the transgene, studies were performed using mice carrying 1 and 3 copies of *Tlr4.* Additionally, we wished to compare these transgenic lines to mice of the same genetic background that would carry 2 natural (non-transgenic) copies of *Tlr4* in their natural genomic context. By generating a double congenic mouse line, B10.Cg-*Nramp1/Tlr4*, we were able to study the *in vivo* host response to *Salmonella* Typhimurium in mice carrying 1, 2 and 3 copies of *Tlr4* on the same genetic background.

Incremental expression of Tlr4 correlates with mouse resistance to *Salmonella* infection and fine regulation of relevant immune genes.

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Running title: Tlr4 overexpression and resistance to *Salmonella*.

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ABSTRACT

The mouse response to *Salmonella* Typhimurium infection is partly controlled through detection of the bacterium LPS by the host pattern recognition receptor, Tlr4. Mice deficient in Tlr4 signaling are extremely susceptible to *Salmonella* infection with a 1000 fold reduction in LD_{50}. In a previous study, we showed, using transgenic mice carrying 1, 3, 6 and >30 copies of *Tlr4*, that the level of expression of this gene influences the outcome of *Salmonella* infection, with a plateau effect starting at 3 copies. In the present study, we further investigate the impact of Tlr4 during *Salmonella* infection in mice expressing Tlr4 at slightly sub-normal, normal and slightly supra-normal levels by comparing host responses in mice carrying 1, 2 and 3 copies of *Tlr4* on the same genetic background. We describe in detail the *in vivo* host response to pathogenic *Salmonella* and show for the first time, in this narrow range of *Tlr4* expression, an incremental protective effect against *Salmonella* due to improved control of bacterial growth in target organs and increased expression of important immune response genes in the spleen.

Keywords: Salmonella, Tlr4, gene expression, intracellular bacteria, infection.

Incremental expression of Tlr4 correlates with mouse resistance to *Salmonella* infection and fine regulation of relevant immune genes.

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INTRODUCTION

Recognition of pathogen invasion in vertebrates occurs initially through binding of conserved microbial motifs, the pathogen associated molecular patterns (PAMPs), with specialized host receptors, the pattern recognition receptors (PRRs).[77] This system of microbial recognition is highly conserved throughout evolution and is found in plants, insects and vertebrates. Binding of PAMPs to signaling PRRs initiates a cascade of events that lead to the expression of a wide range of genes important in the host innate immune response and in the development of its adaptive counterpart. Among the prime initiators of the innate immune system, lipopolysaccharide (LPS) covering the outer wall of gram-negative bacteria is one of the most studied and most potent immune stimulator. Throughout evolution, mammalians have learned to recognize LPS as the very worse of the bad news as colorfully described by Lewis in 1972:[261] "When we sense lipopolysaccharide, we are likely to turn on every defense at our disposal; we will bomb, defoliate, blockade, seal off and destroy all the tissues in the area."

It is true, in fact, that injection of small amounts of LPS to responsive host will recapitulate the whole range of events seen with invading pathogens, leading eventually to fatal septic shock.[262] Beginning with the study of *Drosophila* Toll,[263] the discovery of the human homologue Toll-like receptor 4 (Tlr4)[188] and the cloning of the *Lps* gene,[178,179] our understanding of the molecular mechanisms underlying the host response to LPS has progressed tremendously. Circulating LPS are captured by the lipoprotein binding protein, which transfers them to the host LPS receptor complex believed to comprise CD14, MD-2 and Tlr4. Through its cytoplasmic Toll-Interleukin (IL)-1 receptor (TIR) domain, Tlr4 transduces the LPS signal leading to the recruitment of various adaptors and kinases, resulting in the transcription of wide array of inflammatory response genes. Four adaptor molecules are now recognized as important in full activation of Tlr4 signaling: the myeloid differentiation factor 88 (MyD88), the TIR domain-containing adaptor

protein (TIRAP), the TIR domain-containing adaptor protein inducing interferon-β (TRIF), and TRIF-related adaptor molecule (TRAM).[183] With the help of these 4 adaptor molecules, the Tlr4 signaling cascade follows 2 distinct pathways. On the one hand, the MyD88-dependant signaling pathway, dependant on MyD88 and TIRAP, leads to rapid activation of NFκB and transcription of inflammatory cytokines. On the other hand, the TRIF/TRAM-dependant, MyD88-independent pathway leads to delayed activation of NFκB, specific activation of IRF3, and expression of IFN-β and interferon responsive genes.

In the mouse, the host response to the gram-negative, facultative intracellular bacterium *Salmonella enterica* serovar Typhimurium (or *Salmonella* Typhimurium) is strongly dependant on activation of Tlr4 by LPS. Indeed, mice carrying a proline for histidine substitution at codon 712 of Tlr4 are not only hyporesponsive to the effects of LPS but also extremely susceptible to *Salmonella* Typhimurium infection with a 1000 fold reduction of the LD_{50}.[167,176] Moreover, the gene expression profiles of macrophages infected with *Salmonella* Typhimurium or exposed to LPS overlap widely.[264] Finally, it has been shown that LPS is the main PAMP mediating *Salmonella*-induced production of TNFα and nitric oxide by infected macrophages.[265]

Knowing the importance of Tlr4 in the mouse model of *Salmonella* infection, we are interested in investigating how changes in the level of expression of Tlr4 impacts on the mouse response to this pathogen. To address this question, we previously generated 4 lines of *Tlr4* transgenic mice carrying various copy numbers of this gene on a C57BL10/ScNCr (B10/ScNCr) *Tlr4* null background.[266] We refer to these mice as Tg382 (1copy of *Tlr4*), Tg388 (3 copies), Tg390 (6 copies) and Tg394 (>30 copies). Using these transgenic mice, we showed that *Tlr4* copy number correlates strongly with mRNA and cell surface protein expression. Additionally, we identified a functional correlation with *in vitro* splenocytes proliferative response to LPS and *in vivo* susceptibility to LPS-induced septic shock. Finally we demonstrated that, in the presence of a wild-type allele at *Nramp1* (Natural resistance-associated macrophage protein 1, a gene known to be extremely important in mouse resistance to *Salmonella*[139]), increasing the number of copies of *Tlr4* imparts a survival advantage during *Salmonella* infection in mice with a plateau effect starting at 3 copies of the gene.

In the present study, we further investigated the impact of *Tlr4* during *Salmonella* infection in mice expressing *Tlr4* at slightly sub-normal, normal and slightly supra-normal levels by comparing host responses of mice carrying 1, 2 and 3 copies of *Tlr4* on the same genetic background. We describe in detail the host response to pathogenic *Salmonella in vivo* and show for the first time, in this narrow range of *Tlr4* expression, an incremental protective effect against *Salmonella* due to improved control of bacterial growth in target organs and increased expression of important immune response genes in the spleen.

RESULTS

We have previously shown, using transgenic mice possessing 1, 3, 6 and >30 copies of *Tlr4* that increased Tlr4 expression imparts a survival advantage during infection with *Salmonella* Typhimurium.[266] This protective effect appears, however, to plateau starting at 3 copies of the gene. To investigate further the role of Tlr4 at expression levels that are more biologically relevant we focused our studies on the F1Tg382 and F1Tg388 mice carrying 1 and 3 copies respectively of the *Tlr4* transgene (see Materials and Methods for a detailed description of the mice used). Additionally, we were interested in comparing these two transgenic strains to a strain that carries 2 natural, wild type (non-transgenic) *Tlr4* alleles on a similar B10.C3H-*Nramp1* background. To achieve this, we generated a double congenic mouse strain, B10.Cg-*Nramp1/Tlr4*, carrying 2 wild type alleles at *Tlr4* from the C57BL/10SnJ strain. These 3 strains allowed direct comparison of the effect of 1, 2 and 3 copies of *Tlr4* on the mouse response to *Salmonella* Typhimurium infection in the context of a resistant allele at *Nramp1*. When these mice were infected with ~10^3 CFUs of *Salmonella* Typhimurium intravenously we observed a clear effect of the number of copies of the gene. The F1Tg388 (3 transgenic copies) showed the longest survival time, followed by the B10.Cg-*Nramp1/Tlr4* (2 natural copies), and finally by the F1Tg382 (1 transgene) (Figure 1). The B10.C3H-*Nramp1* mice (no *Tlr4* but wild-type *Nramp1*), B10/SnJ (*Tlr4* but mutated *Nramp1*), and B10/ScNCr (no *Tlr4* and mutated *Nramp1*) were increasingly more susceptible to infection. These results confirm the important role of Tlr4 and Nramp1 during *in vivo* *Salmonella* infection in the mouse and show for the first time an incremental protection

from 0 to 1, 2 and 3 copies of the gene. We, thereafter, sought to understand how increasing *Tlr4* expression translates into improved resistance to *Salmonella* infection.

The increased resistance of Tlr4 competent mice compared to mice carrying a non-functional *Tlr4* allele has been linked to improved control of bacterial growth in the liver.[176,267] We were therefore interested in testing the hypothesis that increasing *Tlr4* expression would also translate into improved control of bacterial growth in the spleen and liver of mice during infection. To verify this hypothesis, we measured the bacterial load in the spleen and liver of our mice at various time points following *Salmonella* infection. As shown in Figure 2, the F1Tg388 mice were able to control the bacterial load in the spleen and the liver for a longer period of time compared to F1Tg382 or B10.Cg-*Nramp1/Tlr4* mice. Significant differences were observed for the splenic and liver CFUs at day 8 for both F1Tg382 and B10.Cg-*Nramp1/Tlr4* compared to F1Tg388 mice. Additionally, a significant difference was detected at day 10 between F1Tg382 and F1Tg388 for splenic CFUs. These results indicate a role for the level of *Tlr4* expression in controlling the bacterial replication during the first 10 days of infection. However, in F1Tg388 mice the spleen and liver bacterial load eventually increased during the late phase of infection to reach lethal numbers around day 15 to 20. Therefore, the increased protection conferred by incremental Tlr4 expression appears to be limited to the early phase of infection suggesting that despite a robust innate immune response, the host is unable to mount protective adaptive immunity resulting in long-term control of the bacterial replication.

Given the differences in survival time and bacterial load observed in our mice we were interested in comparing the histological lesions in the F1Tg382 and the F1Tg388 mice during infection. The intravenous injection of *Salmonella* Typhimurium in these transgenic mice resulted in microscopic changes detected in the liver, spleen, kidney, lung, brain, heart, pancreas and gastrointestinal tract (including the mesenteric lymph nodes) at Day 5, 10, 15 and 19 post-infection. There was, however, no obvious intergenotype difference in the severity or nature of these changes at the time points were both lines were available for study (day 5 and 10, all F1Tg382 mice dying before day 15). The histopathologic lesions were more pronounced in the spleen and the liver where leukocytosis and thrombosis of the venular system were found at all time points,

suggesting priming of endothelial cells, activation of circulating leukocytes and activation of the coagulation cascade as a consequence of the intravenous injection of *Salmonella.* These lesions were characterized by multifocal mixed inflammatory cells adhering to a reactive endothelium with or without clusters of inflammatory cells and/or thrombosis present in the lumen of the affected veins (Figure 3 A through D). Necroinflammatory and inflammatory changes characterized by multifocal foci of coagulation necrosis were also found in the spleen and the liver and through time, these changes evolved from acute to subacute or chronic (Figure 3 A through D). Especially in the liver, bacterial colonies were often seen within these lesions, which most likely represent areas of bacterial localization and replication. Splenic histiocytosis was present as early as day 5 post-infection in some mice. The incidence and severity of this change seemed to increase at day 10 and 15 post-infection and subsided at day 19 (Figure 3 E through H) indicating massive recruitment of tissue macrophages to the spleen of infected mice. Splenic periarteriolar lymphoid sheath atrophy was noted starting at day 5 in some mice but its incidence and severity increased through time to become diffuse and massive at day 19 in the mouse examined (Figure 3 I through L). These lesions could reflect a depletion of T lymphocytes and possibly illustrate the failure of the mice to generate an adequate adaptive response and control the infection. Extramedullary hematopoiesis was also detected in the liver and the spleen of F1Tg388 at day 15 and 19 post-infection. While no intergenotype difference could be detected in the early time points (day 5 and 10) when both strains could be examined, the massive splenic white pulp periarteriolar lymphoid sheath atrophy was observed only in the F1Tg388 mice that survived longer indicating that death occurred in these strains at similar bacterial load but with different histopathologic lesions.

The spleen is a major organ for the development of innate and adaptive immune responses.[268] During *Salmonella* infection, it is one of the main site for bacterial localization and replication. Immune cells are recruited to the spleen during infection and, with increased blood flow and accumulation of inflammatory exsudates, they contribute to the development of a massive splenomegaly. The splenic index of mice infected with *Salmonella* increases more than 3 times from 0.6 in non-infected mice to sometimes more than 2.0 at day 15 post-infection (data not shown). Using flow

cytometry analysis, we characterized the cell populations of the spleen during *Salmonella* infection in F1Tg388 and B10.Cg-*Nramp1/Tlr4* mice using general leukocytes markers with the aim of identifying Tlr4 copy number-induced differences in cell recruitment. As shown in Figure 4, the major cell populations recruited to the spleen during infection expressed Gr1 and Mac1, consistent with the observation of infiltration of neutrophils and macrophages seen on histopathologic examination. The percentage of cells expressing these markers increased in both groups examined throughout infection to finally plateau at day 15 in the F1Tg388. The MHC class II molecule I-Ab was upregulated in a higher percentage of cells and with a higher mean fluorescence intensity (data not shown) at day 4 in both groups, indicating activation of antigen-presenting cells. The percentage of cells expressing the B cell marker IgM declined on day 8 and 15 after a small increase on day 4. The percentage of CD3 positive cells steadily declined throughout infection in both groups, which is in agreement with the depletion of lymphocytes in the periarteriolar lymphoid sheaths seen on histopathology. Dendritic cells were recruited to the spleen during *Salmonella* infection in both groups as shown by the increased percentage of CD11c positive cells. Finally, NK cells did not appear to play a major role in mouse salmonellosis with percentages of Pan-NK postitive cells showing a slight decline throughout infection. In order to detect potential Tlr4-induced differences in the development of an adaptive response, we also studied the up regulation of the co-stimulatory molecules CD40, CD80 and CD86 on Mac1-positive and CD11c-positive cells at day 4 in B10.C3H-*Nramp1*, F1Tg382, B10.Cg-*Nramp1/Tlr4* and F1Tg388. While these molecules were up regulated on both cell types in all 4 strains of mice, we failed to detect any significant difference between the groups (data not shown). These results indicate that the effect of increased Tlr4 expression leading to increased control of bacterial proliferation is not reflected in an obvious difference in the recruitment of effector cells to the spleen.

The systemic activation of the immune system during infection may be reflected by changes in circulating white blood cells. To investigate for potential *Tlr4*-induced differences in the hematological changes that occur during *Salmonella* infection we performed red blood cell and white blood cell counts in F1Tg388 and B10.Cg-*Nramp1/Tlr4* mice (Figure 5). Both strain of mice developed a severe leukopenia at day 4

post-infection (Figure 5A) indicating acute activation of the immune system caused by intravenous *Salmonella* infection. Again no intergenotype differences were observed except for a more pronounced rebound neutrophilia (Figure 5C) and monocytosis (Figure 5D) in the B10.Cg-*Nramp1/Tlr4* mice at day 8. We were, however, surprised to observe the development of anemia in both strains of mice during infection (Figure 5E). This anemia worsened throughout infection to become quite severe at day 15 in F1Tg388 mice, probably explaining the extramedullary hematopoïesis observed at day 15 and 19 in these mice. To the best of our knowledge, development of anemia in mice during *Salmonella* infection as not previously been described and we are currently investigating further the mechanisms underlying this pathology.

In continuing our search for pinpointing a precise mechanism explaining increased resistance to *Salmonella* brought by incremental expression of *Tlr4*, we next compared mRNA expression of immune-related genes in the spleen of our mice during infection using a Tlr4 pathway-specific oligo array (SuperArray Bioscience Corporation). The genes from the Tlr4-specific pathway that were the most regulated within each group during infection (Figure 6 and Table 1 and Supplemental Table 1) were selected for study with qPCR. This technique was expected to be more sensitive to detect small differences in gene expression that could exist between the groups carrying 1, 2 or 3 copies of *Tlr4* at similar time points. The genes selected for qPCR analysis were: chemokine (C-X-C motif) ligand 10 (*Cxcl10*), interferon-γ (*Ifng*), interleukin-12a *(Il12a*), *Il12b*, *Il1b*, interferon-regulatory factor-1 (*Irf1*), *Irf7*, *Myd88*, NFκB inhibitor alpha (*Nfkbia*) and Toll interacting protein (*Tollip*). Additionally, we investigated the expression of *Tlr4* as an internal control for the genotype of our mice and our qPCR technique. We observed incremental *Tlr4* expression across our 3 groups at all time points studied (Figure 7 A, B and C), confirming the validity of our qPCR technique, the genotype of our mice and the correlation between the number of copies of *Tlr4* and its mRNA expression. Additionally, *Tlr4* expression increased in all 3 groups during *Salmonella* infection (Figure 7 D, E and F) reflecting either up-regulation of its expression on cells normally expressing *Tlr4* or the recruitment of *Tlr4* expressing cells to the spleen.

Reasonably good agreement was found between the array and the qPCR results with respect to the overall trend in gene expression during *Salmonella* infection in our

mice. The genes that were found to be up regulated during *Salmonella* infection (Figure 7 D, E and F) all belong to what has been described as the common host response to infection.[269] *Il1b*, encoding the pro-inflammatory cytokine IL-1β, was strongly and similarly up regulated in all 3 groups at day 4 and 8 post-infection. *Il12a*, *Il12b* (at day 4) and *Ifng* were all up regulated during *Salmonella* infection indicating activation of the IL12-IFNγ axis shown to be important in immunity to *Salmonella*.[119] *Cxcl10*, encoding an interferon-inducible chemokine important in T cell recruitment, was also strongly induced by *Salmonella* infection in a pattern similar to *Ifng*. Two transcription factors, *Irf1* and *Irf7*, which mediate the effect of interferons, were also up regulated during infection in all three groups studied. *Myd88*, a gene extremely important in the *Tlr4* signaling cascade leading to rapid production of pro-inflammatory cytokines, was induced by infection in our three groups of mice. *Irf1*, *Irf7* and *Myd88* are good example of genes that may amplify the host immune response through a positive feedback loop. On the other hand, we also detected increased expression of two negative regulator of inflammation in all three groups following infection: *Nfkbia* and *Tollip*. While IκBα (encoded by *Nfkbia*) is important for sequestration of NF-κB to the cytoplasm, Tollip is believed to be important in terminating TLR signaling during inflammation. Our results illustrate the concomitant activation of both positive and negative regulators of the inflammatory response during *Salmonella* infection. In addition, our findings demonstrate activation of both arms of the Tlr4 signaling cascade with *Il1b*, *Il12a* and *Il12b* being good example of Myd88-dependent genes and *Cxcl10*, *Irf1* and *Irf7* representing the activation of the Trif-dependent arm of Tlr4 signaling.

When we compared gene expression across our 3 groups at similar time points, we were able to identify small but significant differences in gene expression (Figure 7 A, B and C). For example, when comparing gene expression in non-infected mice (Figure 7A) we could see a tendency for increasing mRNA expression in almost all the genes studied according to the number of copies of *Tlr4* of the strains. These differences were significant for *Il1b* (F1Tg388 vs B10.Cg-*Nramp1/Tlr4*) and *Ifng* (F1Tg388 vs F1Tg382 and B10.Cg-*Nramp1/Tlr4*). On day 4 (Figure 7B) we found that *Cxcl10* was overexpressed in the F1Tg388 compared to the 2 other groups (significant in F1Tg388 vs F1Tg382). Additionally, mRNA expression of *Myd88* was significantly higher in the

F1Tg388 group compare to both the F1Tg382 and the B10.Cg-*Nramp1/Tlr4*. Finally, on day 8 (Figure 7C), differences in gene expression for *Cxcl10*, *Il1b*, *Ifng* and *Irf7* were observed between the F1Tg388 and the B10.Cg-*Nramp1/Tlr4* strain.

In summary, our results show that while up regulation of these important immune genes during infection occurred with a similar pattern in the three strains studied, some slight but significant differences could be detected between the groups at specific time points. The F1Tg388 strain in particular showed higher constitutive expression of specific immune related transcripts compared to the 2 other groups. These results suggest that increased basal *Tlr4* expression *in vivo* leads to increased expression of downstream immune related genes in the spleen, which appears to provide a survival advantage following *Salmonella* infection in mice.

DISCUSSION

In our original description of the Tlr4 transgenic mice we demonstrated a clear copy number effect on the mouse response to LPS *in vitro* and *in vivo*. However, the effect of *Tlr4* expression on the *in vivo* mouse resistance to *Salmonella* infection showed a plateau starting at 3 copies of the gene.[266] The mice carrying 6 or 30 copies of *Tlr4* were less or equally resistant compared to the strain carrying 3 copies (Bihl *et al*[266] and data not shown). In the present study, we further refined our understanding of the role of expression of *Tlr4* within a narrow, biologically relevant range, on the host response to *Salmonella* infection. We showed, for the first time, an incremental protective response against gram-negative sepsis from 0 to 1, 2 and 3 copies of *Tlr4* (Figure 1). The small differences in survival between the groups carrying 2 and 3 copies of *Tlr4* and the lack of benefit from higher levels of *Tlr4* expression illustrate the fine balance in gene dosage brought about by millions of years of evolution of the innate immune system. Indeed, a recently published phylogenetic analysis of TLRs revealed that most vertebrates have exactly one gene orthologous to each of the TLR family for recognition of specific PAMPs (except for the TLR1 family which comprise Tlr1, Tlr2, Tlr6 and Tlr10).[270] This study suggests that the TLR superfamily of genes is under strong selective pressure for maintenance of the actual repertoire of PAMPs recognition and for maintenance of the gene dosage within each family. These conclusions are in agreement with our findings of

the role of Tlr4 gene dosage on the mouse response to gram-negative infection. The small differences in survival and bacterial load seen in the narrow range of copy number of *Tlr4* from 1 to 3 copies suggests that 2 copies of *Tlr4* per genome represents the most advantageous dosage possible for a diploid organism.

The increased resistance to *Salmonella* infection in the F1Tg388 correlates with improved control of the bacterial load within the spleen and the liver (Figure 2). Previous studies using *Tlr4* wild-type and natural functional knockout mice demonstrated the importance of a functional Tlr4 for the early control of bacterial proliferation[167,176,267] and establishment of a plateau phase.[75] In our study, we show a Tlr4 expression level-dependent mechanism for control of bacterial growth at later time points, approximately from day 5 to day 10 post-infection (prolongation of the plateau phase). The exact mechanism underlying this phenomenon remains unclear. We first believed that increased *Tlr4* expression would result in a more robust inflammatory response that would be visible on histopathologic examination. However, no obvious differences could be detected. Given the low sensibility of histopathologic examination to subtle changes in cell recruitment, we performed FACS analysis to identify potential Tlr4-induced differences. Again, we could not detect any major differences between the F1Tg388 and the other strains examined. Therefore, the increased resistance and better control of bacterial load probably resides not in the type or quantity of cells recruited to the spleen but in their activation state and the type of cytokine, chemokines and antibacterial molecules they produce.

Using oligoarrays and qPCR for mRNA expression analysis of important immune effectors in the whole spleen of our mice, we found some interesting Tlr4 copy number-induced differences. In particular, we detected increased basal expression of IFNγ in the F1Tg388 mice compared to the 2 other strains indicating that constitutive supra-normal expression of Tlr4 also leads to constitutive supra-normal expression of IFNγ (Figure 7A). Activated T cells synthesize IFNγ following IL-12 stimulation. Although no significant differences were detected for IL-12 expression, we observed a Tlr4 copy number trend for increased constitutive IL-12a and IL-12b expression, possibly explaining the significant increase in basal IFNγ expression. The importance of the IL-12/IFNγ axis in the host response to *Salmonella* is well known in mice and in humans.

Wild type mice infected with attenuated *Salmonella* Typhimurium are capable of controlling the infection while IFNγ deficient mice succumb to infection due to unrestricted growth of the bacteria.[129,235] Administration of monoclonal antibodies directed against IL12 exacerbates the mild disease caused by attenuated *Salmonella*[237] and mice carrying targeted disruption of *Il12a* or *Il12b* are more susceptible to *Salmonella* Enteritidis.[238] Moreover, the syndrome of Mendelian susceptibility to mycobacterial disease illustrates the importance of IL12 and IFNγ in the host response to *Salmonella* in humans.[119] Affected patients carrying a pathogenic mutation in one of the genes coding for the IFNγ receptor, the IL-12 receptor or the IL-12 p40 subunit show increased susceptibility to environmental mycobacterial species and *Salmonella*. The IL-12/IFNγ axis is therefore extremely important in immunity against *Salmonella* most likely through its effect on the generation of a type 1 adaptive immune response. Our observations go along these lines and suggest that increased constitutive expression of Tlr4 leads to increased basal expression of IL-12 and IFNγ, which seems to confer a survival advantage when the animal is suddenly confronted to an infectious agent such as *Salmonella*.

Despite increased expression of Tlr4, improved control of bacterial growth and increased survival time, the F1Tg388 mice fail to completely control the infection, indicating that a robust innate immune response against *Salmonella* infection is not sufficient for survival. It is conceivable that the host-pathogen interactions in this model are such that the host fails to mount a protective adaptive immune response. While mortality from sepsis has long been linked to excessive activation of systemic inflammation, it is now believed that excessive anti-inflammatory response with accompanying immunosuppression certainly contributes to mortality in severe sepsis.[271] Along these lines, we observed in our mice a severe and diffuse depletion of lymphocytes in the splenic white pulp (Figure 3 J, K and L), decreasing percentages of CD3+ and IgM+ cells (Figure 4), and a severe and persistant lymphopenia (Figure 5B) at a time where one would expect to see clonal expansion of lymphocytes. Depletion of lymphocytes from the splenic white pulp and lymphopenia have been reported in human patients dying from severe sepsis.[272] The mechanism underlying lymphocyte depletion during sepsis is believed to be apoptosis, and treatment with apoptosis inhibitor or use of

mice with genetic modifications rendering them less susceptible to apoptosis improve survival from polymicrobial sepsis.[273,274] It is conceivable that sepsis-induced apoptosis could contribute to the failure of our mice to resolve the infection. In addition, we also observed a drop in *Il12b* expression on day 8, below the level of expression seen in non-infected mice. Given the importance of IL-12 in the mouse response to *Salmonella*, we could hypothesize that the failure of sustaining IL-12 expression is detrimental to the host. Indeed, John *et al* have showed that IL-12p40 is essential for a sustained IFNγ production by T cells in mice immunized with live *Salmonella* Typhimurium aroA.[275]

The study of the effect of the level of expression of Tlr4 within a biologically relevant range is important in light of recent findings regarding the role of human polymorphisms within the TLR4 gene in association with various diseases. Two polymorphisms, Asp299Gly and Thr399Ile, were originally described as hyporesponsive in regards to inhaled LPS in healthy human volunteers.[191] Following this publication, numerous studies have found an association between these polymorphisms and susceptibility to a wide range of infectious and non-infectious diseases. For example, the hypomorphic TLR4 alleles have been associated with increased risk of gram-negative infection[192] or septic shock due to gram-negative organisms,[193] severe malaria,[194] brucellosis,[195] Crohn's disease,[196] and severe sepsis following burn injury.[197] On the other hand, the same alleles have shown protective effects against various diseases such as atherosclerosis,[198] Legionnaire's disease[199] and late-onset Alzheimer's disease.[200] In light of these findings, the mice characterized in the present study could represent invaluable tools to study in more depth the role of slightly different levels of Tlr4 expression on specific diseases outcome and pathophysiology.

In conclusion, we provide evidence for a *Tlr4* expression level-induced difference in mouse survival following *Salmonella* infection due to improved control of bacterial growth in the spleen and the liver and increased expression of relevant immune genes at the site of infection. The lack of striking differences in some of the specific measurements made during infection in our 3 mice strains should not be surprising. We were in fact looking at 3 almost identical mice strains that differed only by 1 or 2 copies of the Tlr4 gene, each of them possessing at least 1 copy. Therefore, it was probably only possible to detect differences by using methods that are very sensitive to small changes

such as mRNA qPCR expression analysis. The differences in survival and bacterial load were however noticeable and must be explained by some subtle *Tlr4* copy number-induced change in the host response to the invasive pathogen occurring at the cellular level. The subtle changes we detected in whole spleen mRNA expression give an indication of the possible mechanisms underlying the increased resistance in the F1Tg388 mice. Further experiments should allow a better understanding of the real biological impact of these subtle changes in gene expression.

MATERIALS AND METHODS

Animal used: Transgenic mice carrying different copy numbers of *Tlr4* were generated in our laboratory[266] and were maintained at the Montreal General Hospital Research Institute (MGHRI) animal facility under conditions specified by the Canadian Council on Animal Care (CCAC). These mice carry a 129S6/SvEvTac *Tlr4* BAC transgene on a B10/ScNCr background. The original B10/ScNCr strain has no Tlr4 because of a 75kb chromosomal deletion encompassing the *Tlr4* locus[180] and carries a mutated, non-functional allele at *Nramp1* (*Nramp1*^{Asp169})).[150] Because of the known major effect of *Nramp1* on mouse salmonellosis, we generated a congenic strain, B10/ScNCr.C3H-*Nramp1* (or B10.C3H-*Nramp1*) through 10 consecutives backcross generations to be used in a cross with our transgenic strain. The B10.C3H-*Nramp1* are now maintained in a wild type *Nramp1*^{Gly169} homozygote state at the MGHRI animal facility. (Tg x B10.C3H-*Nramp1*)F1 mice carrying the transgene and one wild type allele at *Nramp1* are used in experimental infection with *Salmonella* Typhimurium. We refer to these mice as F1Tg382 (1 copy of the *Tlr4* transgene and 1 wild-type allele at *Nramp1*) and F1Tg388 (3 copies of the *Tlr4* transgene and 1 wild-type allele at *Nramp1*). A double congenic strain, C57BL/10ScNCr.Cg-*Nramp1*^{Gly169}*Tlr4*^{Pro712} (abbreviated B10.Cg-*Nramp1/Tlr4*), was generated through backcrossing of *Tlr4* from C57BL/10SnJ onto a B10.C3H-*Nramp1* background. The mice used in our infections are homozygote wild type at *Tlr4* and heterozygote at *Nramp1* (Gly169/Asp169) in concordance with the F1Tg mice. C57BL/10ScNCr (maintained at the MGHRI animal facility) and C57BL/10SnJ (obtained from the Jackson Laboratory) were used as controls. At the time of infection, all mice were aged between 2 and 6 months.

In vivo Salmonella infection: All animal procedures were performed in accordance with the regulations of the CCAC. *Salmonella* Typhimurium strain Keller was grown in trypticase soy broth at 37°C for 1.5 hour followed by enumeration of the colony-forming units (CFUs) by incubating 10-fold serial dilutions on trypticase soy agar at 37°C for 16 hours. Each mouse was infected with 0.2ml of 0.9% saline containing ~10^3 CFUs of *Salmonella* Typhimurium by intravenous injection in the tail vein. The inoculation dose was verified by plating 10-fold serial dilutions of the dose on agar plates. For survival analysis, animals were monitored twice a day and moribund animals were sacrificed by CO_2 asphyxiation. For determination of the splenic and hepatic bacterial loads, mice were sacrificed at various time points after infection and half of the spleen and the left lateral lobe of the liver were collected aseptically, weighed and homogenized with a Polytron homogenizer in isotonic saline. Serial dilutions of each homogenate were plated on trypticase soy agar to enumerate the CFUs within each organ. For histopathologic examination, mice were anesthetized with ketamine and xylazine intramuscularly and perfused first with 20ml of physiologic saline followed by 20ml of fresh 4% paraformaldehyde. The organs were harvested, placed in 4% paraformaldehyde for several hours, washed in PBS twice and stored in 20% sucrose-PBS at 4°C. Tissue processing and histopatlogic examinations were performed by CTBR Bio-Research Inc (Senneville, Québec). The splenic index was calculated as follow: SI = $\sqrt{}$ (spleen wt x 100 / body wt).

Flow cytometry: Single cell suspensions of spleens harvested from mice sacrificed before or at various time points following infection with *Salmonella* were prepared as previously described.[276] The various cell populations were determined by single-color staining of the cells with fluorescein isothiocyanate (FITC) or phytoerythrin (PE)-conjugated monoclonal antibodies against Mac-1 (CD11b, clone M1/70, rat IgG2b), Gr-1 (Ly-6G and Ly-6C, clone RB6-8C5, rat IgG2b), CD3e (clone 145-2C11, hamster IgG1), CD11c (clone HL3, hamster IgG1), CD49b/Pan-NK (clone DX5, rat IgM), I-A^b (clone AF6-120.1, mouse (BALB/c) IgG2a) and IgM (clone R6-60.2, rat IgG2a). CD16/CD32 (clone 2.4G2, rat IgG2b) was used prior to staining to block the non-specific binding by Fc receptors. All antibodies were purchased from BD Biosciences. Cell acquisition and

data analysis was done with CellQuest software (BD Biosciences). Results are shown as the mean percentage of positively stained cells per spleen.

Hematologic parameters: Red blood cells were counted manually after dilution in PBS. White blood cells counts were also done manually after dilution in 3% acetic acid. Leukocyte differentials were determined by counting 400 leukocytes on Diff Quick-stained blood smears.

Tlr4 pathway-specific oligoarray: Half of the spleens were harvested immediately after the mice were sacrificed by CO_2 asphyxiation and snap frozen in liquid nitrogen before being stored at -80°C. Approximately 50 mg of spleen was homogenized using a mortar and pestle kept on dry ice and RNA was extracted with Trizol Reagent (Invitrogen) according to the manufacturer instructions. cRNA target for microarray hybridization were synthesized using the TrueLabeling-AMP Linear RNA Amplification kit from SuperArray Bioscience Corporation according to the manufacturer instructions using biotin-16-dUTP for labeling. The synthesized probes were purified using RNeasy minikit (Qiagen). The probes were hybridized to the OligoGEArray Mouse Toll-like Receptor Signaling Pathway Microarray (SuperArray Bioscience Corporation) according to the manufacturer instructions. RNA from infected (3 mice per group per time point) and non-infected (2 mice per group) male mice was used to hybridize the arrays (RNA from a single mouse per array for a total of 24 arrays). The array images were acquired by exposing X-ray films and digitalized with a desktop scanner. The images and the data were analyzed using the GEArray Expression Analysis Suite. Background correction was done by subtracting the local background and normalization was performed using 4 housekeeping genes present on the arrays.

Quantitative real-time RT-PCR: The same RNA samples used for array hybridization were used for quantitative real-time RT-PCR (qPCR) analysis. cDNA were synthesized all at once using M-MLV reverse transcriptase (Invitrogen). All primers were designed to overlap two exons and primer specificity was verified by ensuring a single band of the expected size on ethidium bromide stained agarose gel. Standards were prepared from 10-fold serial dilutions of the gel extracted PCR product for all genes tested. qPCR was performed on Chromo4 Real-Time PCR Detection System (Bio-Rad Laboratories) using Brilliant SYBR Green QPCR Master Mix (Stratagene). All samples were amplified in

triplicates during the same PCR run on 96 wells plates. Three potential housekeeping genes were tested (Polymerase (RNA) II (DNA directed) polypeptide A (*Polr2a*), TATA box binding protein (*Tbp*) and Glucose-6-phosphate dehydrogenase X-linked (*G6pdx*)) and the best housekeeping genes were chosen after analysis in Bestkeeper.[277] *Polr2a* and *Tbp* were found to be stable across our groups and experimental conditions and they were incorporated into a Bestkeeper Index used for relative quantification. PCR amplification efficiencies were measured on individual amplification plots using LinReg PCR.[278] The software REST 384 (http://www.gene-quantification.info/)[279] was used to analyze the relative expression of our measured transcripts using an efficiency corrected ratio. Error estimations of the calculated ratios were obtained using a Taylor's series as implemented in REST. The significance of the difference in expression ratios across our groups or experimental time points was investigated using pair wise fixed randomization tests implemented in REST (2000 randomizations for each test).

ACKNOWLEDGMENTS

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TABLES

	F1Tg382								
	Day 4/0	Day 8/0	Day 8/4						
Cd86				3.9		0.2			
Cxcl10	37.6	34.1		20.0	18.2		21.4	23.7	
Hspa4				2.5	2.1				
Hspd1	2.3		0.5	4.5	3.4		1.8		0.5
Ifng	8.4	6.6		27.7		0.5	12.7		
Il12a								2.4	9.2
Il1b	7.0	5.3		9.3	5.6	0.6	6.5	8.9	
Irf1	2.0	1.9		2.1	2.5			1.6	
Irf7	5.5	2.9	0.5	7.2	5.2		2.4	1.5	0.6
Map2k3			0.5						
Mapk14			0.6	2.0	1.6				0.7
Myd88	3.0	2.1		5.0	2.8	0.6	1.7	1.8	
Nfkbia							2.4		0.6
Tollip		2.4	1.7		0.5			2.1	

Table 1: Genes upregulated during *Salmonella* infection: Gene expression ratios comparing various time points after *Salmonella* infection (day 4 vs 0; day 8 vs 0; day 8 vs 4) within our 3 groups. Each array was hybridized with RNA from a single mouse. n = 3 per group on day 4 and 8 and n = 2 per group for day 0. A total of 24 arrays were used.

SUPPLEMENTAL TABLE

Gapd	Btk	Casp8	Ccl2	Cd14	Cd80	Cd86	Chuk
Clecsf9	Csf2	Csf3	Cxcl10	Elk1	Fadd	Fos	Gpc1
Hmgb1	Hras1	Hspa4	Hspd1	Ifna1	Ifnb	Ifng	Ikbkb
Ikbkg	Il10	Il12a	Il12b	Il1a	Il1b	Il2	Il6
Irak1	Irak2	Irak3	Irak4	Irf1	Irf3	Irf7	Jun
Lta	Ly64	Ly86	Ly96	Mal	Map2k3	Map2k4	Map2k6
Map3k1	Map3k14	Map3k7ip1	Map3k7ip2	Map4k4	Mapk10	Mapk11	Mapk12
Mapk13	Mapk14	Mapk8	Mapk8ip3	Mapk9	Myd88	Nfkb1	Nfkb2
Nfkbia	Nfkbib	Nfkbie	Nfkbil1	Nr2c2	Peli1	Peli2	Pglyrp
Pglyrp3	Prkra	PTGES	Ptgs2	Rel	Rela	Relb	Ripk2
Sitpec	Tbk1	Tirap	Tlr1	Tlr2	Tlr3	Tlr4	Tlr5
Tlr6	Tlr7	Tlr8	Tlr9	Tnf	Tollip	Traf6	Ube2n
Ube2v1	Blank	PUC18	Luc1	Luc2	AS1R2	AS1R1	AS1
Rps27a	B2m	Hspcb	Hspcb	Ppia	Ppia	BAS2C	BAS2C

Supplemental Table 1: Array layout with gene symbols from the Oligo GEArray mouse Toll-like receptor signaling pathway microarray (Superarray Bioscience Corporation).

FIGURES

Figure 1

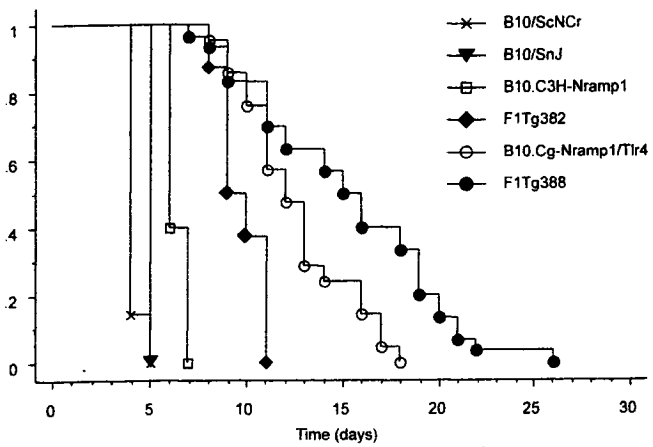

Figure 1: Cumulative survival in mice following intravenous infection with 10^3 CFUs of *Salmonella* Typhimurium. The results shown are combined from two separate infections. Ten to twenty mice (both male and female) per group were used in both experiments. Kaplan Meir survival analysis was performed on the data from the individual infections datasets and showed a significant difference in survival ($p < 0.05$) between the F1Tg382 and the B10.Cg-*Nramp1/Tlr4* compared to the F1Tg388. The B10.C3H-*Nramp1*, B10/SnJ and B10/ScNCr were increasingly susceptible to infection. (Kaplan Meir survival analysis with Tarone-Ware rank test performed with StatView 5.0.)

Figure 2

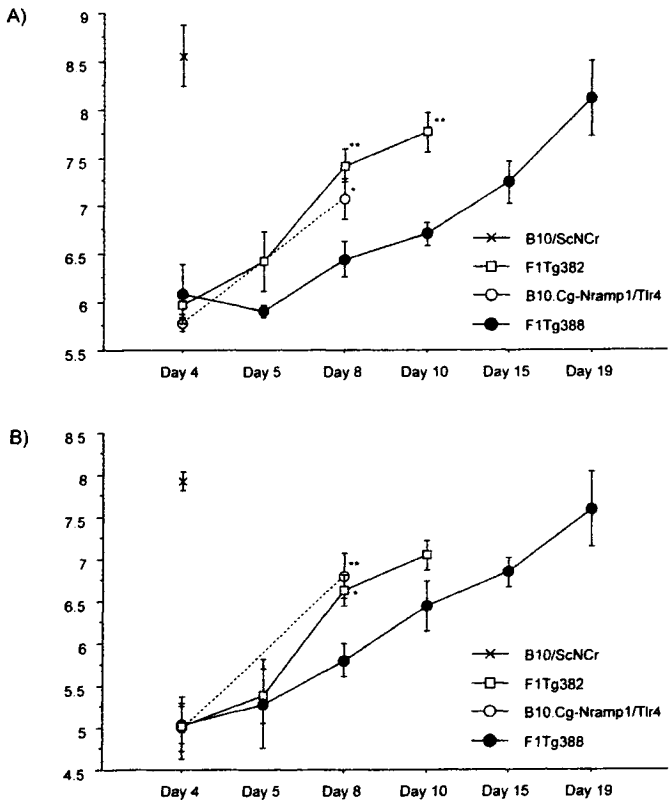

Figure 2: Splenic and hepatic bacterial load in mice following infection with 10^3 CFUs of *Salmonella* Typhimurium. Mean +/- SEM of the log of CFU per gram of spleen (A) or liver (B) of mice at various time points following intravenous infection with 10^3 CFUs of *Salmonella* Typhimurium. The results shown are combined from two separate infections. Six mice (males and females) per group and per time point were used in both experiments. * $p < 0.05$ and ** $p < 0.01$ compared to F1Tg388 (ANOVA with Fisher's PLSD performed with StatView 5.0 on the separate infections datasets).

Figure 3

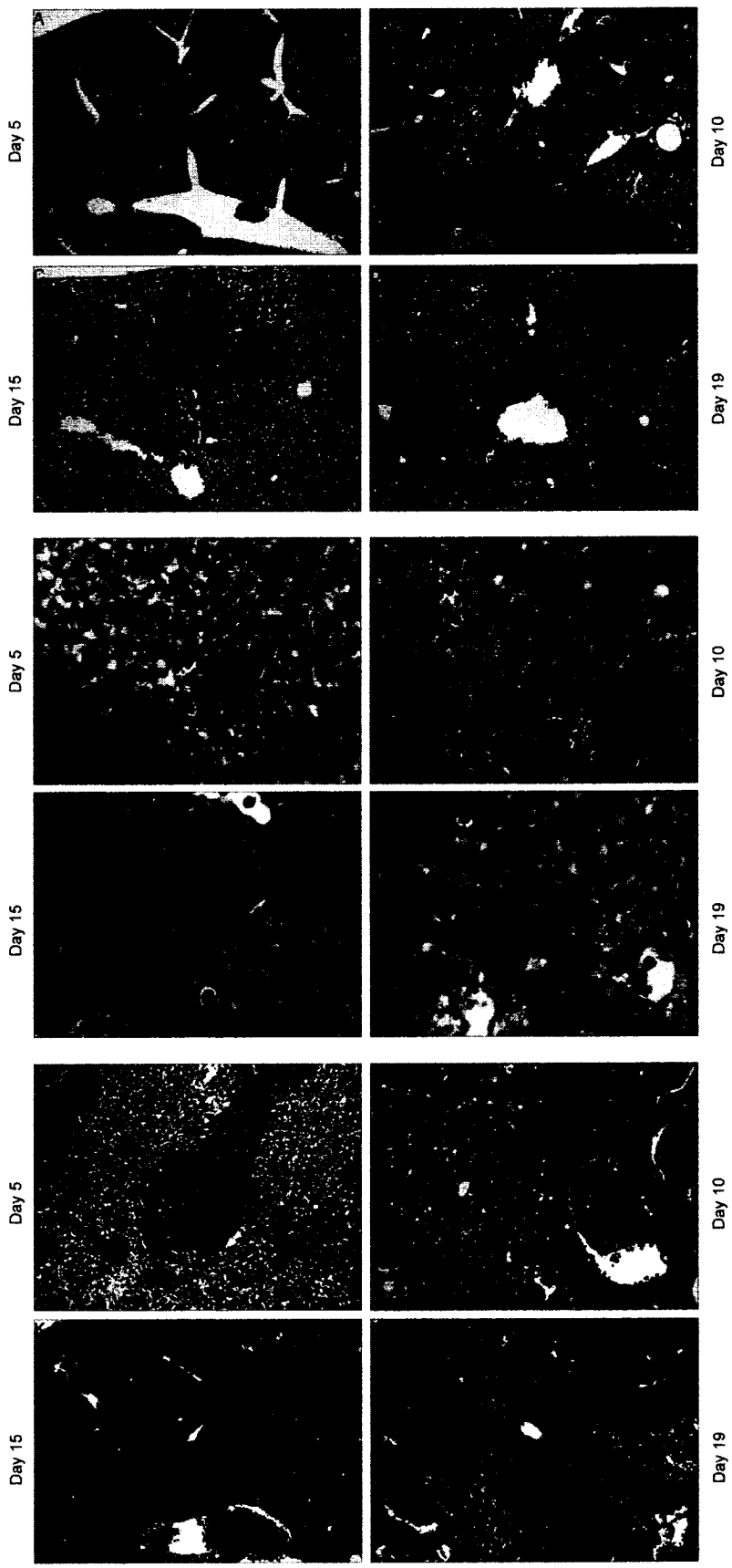
Day 5
Day 10
Day 15
Day 19
Day 5
Day 10
Day 15
Day 19
Day 5
Day 10
Day 15
Day 19

Figure 3 (previous page): Progression of the lesions in the spleen and liver of mice infected with *Salmonella* Typhimurium (Hematoxylin and eosin staining). A, B, C and D: liver, objective 4X. Hepatic necrosis and inflammation (denoted by "N") progressing from moderate and acute (A) to marked and subacute (B) and finally moderate and subacute to chronic (C and D). The leukocytosis and thrombosis of the venular system is shown by the arrows. E, F, G and H: splenic red pulp, objective 40X. Acute inflammation and necrosis of the splenic red pulp with no evidence of histiocytosis (E). Progression of the splenic histiocytosis from slight (F) to moderate (G) and finally minimal (H). I, J, K and L: splenic white pulp, objective 10X. Normal splenic white pulp showing the periarteriolar lymphoid sheath (I). Progression of the periarteriolar lymphoid sheath atrophy from slight (J) to moderate (K) and finally massive (L). Moderate leukocytosis and thrombosis of the venular system is also show in J and K (arrows).

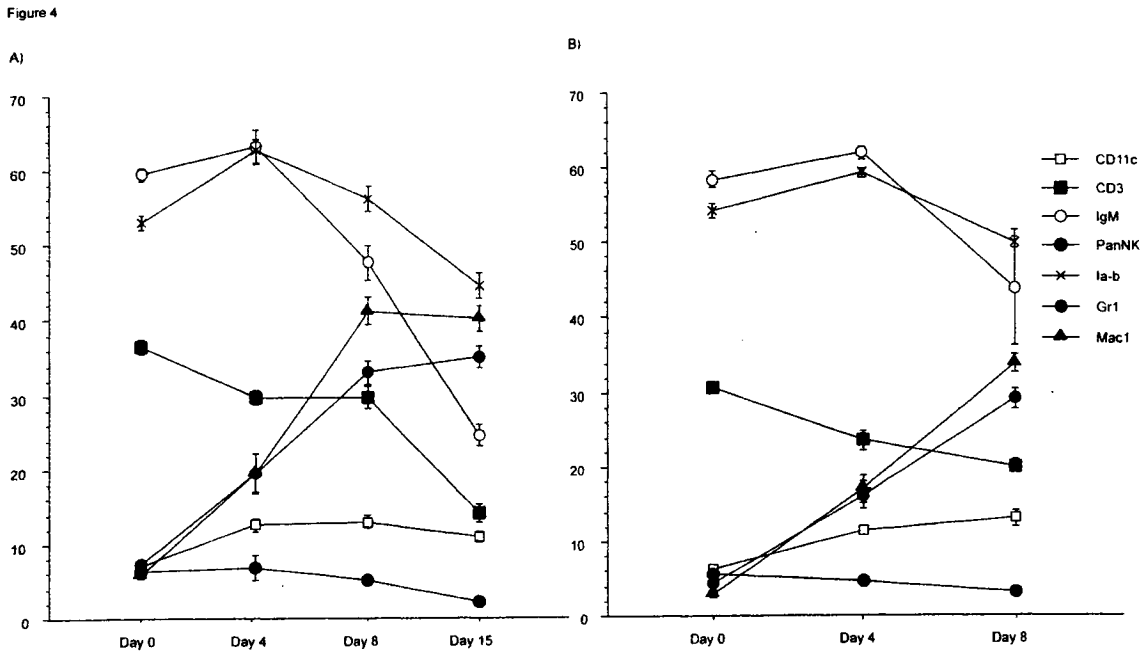

Figure 4: Flow cytometry analysis of the cell population recruited to the spleen during *Salmonella* infection. Mean +/- SEM percent of cell with positive staining for the various markers. Six mice per group and per time point were used. A) Cell populations of the spleen during *Salmonella* infection in F1Tg388 mice. B) Cell populations of the spleen during *Salmonella* infection in B10.Cg-*Nramp1/Tlr4*.

Figure 5

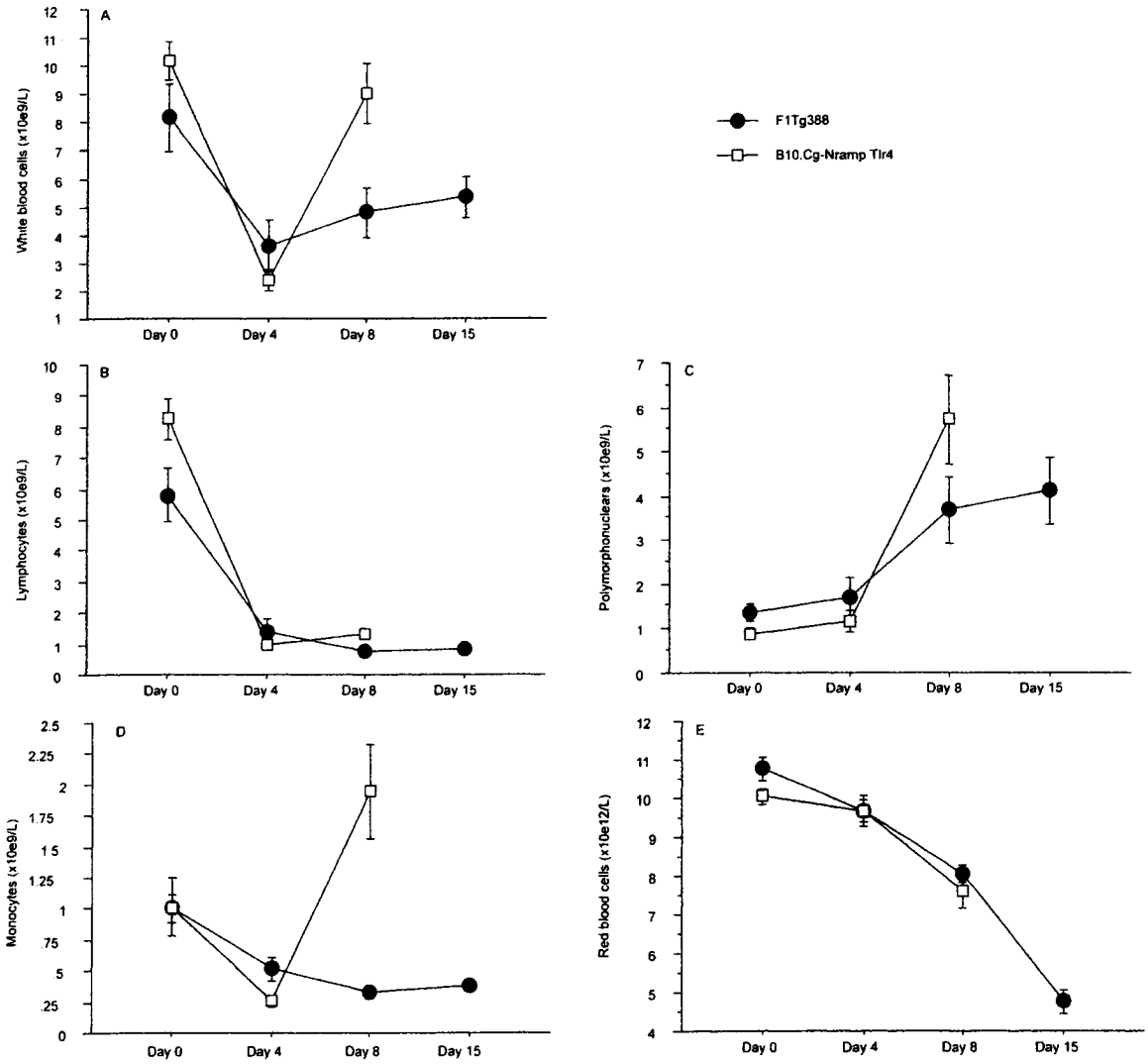

Figure 5: Red blood cell and white blood cell counts in mice infected with *Salmonella* Typhimurium. Mean +/- SEM are shown for the F1Tg388 and the B10.Cg-*Nramp1/Tlr4*. Six mice per group and per time point were used.

Figure 6

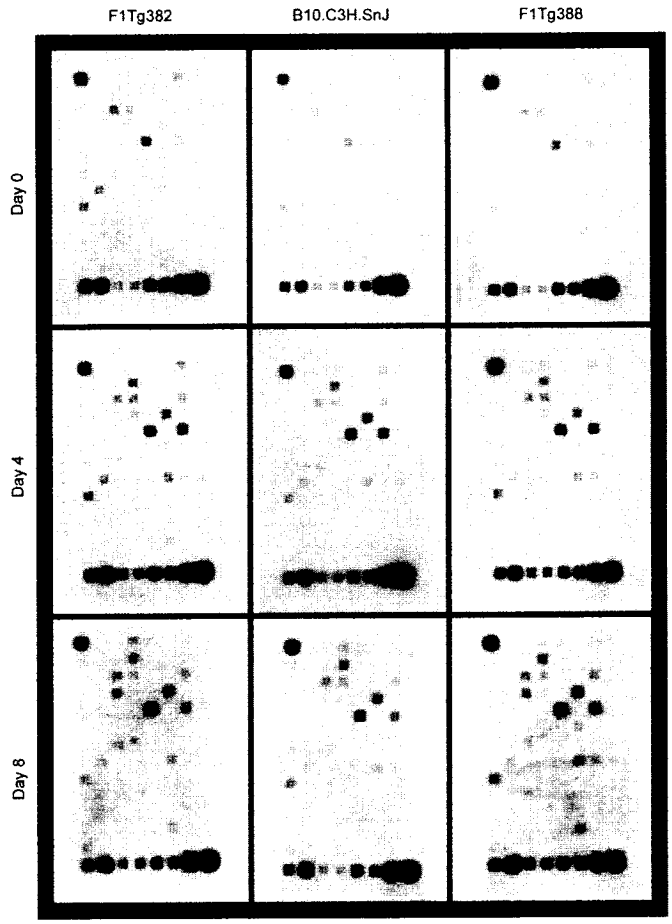

Figure 6: mRNA expression in whole spleen during *Salmonella* infection in mice as measured by oligoarray analysis. Typical arrays for each group and each time point are shown. Whole spleen mRNA from single mouse were hybridized to each array. We had three arrays per group on day 4 and 8 and two arrays per group for non-infected mice. (Refer to Supplemental Table 1 for the list and position of the genes on the array.)

Figure 7

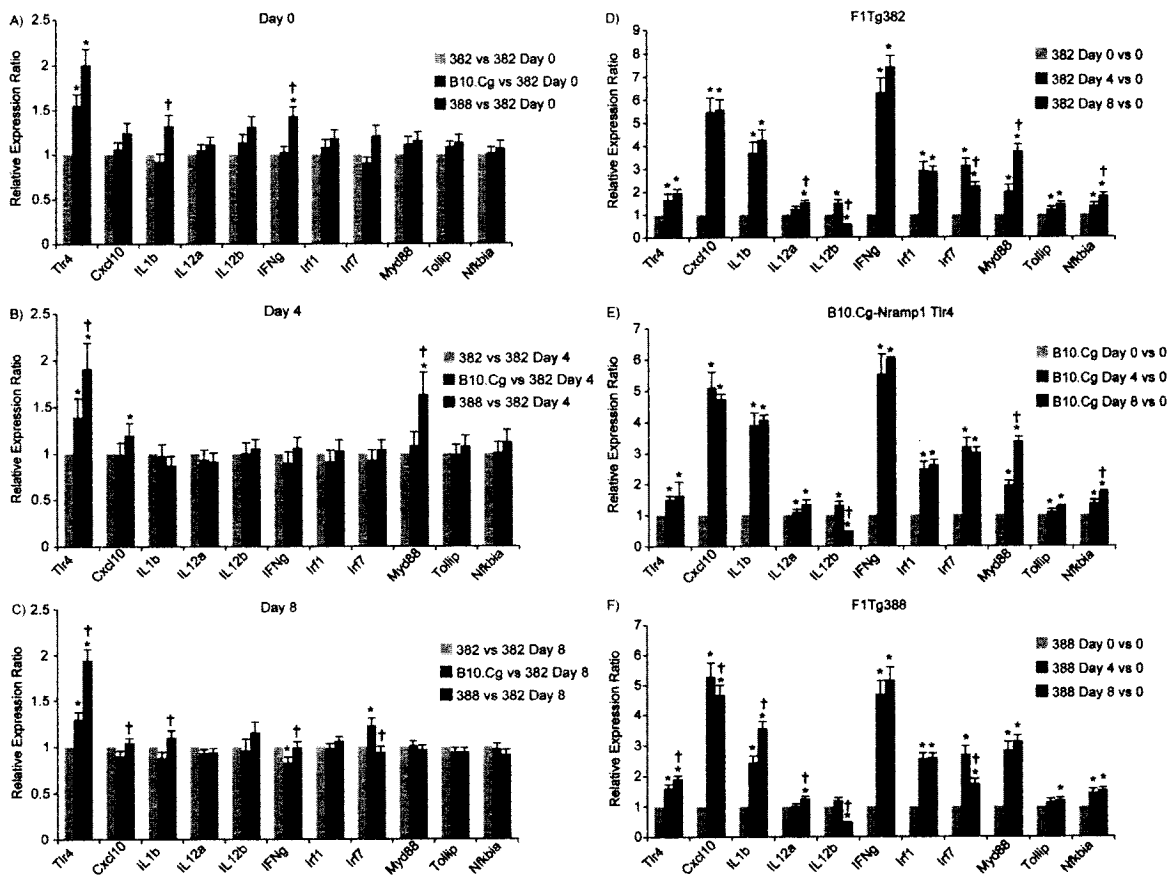

Figure 7: mRNA expression in whole spleen during *Salmonella* infection in mice as measured by quantitative real-time RT-PCR. A, B and C) Relative expression ratios of various transcripts comparing the response of each groups within a similar time point. D, E and F) Relative expression ratios of various transcripts illustrating the effect of *Salmonella* infection in each mouse strain. * indicates significantly different ($p < 0.05$ after 2000 randomizations in REST 384) from the reference group (F1Tg382 (A through C) or Day 0 (D through F)). † indicates significantly different ($p < 0.05$ after 2000 randomizations in REST 384) from the intermediate group (B10.Cg (A through C) or Day 4 (D through F)).

CHAPTER III: Complexity in the host response to *Salmonella* Typhimurium infection in AcB and BcA recombinant congenic strains.

PROLOGUE

The dissection of complex genetic traits, such as the susceptibility to *Salmonella* Typhimurium infection in mice, may be facilitated by the use of specialized mouse ressources such as the recombinant congenic strains (RCS). RCS are derived from reciprocal double backcrosses followed by inbreeding for several generations. The resultant newly inbred RCS therefore possess ~12.5% of the donor genome onto the recipient genome. A set of A/J and C57BL/6J RCS was generated and consisted of 14 AcB and 22 BcA strains. These strains can be used for the study of various phenotypes for which the two parental strains differ. When infected with *Salmonella* Typhimurium, A/J shows an intermediate resistance while C57BL/6J are extremely susceptible. Previous studies have shown that this difference is mainly due to the fact that C57BL/6J carries a mutated, non-functional allele at *Nramp1*. *Nramp1* is implicated in the transport of divalent cations at the phagolysosomal membrane and has a tremendous impact on the growth of *Salmonella* in the spleen and liver early during infection.

In the present study, we undertook a systematic screening of the 36 A/J and C57BL/6J RCS for their susceptibility to acute infection with *Salmonella* Typhimurium. While we knew before hand that *Nramp1*, which segregates in these lines, would be the main determinant of the susceptibility or resistance of the mice, we hypothesized that additional genes influencing the outcome of *Salmonella* infection would segregate in this RCS set. The strains identified as having a deviant phenotype from their known *Nramp1* genotype would then be used in the generation of fully informative crosses for QTL mapping and gene identification.

Complexity in the host response to *Salmonella* Typhimurium infection in AcB and BcA recombinant congenic strains.

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Running title: Recombinant congenic strains and *Salmonella*.

Disclosure: The authors have no competing financial interests.

ABSTRACT

The host response to *Salmonella* infection is controlled by its genetic makeup. Using the mouse model of typhoid fever, several genes were found to influence the outcome of *Salmonella* infection, including *Nramp1* (*Slc11a1*). In order to improve our knowledge of genetic determinants of the mouse response to acute *Salmonella* Typhimurium infection, we performed a systematic screening of a set of A/J and C57BL/6J recombinant congenic strains (RCS) for their resistance to infection. While we knew that the parental strains differ in their susceptibility to *Salmonella* because C57BL/6J mice carry a non-functional allele at *Nramp1*, we hypothesized that other genes would influence the response to *Salmonella* and segregate in the RCS. We identified several RCS that showed a non-expected phenotype given their known *Nramp1* genotype proving that the response to *Salmonella* in A/J and C57BL/6J mice is complex. Based on these findings, we selected two RCS for generation of fully informative F2 crosses, (AcB61 x 129S6) and (AcB64 x DBA/2J). Genetic analyses performed on these crosses identified five novel *Salmonella* susceptibility QTL mapping to chromosomes 3 (*Ity4*), 2 (*Ity5*), 14 (*Ity6*), 7 (*Ity7*) and 15 (*Ity8*). These results illustrate the genetic complexity associated with the mouse response to *Salmonella* Typhimurium.

Key words: *Salmonella*, host resistance, recombinant congenic strains, QTL, infection.

Complexity in the host response to *Salmonella* Typhimurium infection in AcB and BcA recombinant congenic strains.

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INTRODUCTION

Salmonella spp are gram-negative, facultative intracellular pathogens that infect a wide range of host species such as mammals, reptiles and birds.[12] Several serovars are recognized but they almost all belong to the same species designated *Salmonella enterica*.[3] In humans, two main clinical syndromes are recognized, according to the host specificity of the infecting serovar. *Salmonella enterica* serovars Typhi and Paratyphi (*Salmonella* Typhi or Paratyphi) are the causative agents of enteric fever, a systemic illness found only in humans, where the bacteria localizes to the spleen and liver.[13,14] These host specific serovars continue to be a major threat to public health in developing countries where access to clean water is problematic, and in travelers returning from endemic areas. The non-host specific serovars such *Salmonella enterica* serovars Typhimurium and Enteritidis (*Salmonella* Typhimurium and Enteritidis) are causative agents of a more localized gastrointestinal illness, commonly known as salmonellosis. Isolated cases or outbreaks of salmonellosis are most often seen in industrialized countries, usually associated with food contamination[280] or contact with domestic animals that are shedding *Salmonella*.[10] The emergence of antimicrobial resistance among the various *Salmonella* serovars noted over the past few decades greatly complicates the treatment of infected patients and these resistant isolates are associated with increased bloodstream infection, hospitalization and mortality (reviewed in Molbak[281]). A better understanding of the host-pathogen interactions and of the pathogenesis of both enteric fever and salmonellosis will permit elaboration of new anti-*Salmonella* strategies that rely less on antimicrobials such as more effective vaccines, breeding of more resistant animal stock or prophylactic identification of genetically susceptible host.

Salmonella Typhimurium infection in mice recapitulates the disease found in humans infected with *Salmonella* Typhi or Paratyphi.[66] Following infection of mice, either orally or parenterally, the bacterium rapidly localizes to the spleen and the liver where it replicates. This experimental model is an invaluable tool for the study of the

host genes that may influence the outcome of infection and it was used to show that the mouse genetic background has a major impact on the course of infection. Using a positional cloning approach, two major genes influencing the innate immune response of mice to *Salmonella* Typhimurium infection have been identified: the natural resistance associated macrophage protein 1 gene (*Nramp1*, also known as *Slc11a1*)[139,140,150] and the Toll-like receptor 4 (*Tlr4*) gene.[178,179] *Nramp1* has a tremendous impact on the exponential growth of the bacteria in the spleen and liver during the first 5 days of infection and mice carrying a point mutation in *Nramp1* (Gly169Asp) are extremely susceptible to *Salmonella*.[121] The mouse *Tlr4* is also involved in the early mouse response to *Salmonella* infection and mice carrying a non-functional allele show a 1000 fold reduction in LD_{50} compared to wild type animals.[167] The mouse Bruton's tyrosine kinase gene (*btk*) has also been shown, through positional cloning, to control the late response to *Salmonella* Typhimurium.[81,205] In addition to these positionally cloned genes, studies done using congenic and knockout mice have shown a role for several additional genes including the ones encoding for interferon gamma, interleukin-12, NADPH oxidases, inducible nitric oxide synthase, lipoprotein binding protein, CD14 and the mouse MHC complex (reviewed in Roy and Malo[282]).

It is clear that the mouse response to *Salmonella* Typhimurium is most likely complex and under the influence of several genes. While the genes described above seem to have a major impact on the host response to *Salmonella* it is conceivable that additional gene variants of smaller effect also make a significant contribution to disease outcome in mice. These may be ancient functional variants that have been fixed in the various inbred strains or more recent mutations that occurred during the breeding of the strains. Polymorphisms in orthologous genes could potentially also exist in the human population and have a similar impact on the outcome of enteric fever or diseases caused by other intracellular pathogens. To date, two quantitative trait loci (QTL), *Ity2* and *Ity3* (Immunity to Typhimurium 2 and 3), have been identified as influencing the outcome of acute *Salmonella* Typhimurium infection in a (C57BL/6J x MOLF/Ei)F2 cross.[123] Additionally, in a chronic model of infection with *Salmonella* Enteritidis, several QTL were found to be involved in persistence of the infection in target organs.[124,125] It is likely that several additional QTL influence the mouse resistance to *Salmonella* infection and

that identification of the genes underlying these QTL will be good candidate to test for their impact on *Salmonella* infection in human or veterinary important species.

Recombinant congenic strains (RCS) have been used in an attempt to genetically dissect complex trait.[283-286] RCS are generated through reciprocal double backcrosses followed by inbreeding for several generations. In a typical RCS set, one has transferred a fixed proportion of the genome from one strain (the donor strain) onto the background of a second strain (the recipient strain) and has generated a set of new inbred strains, each carrying a small proportion of the donor strain in various combinations. If the parental strains differ significantly in a quantitative trait of interest, then the study of the strain distribution pattern (SDP) in relation to the phenotype should facilitate the identification of genomic regions influencing the studied trait. The mapping of the gene is usually not possible by the sole study of the SDP unless the gene has a strong, Mendelian-type effect on the phenotype. The usual approach to gene identification is to cross the interesting RCS back to their background strain and limit the search for linkage to the already known donor regions.

In order to identify additional QTL influencing the outcome of acute *Salmonella* Typhimurium infection in mice, we undertook a systematic screening of a set of A/J and C57BL/6J RCS (AcB/BcA RCS).[285] While it is well known that these two parental strains differ in their susceptibility to acute *Salmonella* Typhimurium infection because of *Nramp1* (C57BL/6J mice carry a non-functional *Nramp1* gene), we hypothesized that additional genes, influencing the outcome of *Salmonella* infection would segregate in the AcB and BcA RCS. Using this approach, we have identified several strains that showed a deviant phenotype from what was expected given their known *Nramp1* genotype. Two of these strains were selected for generation of two fully informative F2 crosses. Linkage analysis in these crosses revealed five novel QTL that influence the outcome of typhoid fever in mice. These findings show that the phenotypic difference in resistance to *Salmonella* between A/J and C57BL/6J cannot be explained by *Nramp1* alone and that overall, the mouse response to *Salmonella* Typhimurium is complex and under the influence of several QTL and environmental factors.

RESULTS

Screening of the AcB/BcA RCS

The parental strains, A/J and C57BL/6J, differ in their susceptibility to *Salmonella* Typhimurium infection mainly because C57BL/6J carries a non-functional allele at *Nramp1*, rendering it extremely susceptible to *Salmonella* infection. We hypothesized, however, that additional genes, besides *Nramp1*, have an impact on the mouse response to *Salmonella* and segregate in the AcB/BcA RCS. To test this hypothesis, we undertook a systematic screening of the thirty-six AcB/BcA RCS and tested their susceptibility to *Salmonella* Typhimurium infection by measuring survival and splenic and hepatic bacterial load at day 4. When infected with 10^3 CFUs of *Salmonella* Typhimurium intravenously, most RCS behaved as expected from their known *Nramp1* genotype. All the strains carrying a non-functional allele at *Nramp1* (*Nramp1* susceptible) were extremely susceptible to *Salmonella* and died on day 4, 5 or 6 post-infection (Figure 1). Similarly, most of the RCS carrying a wild-type allele at *Nramp1* (*Nramp1* resistant) were more resistant to *Salmonella* infection and showed a phenotype almost identical to their resistant parent, A/J. Interestingly, some of the *Nramp1* resistant RCS showed a deviant phenotype. The strains AcB61 and AcB62, despite carrying a resistant allele at *Nramp1*, were extremely susceptible to infection and did not survive past day 7. Such an extreme susceptibility is rarely seen in inbred strains of mice and when seen, it has usually been attributed to spontaneous mutation either in *Nramp1* (e.g. C57BL/6J, C57BL10/J or BALB/c)[121,140] or *Tlr4* (e.g. C3H/HeJ or C57BL/10ScNCr).[167,178,179] One exception is the wild-derived inbred strain MOLF/Ei with a low mean survival time of 6 days apparently attributable to the effect of several genes.[123] Two other RCS, AcB60 and AcB64, were found to be more resistant than their resistant parent, A/J. Here again, this increased resistance to virulent *Salmonella* Typhimurium with a fair percentage of mice being able to survive the infection is rarely seen in inbred mouse strains with the exception of 129S6/SvEvTac (129S6, completely resistant) and CAST/Ei.[282] Finally, AcB56, AcB63 and BcA69 showed an intermediate phenotype between that of C57BL/6J and A/J. These results illustrate the complexity underlying the mouse response to acute *Salmonella* infection and indicate that additional genes, besides *Nramp1*, control the mouse survival following *Salmonella* infection and segregate in the AcB/BcA RCS.

We were also interested in studying the genetic control of the bacterial growth in the target organs by measuring the spleen and liver bacterial loads at day 4 following infection in each of the available RCS. Figure 2 shows that, as expected, the RCS segregate into two major groups according to their genotype at *Nramp1*. All *Nramp1* susceptible strains show bacterial loads similar to their C57BL/6J parental strain and much higher than A/J. Likewise, most of the *Nramp1* resistant strains showed lower bacterial loads, corresponding to the levels measured in A/J. Interestingly, some strain-to-strain variations in bacterial load could be observed within the two *Nramp1* groups. Especially, we observed that two *Nramp1* resistant strains, AcB61 and AcB62, showed an intermediate level of CFUs, between that of the two parental strains. As mentioned earlier, these two strains were also found to be extremely susceptible to *Salmonella* infection in terms of survival (Figure 1). Additionally, a few *Nramp1* susceptible strains (BcA72, BcA73, BcA76, BcA79, AcB51 and AcB53) presented lower or higher CFUs compared to the C57BL/6J parent. Finally, two groups, BcA66 and BcA80 showed a sex effect with the males having lower CFUs in the spleen and the liver compared to the females.

Taken together, the survival and bacterial load measurements made in the thirty-six AcB/BcA RCS revealed the existence of *Nramp1*-independent genetic mechanisms influencing the host response to *Salmonella* Typhimurium infection in C57BL/6J and A/J.

Segregation analysis of the survival phenotype in AcB61 and AcB64 F2 crosses

Following identification of a few RCS that showed a deviant phenotype from what would have been expected given their known *Nramp1* genotype, we selected two strains, AcB61 (extremely susceptible) and AcB64 (more resistant), for further genetic analysis. We decided to generate two fully informative segregating F2 populations by intercrossing these RCS with unrelated inbred strains. While it could have been advantageous to cross these strains to A/J and concentrate our search for linkage to the already known C57BL/6J congenic fragments, we decided to do otherwise for two main reasons. First, spontaneous mutations influencing the phenotype of interest can occur during the generation of the RCS and these would go undetected in a cross with one of the parental strain unless the mutation occurred at or close to a congenic fragment.[287] Second, the genomic structure of the RCS is not completely known and small congenic fragments

could have been missed during the initial genotyping of these strains. We therefore generated two fully segregating F2 populations, (AcB61 x 129S6) and (AcB64 x DBA/2J). The inbred partners were chosen so they would differ as much as possible in their phenotype from the AcB strain and also on the basis that they did not carry mutation in genes known to be important in *Salmonella* resistance. 247 (AcB61 x 129S6) and 249 (AcB64 x DBA/2J) F2 mice were generated and the survival of each mouse following infection with 10^3 CFUs of *Salmonella* Typhimurium was recorded. Figure 3a and b show the cumulative survival of the F1s, F2s and the parental strains for both crosses. Both F1 populations showed a survival phenotype almost identical to their resistant parent while the F2 populations showed a continuous distribution in their phenotype, intermediate between that of their respective parental strain. On closer examination, however, the (AcB61 x 129S6)F2 mice appear to segregate into more or less 3 groups: 1) a susceptible group dying before day 12; 2) an intermediate group dying between day 12 and 40 and 3) a resistant group surviving the infection past day 50. For both crosses, some mice survived the infection past day 50, although in greater number for the (AcB61 x 129S6)F2 cross. Some of these mice were followed for more that 90 days and showed no signs of illness although they still carried *Salmonella* in their spleen and liver (data not shown) at that time. These results suggest that for both crosses, the mouse response to *Salmonella* infection behaves as a complex trait and is under the influences of more than one gene and potentially additional environmental factors. No effect of the infection day or of the sex was observed on the survival of the F2 mice and therefore, we could analyze together the F2 mice from each individual cross.

Genetic analysis of the survival time in (AcB61 x 129S6)F2 mice

As a first exploration for potential survival-associated genomic regions in the (AcB61 x 129S6)F2 cross, we performed a one locus interval mapping in R/qtl[288] under a non-parametric model[289] which was indicated given the distribution of the survival time in this cross (Figure 3c). Supplemental Figure 1a shows the genetic map used and the names and physical positions of the markers are listed in Supplemental Table 1. Two loci were found to be significantly associated with survival in this cross (Figure 4a). A highly significant LOD score of 28.8 was detected at position 44cM on chromosome 3. We will refer to this locus as *Ity4*. A second significant peak was detected on the distal end of

chromosome 2 at position 117cM with a LOD score of 4.6 (*Ity5*). The p = 0.05 genome wide significance threshold for this cross, established following 1000 permutations was 3.4.

Because of concerns that the extremely high LOD score on chromosome 3 might interfere with detection of additional QTL or lead to spurious association, we repeated the analysis after having removed all the mice that were AcB61 homozygous for the marker most closely associated with *Ity4*. One hundred and ninety individuals were left for analysis and interval mapping under the non-parametric model revealed that the distal chromosome 2 locus continued to be strongly associated with survival following *Salmonella* infection in this cross with a LOD score that increased to 6.5 (maximum LOD score position at 121cM). These results show that both *Ity4* and *Ity5* independently contribute to the mouse response to acute *Salmonella* Typhimurium infection in this cross.

Examination of the distribution of the survival time in the (AcB61 x129S6)F2 population reveals that approximately 50% of the mice survived the infection beyond day 50, thus this distribution was heavily censored (Figure 3c). Because the interval mapping approach described above does not properly account for censored data, we re-analyzed our data using a survival analysis approach. A Weibull distribution for the survival times was assumed and a parametric survival regression at the markers was carried out using the statistical program R.[290] Two loci, co-localizing with the ones detected in the initial analysis (*Ity4* and *Ity5*, Figure 4a) were found to be significantly associated with survival time in this cross (Figure 5a, Table 1 and Supplemental Table 1). First, a significant association was found on a region of chromosome 3 (*Ity4*) extending from 65.7 to 124.3Mb with genome wide p-value < 0.05. The peak LOD score was 27.3 under a dominant model at the typed marker 03-098415492-M (position = 98.4Mb, p-value < 0.0001, 1.5-lod support interval = 72.7-100.5Mb) with the 129S6 allele conferring protection against *Salmonella* infection. A second significant association was detected on the distal end of chromosome 2 (*Ity5*) with a genome wide p-value < 0.05 from 155.5 to 180.0Mb. The peak LOD score was 5.4 under an additive model at 02-167943959-M (position = 168.8Mb, p-value = 0.0005, 1.5-lod support interval = 165.2-180.0Mb) with the protective effect associated with the 129S6 allele. The proportion of the phenotypic

variance explained was 42.1% and 7.5% for *Ity4* and *Ity5*, respectively. The survival curves with the F2 mice grouped according to their genotype at 02-167943959-M and 03-098415492-M, the typed markers most strongly associated with survival time, are shown on Figure 6a and b. No interaction effect between these loci was detected (Figure 6c).

Genetic analysis of the survival time in the (AcB64 x DBA/2J)F2 mice

A similar approach was used to analyze the survival data from the (AcB64 x DBA/2J)F2 cross. Exploratory one locus interval mapping of the survival phenotype was performed in R/qtl. Given the survival time distribution (Figure 3d) we used the two-part model described by Karl Broman[289] and implemented in R/qtl. Under the two-part model, two separate analyses are performed: one for a binary trait (the mice that died versus the mice that survived) and one for the normal trait conditional on a non-censored phenotype (mice that did not survive the infection). The final LOD score for the two-part model is the sum of the two LOD scores obtained on the separate analyses. The genetic map used is shown in Supplemental Figure 1b and the names and physical positions of the markers are listed on Supplemental Table 2. Two loci reached the genome wide ($p = 0.05$) significance threshold established following 1000 permutations (LOD = 4.2) (Figure 4b). *Ity6*, at position 27cM on chromosome 14, with a LOD score of 5.3 and *Ity7*, at position 52.8cM on chromosome 7, with a LOD score of 4.3. Several additional loci, which did not reach the $p = 0.05$ significance threshold but that presented a LOD score ≥ 3 were also detected: chromosome 3 at position 51cM with a LOD score of 3.5; chromosome 10 at 7.5cM with a LOD score of 3.8; chromosome 11 at 58cM with a LOD score of 3.0; chromosome 12 at 68.7cM with a LOD score of 3.4; chromosome 15 at 51.6cM with a LOD score of 3.6; chromosome 19 at 38cM with a LOD score of 3.6; and chromosome X at position 45cM with a LOD score of 3.2. These results illustrate the genetic complexity associated with the mouse response to *Salmonella* Typhimurium infection in this cross.

The survival phenotype in the (AcB64 x DBA/2J)F2 cross was also heavily censored since about 25% of mice survived beyond day 50 (Figure 3d). As for the previous cross, and in order to properly account for the incomplete observation of the data, inference was carried out through parametric survival regression at the markers under a Weibull model for the survival times. Three loci having a small effect on the survival times reached the genome wide significance (Figure 5b, Table 2 and

Supplemental Table 2), two co-localizing with the previously detected *Ity6* and *Ity7*, and the third one co-localizing with the locus detected on chromosome 15. The first locus, *Ity6*, was detected on chromosome 14 at the marker 14-055172074-M with a LOD score of 4.8 under a recessive model (position = 54.7Mb, p-value = 0.0289, 1.5-lod support interval = 44.4-84.4Mb). The resistant allele for *Ity6* was inherited from the susceptible strain, DBA/2J, and behaved recessively, with only the mice homozygous for the DBA/2J allele showing increased resistance. This locus explained 7.4% of the phenotypic variance. A second significant association was found on chromosome 7 at the marker 07-096980068-M (*Ity7*) with a LOD score of 5.8 under a recessive model (position = 95.7Mb, p-value = 0.0076, 1.5-lod support interval = 87.9-105.0Mb) and explaining 5.7% of the phenotypic variance. The third locus, *Ity8*, was located on the distal end of chromosome 15 at the marker 15-096231715-M with a LOD score of 5.4 under a recessive model (position = 95.7Mb, p-value = 0.0134, 1.5-lod support interval = 88.3-102.6Mb) and explaining 4.0% of the phenotypic variance. For both *Ity7* and *Ity8*, the protective allele was inherited from the resistant AcB64 parent and behaved in a dominant fashion. The survival curves with the F2 mice grouped according to their genotype at 14-055172074-M, 07-096980068-M and 15-096231715-M, the typed markers most strongly associated with survival time, are shown on Figure 7a-c. No interaction among these three loci was detected as seen in Figure 7d for *Ity6* and *Ity7*.

DISCUSSION

The susceptibility to infectious diseases in the human population is partially controlled by the genetic constitution of the infected host.[111,291,292] The host response to *Salmonella* spp also follows this rule, as evidenced by several studies that identified genetic variants responsible for increased susceptibility to this pathogen. For instance, patients with mutations in the interleukin-12/interferon-gamma axis show increased susceptibility to mycobacteria and *Salmonella*.[119] The same is true for patients with defect in the NADPH oxidase system (chronic granulomatous disease[117]) or sickle cell anemia patients,[116] which often present with *Salmonella* infections. Finally, the susceptibility to typhoid fever in humans has also been linked to specific MHC genes.[120] While these studies give some insights into the genetic control of the host response to

Salmonella, there is most likely a wealth of other genetic variants that influence the outcome of an encounter between *Salmonella* and its host.

Because of the inherent difficulties associated with the genetic dissection of susceptibility to infectious diseases in the human population, the experimental model of typhoid fever in the mouse has been used extensively. Using forward (positional cloning) or reverse (knockout) genetic approaches, several genes have been shown to influence the outcome of *Salmonella* Typhimurium infection in mice (reviewed in Roy and Malo[282]). While most of the genes identified so far have a clear, Mendelian-type effect on the phenotype, there are most likely several additional variants of lesser effect that also contribute to the response of mice to *Salmonella* Typhimurium. These variants may be highly relevant candidates to be tested for their effect in human typhoid fever or other infections with intracellular pathogens. Using a (C57BL/6J x MOLF/Ei)F2 cross, Sebastiani *et al*[123] have indeed identified two QTL (*Ity2* on chromosome 11 and *Ity3* on distal chromosome 1) that each have a small effect on the mouse response to acute *Salmonella* infection. In the present study, we have used a specialized tool of mouse genetics, the RCS, to further increase our understanding of the genetic basis of the host response to *Salmonella* Typhimurium infection in mice.

RCS have been used before in the identification of QTL underlying complex traits. This approach is based on the premise that the isolation of the various genes underlying a quantitative trait in the individual RCS will facilitate their identification. Additionally, the mapping of QTL with RCS is further facilitated by the unlimited supply of each individual RCS (permitting accurate phenotyping), and by the complete genotypes known for each strain (limiting the cost and labor associated with the project). Detection of single gene effect may be possible by the sole study of the strain distribution pattern in relation to the phenotype of interest.[285] In our case, a simple marker regression of the mean phenotype for each of the thirty-six RCS against their known genotypes was powerful enough to detect the effect of *Nramp1* (LOD score of 3.9, 12.8 and 11.5 at *D1Mit532* for survival, splenic and hepatic bacterial loads respectively), a gene known to have a strong, Mendelian-type effect on the mouse response to *Salmonella* Typhimurium. However, as would have been expected, no other loci having an impact on one or the other measured phenotypes could be detected and further breeding was necessary for

identification of additional QTL. The usual approach when using RCS for the genetic dissection of complex trait is to first identify the individual RCS that show an unexpected or interesting phenotype and then generate a new cross involving these RCS. One may cross the RCS back to the background strain and then limit the search for linkage to the known congenic fragments.[284] This approach presents the advantages of limiting the cost and workload associated with genotyping and prevents the introduction of a new strain with additional polymorphisms that will complicate the mapping of the QTL. A disadvantage of this approach is that one will not be able to identify new mutations that may have occurred during the generation of the RCS unless the mutation occurred by chance close to or within a congenic fragment.[287] Additionally, one may also miss some small congenic fragments that have escaped detection during the initial genotyping of the mice. We, therefore, chose a different approach where the selected RCS would be crossed to an unrelated inbred partner thereby maximizing the chance of finding the QTL. In addition to polymorphisms already segregating in the RCS, introduction of a new unrelated inbred mouse strain increases the polymorphisms content of the cross and maximizes the chance of identifying QTL important in the studied phenotype.

The systematic screening of the AcB/BcA RCS allowed us to identify several strains that showed a deviant phenotype from their known *Nramp1* genotype. Two of these deviant strains were selected for further genetic analysis, which led to the identification of five new QTL involved in the mouse response to acute *Salmonella* infection. In the (AcB61 x 129S6)F2 cross, two QTL were detected, which together explain almost half of the phenotypic variance in this cross. *Ity4* by itself accounts for 42.1% of the phenotypic variance indicating that the gene or genes underlying this QTL exert a major, Mendelian-type effect on the host response to *Salmonella* in this mouse model. The genomic region underlying this QTL is gene-rich and contains more than 300 genes with several potential candidates, including a malaria resistance gene, the liver and red blood cell specific pyruvate kinase gene (*Pklr*).[287] We are currently in the process of fine mapping this region and are evaluating good positional candidates. *Ity5* also appears to have, by itself, a major impact on the mouse response to *Salmonella* as evident from Figure 6b. The genomic region underlying the confidence interval for *Ity5* contains 97 genes. Generation of reciprocal congenic strains carrying this region should allow us to

confirm its impact on mouse salmonellosis and help in the fine mapping of this QTL. These two loci do not co-localize with C57BL/6J congenic fragments indicating that we are either mapping a new spontaneous mutation that occurred during the generation of the RCS or a variant between A/J and the 129S6 strain.

The situation appears more complex for the (AcB64 x DBA/2J) cross with several loci possibly each controlling a small percentage of the phenotypic variance. Using interval mapping and survival analysis, three loci, *Ity6*, *Ity7* and *Ity8*, were found to be significantly associated with the mouse response to *Salmonella* infection. Additional QTL are most likely segregating in this cross but did not reach the significance threshold. The generation, genotyping and phenotyping of a few hundreds additional mice will be necessary in order to get a clear understanding of the genetic contribution to the phenotype in this case. The QTL mapping in this cross is further complicated by non-genetic, environmental factors, which appear to play an important role in the outcome of *Salmonella* infection in this case. As noted on Figure 1 and 3b, even in the genetically identical AcB64 and (AcB64 x DBA/2J)F1 mice, the variance of the survival phenotype is quite large implicating additional factors beside genetic effects. It may be that, in these intermediately resistant strains, factors such as the inoculation dose or the state of the immune system can exert a greater influence than in mice that are extremely resistant (e.g. 129S6) or extremely susceptible (e.g. C57BL/6J or AcB61). As observed for *Ity4* and *Ity5*, *Ity6* and *Ity7* do not map to C57BL/6J congenic fragments, indicating again that we are either mapping new mutations or DBA/2J versus A/J variants. However, *Ity8* and the potential loci on chromosome 3 do map to a C57BL/6J congenic fragment, which may facilitate the mapping of these QTL.

In addition to the AcB61 and AcB64 strains that were selected for further genetic analysis, the systematic screening of the thirty-six RCS identified a few additional strains that may be worth further investigation. In particular, the AcB62 strain presents a phenotype quite similar to the AcB61 strain with a very short survival time and higher bacterial load in the spleen and liver at day 4. We are currently investigating the possibility that the same genomic region confers susceptibility to *Salmonella* in both AcB61 and AcB62. Another very interesting strain that may be worth further investigation is the AcB60 strain. AcB60 mice showed increased survival following

Salmonella infection and in this regard, resemble the AcB64 strain. Examination of the C57BL/6J congenic fragments present in these strains revealed a single common C57BL/6J fragment at position 37 to 43cM on chromosome 18. While this locus was not identified in the survival or interval mapping analysis of the (AcB64 x DBA/2J)F2 cross, it may still be involved in the response to *Salmonella* Typhimurium in AcB/BcA RCS. In fact comparison of the mean survival time between the *Nramp1* resistant RCS that carry a C57BL/6J congenic fragment at this locus with the mice that are A/J at this same location revealed a higher mean survival time associated with the C57BL/6J genotype (mean survival time of 19 for the C57BL/6J genotype versus 11 for the group carrying the A/J genotype). Finally, we also identified a sex effect for the bacterial load at day 4 in the BcA66 and BcA80. Interestingly, in these two cases, the male mice present lower CFUs in the spleen and liver compared to the female mice. This situation is unusual for infectious diseases where the females are usually found to be more resistant than the males (e.g. resistance to malaria [284] or bacterial clearance in chronic *Salmonella* Enteritidis infection[124]).

In conclusion, the study of the thirty-six AcB/BcA RCS for various *Salmonella* Typhimurium related phenotypes clearly demonstrated that the phenotypic difference between the parental strains C57BL/6J and A/J cannot be explained solely based on their different allele at *Nramp1*. Genetic analysis involving fully informative crosses derived from two RCS and unrelated inbred partners allowed us to identify five novel QTL, including a locus with major effect, associated with the mouse survival following *Salmonella* infection. *Ity5* and especially *Ity4* detected in our (AcB61 x 129S6)F2 cross appear to have a strong effect on the phenotype and should be amenable to positional cloning within a reasonable time frame. The situation for the (AcB64 x DBA/2J)F2 cross seems much more complex with potentially several loci and additional environmental factors contributing to the outcome of *Salmonella* infection. Increasing the number of mice in our analysis may allow a clearer understanding of the real genetic factors underlying the survival phenotype in this cross. Such confirmation will be necessary before one undertakes confirmation of these QTL in congenic strains.

MATERIALS AND METHODS

Animals used: thirty-six A/J and C57BL6/J RCS have been generated[285] and were purchased from Emerillon Therapeutics Inc. (Montreal, QC, Canada). These strains were produced through reciprocal double backcrosses followed by inbreeding for more than 20 generations and consist of 14 AcB and 22 BcA strains. A/J, C57BL/6J and DBA/2J were purchased from the Jackson Laboratory (Bar Harbor, ME, USA) while 129S6 were purchased from Taconic (Hudson, NY, USA). (AcB61 x 129S6)F2 and (AcB64 x DBA/2J)F2 mice were generated at the Montreal General Hospital Research Institute animal facility.

In vivo Salmonella infection: All animal procedures were performed in accordance with the regulations of the Canadian Council on Animal Care. The *Salmonella* infections were performed as previously described.[293] Briefly, *Salmonella* Typhimurium strain Keller was grown in trypticase soy broth and each mouse was infected intravenously with approximately 10^3 colony forming units (CFUs) diluted in 200ul of 0.9% saline. The infectious dose was verified by plating of serial dilutions on trypticase soy agar. For survival analysis, the mice were monitored twice daily and moribund animals were sacrificed by CO_2 asphyxiation. Enumeration of the liver and splenic bacterial load was done on day 4 post-infection by plating of serially diluted spleen or liver homogenates. For each RCS, we infected approximately 10 to 12 mice (males and females) and recorded the survival time and the spleen and liver bacterial load at day 4. A/J and C57BL/6J mice were included as controls at each infection. The F2 mice were infected by groups of 50 to 100 mice and each time the parental strains were included as controls. All mice were aged between 2 and 6 months at the time of infection.

Genotyping: DNA was extracted from biopsies of mice tail with overnight digestion in lysis buffer and proteinase K, followed by a chloroform extraction. DNA concentrations were measured with Quant-iT™ DNA Assay Kit (Molecular Probes, Invitrogen, Burlington, ON, Canada) and adjusted to 10ng/ul. Single nucleotide polymorphisms (SNPs) were chosen preferably from the KBiosciences mouse SNP panel[294] or from publicly available SNP database (Wellcome Trust (www.well.ox.ac.uk/mouse/INBREDS/) or Jackson Laboratory (www.informatics.jax.org/) databases). SNP genotyping was performed by KBiosciences

(Hoddesdon, Herts, UK). In house genotyping was performed using simple sequence length polymorphisms. Microsatellite markers were selected from public databases and PCR products were resolved either on ethidium bromide stained high-resolution agarose gels (MetaPhor, Cambrex, Walkersville, MD, USA) or on polyacrylamide gel following labeling with ^{33}P.

Genetic analysis: An exploratory one locus interval mapping for the survival phenotype was performed in R/qtl[288] using the expectation-maximization algorithm under a non-parametric model[289] (for the (AcB61 x 129S6)F2 mice) or under the two-part model[289] (for the (AcB64 x DBA/2J)F2 cross) on a grid of genotypes estimated every 1cM. Significance thresholds were established following 1000 permutations. One locus mapping for the survival phenotype for both, (AcB61 x 129S6) and (AcB64 x DBA/2J), F2 mice was performed using a parametric survival regression at the markers under a Weibull model for the survival time distribution. The significance was assessed via 10 000 bootstrapped resamples controlling the genome wide type 1 error. 1.5-lod support intervals were obtained by interpolation on the physical map. The mode of inheritance was inferred according to the genome wide p-values of the lod scores under the different models and graphic inspection.

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TABLES

Table 1

Marker		Full model		Additive model		Recessive model		Dominant model	
JAX SNP ID or Mit marker	rs #	LOD score	Corrected p-values	LOD score	Corrected p-values	LOD score	Corrected p-values	LOD score	Corrected p-values
02-145828186-N	rs3023694	3.0000	0.2823	2.9322	0.0791	2.2234	0.3128	1.6939	0.7041
02-148389584-N	rs3022940	2.9146	0.3262	2.8636	0.0917	2.1190	0.3798	1.7070	0.6940
02-154428186-N	rs4223578	3.3032	0.1672	**3.2473**	**0.0426**	2.3944	0.2255	1.9320	0.5126
02-162979695-M	rs3700147	**4.7392**	**0.0116**	**4.6029**	**0.0024**	1.9277	0.5141	**4.0171**	**0.0106**
02-167943959-M	**rs3687512**	**5.4800**	**0.0028**	**5.4356**	**0.0005**	**3.5438**	**0.0227**	**3.5501**	**0.0242**
02-172943830-M	rs3710327	**5.1963**	**0.0051**	**5.1897**	**0.0007**	2.8377	0.0968	**3.9929**	**0.0111**
D2Mit457		3.6648	0.0871	**3.6492**	**0.0177**	2.3510	0.2463	2.4797	0.1961
02-179086475-M	rs3680965	3.3899	0.1436	**3.3656**	**0.0331**	2.3038	0.2689	2.2442	0.3033
03-033933315-N	rs4223883	3.8571	0.0625	**3.7551**	**0.0142**	1.8365	0.5863	3.1552	0.0528
03-065861493-N	rs3022960	**19.1213**	**0.0000**	**16.5559**	**0.0000**	**4.2802**	**0.0048**	**18.2525**	**0.0000**
03-073214161-M	rs3681493	**27.3309**	**0.0000**	**21.1722**	**0.0000**	**4.5504**	**0.0026**	**26.2591**	**0.0000**
03-098415492-M	**rs3672755**	**27.3627**	**0.0000**	**20.4602**	**0.0000**	**3.4433**	**0.0282**	**27.3289**	**0.0000**
03-115468851-N	rs4224164	**15.4721**	**0.0000**	**12.4746**	**0.0000**	2.8619	0.0915	**15.2473**	**0.0000**
03-124103177-M	rs3720182	**9.2841**	**0.0000**	**7.5783**	**0.0000**	1.6792	0.7177	**9.1392**	**0.0000**
03-136177436-M	rs4136498	3.2830	0.1737	**3.2409**	**0.0432**	1.7365	0.6686	2.5317	0.1784

Table 1: LOD scores and corrected p-values for the survival analysis under the Weibull model for the (AcB61 x 129S6)F2 mice. All results with a corrected p value < 0.1 are listed. Results with a corrected p value < 0.05 are shown in bold as are the markers most strongly associated with survival. The complete list of results and the physical position of the markers can be found on Supplemental Table 1.

Table 2

Marker		Full model		Additive model		Recessive model		Dominant model	
JAX SNP ID or Mit marker	rs#	LOD score	Corrected p-values	LOD score	Corrected p-values	LOD score	Corrected p-values	LOD score	Corrected p-values
07-096980068-M	**rs3726275**	**6.0708**	**0.0300**	4.3444	0.0610	**5.8028**	**0.0076**	1.1279	0.9999
12-107541607-M	rs3682260	4.2167	0.3062	2.1592	0.8089	4.1792	0.0763	0.1484	1.0000
14-055172074-M	**rs3659450**	5.4586	0.0637	**4.9732**	**0.0247**	**4.8115**	**0.0289**	2.1844	0.7992
14-080955404-M	rs3699634	4.6296	0.1883	4.4264	0.0535	3.7842	0.1356	2.1361	0.8194
15-096231715-M	**rs3676400**	5.3904	0.0707	3.1140	0.3149	**5.3513**	**0.0134**	0.2214	1.0000
19-038092479-M	rs3695591	4.7009	0.1707	4.3620	0.0597	3.9814	0.1015	1.9641	0.8935

Table 2: LOD scores and corrected p-values for the survival analysis under the Weibull model for the (AcB64 x DBA/2J)F2 mice. All results with a corrected p value < 0.1 are listed. Results with a corrected p value < 0.05 are shown in bold as are the markers most strongly associated with survival. The complete list of results and the physical position of the markers can be found on Supplemental Table 2.

Supplemental Table 1

Chr	Marker ID	rs #	Position (Mb)	LOD scores				Corrected p-values			
				full	additive	recessive	dominant	full	additive	recessive	dominant
1	01-009072542-M	rs3714728	9.04	0.497	0.008	0.202	0.125	1.000	1.000	1.000	1.000
1	D1Mit212		40.00	1.120	0.145	0.057	0.772	1.000	1.000	1.000	1.000
1	01-072993159-M	rs3716722	73.00	0.389	0.003	0.110	0.159	1.000	1.000	1.000	1.000
1	01-102073421-M	rs3720366	101.80	0.058	0.035	0.058	0.004	1.000	1.000	1.000	1.000
1	01-129114100-M	rs3661721	128.58	0.210	0.051	0.177	0.002	1.000	1.000	1.000	1.000
1	01-162977516-M	rs3706326	162.45	0.387	0.381	0.192	0.300	1.000	1.000	1.000	1.000
1	D1Mit116		178.00	0.462	0.357	0.088	0.455	1.000	1.000	1.000	1.000
1	01-193173300-M	rs3715125	192.35	1.492	1.150	0.343	1.458	0.999	0.985	1.000	0.872
2	02-012171555-M	rs4137557	12.06	0.095	0.086	0.086	0.033	1.000	1.000	1.000	1.000
2	02-050206336-M	rs3684870	50.47	0.027	0.027	0.021	0.015	1.000	1.000	1.000	1.000
2	02-072065558-N	rs4223212	72.22	0.175	0.027	0.125	0.008	1.000	1.000	1.000	1.000
2	02-108953011-M	rs3657882	109.55	1.534	0.424	1.324	0.010	0.998	1.000	0.940	1.000
2	02-122995884-M	rs3726142	123.70	1.904	0.810	1.817	0.015	0.946	1.000	0.601	1.000
2	02-133158154-M	rs3724080	133.80	2.002	1.541	1.940	0.422	0.909	0.817	0.505	1.000
2	02-145828186-N	rs3023694	146.47	3.000	2.932	2.223	1.694	0.282	0.079	0.313	0.704
2	02-148389584-N	rs3022940	149.10	2.915	2.864	2.119	1.707	0.326	0.092	0.380	0.694
2	02-154428186-N	rs4223578	155.50	3.303	3.247	2.394	1.932	0.167	0.043	0.226	0.513
2	02-162979695-M	rs3700147	163.90	4.739	4.603	1.928	4.017	0.012	0.002	0.514	0.011
2	02-167943959-M	rs3687512	168.80	5.480	5.436	3.544	3.550	0.003	0.001	0.023	0.024
2	02-172943830-M	rs3710327	173.66	5.196	5.190	2.838	3.993	0.005	0.001	0.097	0.011
2	D2Mit457		180.00	3.665	3.649	2.351	2.480	0.087	0.018	0.246	0.196
2	02-179086475-M	rs3680965	180.20	3.390	3.366	2.304	2.244	0.144	0.033	0.269	0.303
3	03-007561998-N	rs4223706	7.58	1.247	0.973	1.235	0.219	1.000	0.998	0.970	1.000
3	03-033933315-N	rs4223883	33.92	3.857	3.755	1.836	3.155	0.063	0.014	0.586	0.053
3	03-065861493-N	rs3022960	65.68	19.121	16.556	4.280	18.253	0.000	0.000	0.005	0.000
3	03-073214161-M	rs3681493	73.50	27.331	21.172	4.550	26.259	0.000	0.000	0.003	0.000
3	03-098415492-M	rs3672755	98.40	27.363	20.460	3.443	27.329	0.000	0.000	0.028	0.000
3	03-115468851-N	rs4224164	115.60	15.472	12.475	2.862	15.247	0.000	0.000	0.092	0.000
3	03-124103177-M	rs3720182	124.25	9.284	7.578	1.679	9.139	0.000	0.000	0.718	0.000
3	03-136177436-M	rs4136498	136.30	3.283	3.241	1.737	2.532	0.174	0.043	0.669	0.178
3	03-157197990-M	rs3697892	157.31	1.871	1.306	1.861	0.255	0.956	0.950	0.567	1.000
4	04-007200424-M	rs3692198	7.18	0.457	0.365	0.075	0.451	1.000	1.000	1.000	1.000
4	D4Mit41		35.00	0.154	0.056	0.144	0.001	1.000	1.000	1.000	1.000

4	04-067425585-N	rs3023981	67.53	0.142	0.067	0.136	0.002	1.000	1.000	1.000	1.000
4	04-101002669-M	rs3672377	101.27	0.103	0.063	0.103	0.008	1.000	1.000	1.000	1.000
4	D4Mit308		123.00	0.217	0.150	0.033	0.216	1.000	1.000	1.000	1.000
4	04-141084977-M	rs3718220	141.48	0.385	0.003	0.103	0.154	1.000	1.000	1.000	1.000
5	05-015030133-M	rs3692583	14.73	2.245	0.698	0.011	1.981	0.773	1.000	1.000	0.471
5	05-038394376-M	rs3716546	38.10	0.975	0.971	0.642	0.610	1.000	0.998	1.000	1.000
5	05-088937905-M	rs3690045	88.60	0.109	0.001	0.047	0.026	1.000	1.000	1.000	1.000
5	05-113995253-M	rs3668084	113.26	0.105	0.025	0.002	0.085	1.000	1.000	1.000	1.000
5		rs3701266	125.85	1.217	0.606	1.175	0.028	1.000	1.000	0.983	1.000
5	D5Mit244		127.00	1.604	0.944	1.588	0.101	0.994	0.999	0.784	1.000
5		rs3661159	129.80	1.452	0.994	1.452	0.162	0.999	0.997	0.875	1.000
5	05-136184664-M	rs3141573	135.00	1.686	1.357	1.646	0.299	0.990	0.927	0.742	1.000
5	05-144239610-M	rs3716217	142.80	1.239	0.662	1.237	0.047	1.000	1.000	0.970	1.000
5	05-147904991-M	rs3710365	146.77	1.587	1.211	1.575	0.189	0.996	0.975	0.795	1.000
5		rs6257511	150.30	1.407	1.218	1.340	0.321	1.000	0.974	0.932	1.000
6	06-008096966-M	rs3656818	8.09	0.804	0.618	0.790	0.157	1.000	1.000	1.000	1.000
6	D6Mit346		19.74	0.302	0.232	0.297	0.060	1.000	1.000	1.000	1.000
6	06-052633670-N	rs3023069	52.29	0.074	0.002	0.013	0.039	1.000	1.000	1.000	1.000
6	06-080041434-M	rs3725568	79.60	1.267	0.110	0.765	0.138	1.000	1.000	1.000	1.000
6	06-090142535-M	rs3708822	89.83	0.904	0.735	0.891	0.195	1.000	1.000	1.000	1.000
6	06-098102692-N	rs3023081	97.90	1.086	1.058	0.922	0.517	1.000	0.994	0.999	1.000
6	06-107618320-N	rs4226165	107.40	0.737	0.651	0.706	0.229	1.000	1.000	1.000	1.000
6	06-115164803-M	rs3711196	115.06	1.277	1.132	1.212	0.400	1.000	0.989	0.976	1.000
6	06-121280548-M	rs3710839	121.20	0.304	0.303	0.195	0.222	1.000	1.000	1.000	1.000
6	06-131929438-M	rs3721822	131.80	1.029	0.897	0.361	0.964	1.000	1.000	1.000	0.999
6	06-139951073-M	rs3711613	139.90	1.420	1.134	0.363	1.386	1.000	0.988	1.000	0.911
7	07-007210802-M	rs3673010	7.04	0.016	0.009	0.001	0.016	1.000	1.000	1.000	1.000
7	D7Mit117		25.34	0.080	0.005	0.044	0.011	1.000	1.000	1.000	1.000
7	07-039020313-M	rs3680765	38.02	1.006	0.264	0.835	0.010	1.000	1.000	1.000	1.000
7	07-073958894-N	rs3023147	72.66	0.570	0.424	0.567	0.083	1.000	1.000	1.000	1.000
7	07-096980068-M	rs3726275	95.65	0.931	0.296	0.833	0.001	1.000	1.000	1.000	1.000
7	07-127943093-M	rs3690034	126.85	0.850	0.003	0.239	0.343	1.000	1.000	1.000	1.000
8	08-006942658-M	rs3661145	6.99	0.688	0.205	0.007	0.568	1.000	1.000	1.000	1.000
8	D8Mit4		30.86	0.262	0.137	0.261	0.014	1.000	1.000	1.000	1.000
8	08-065245444-M	rs3694940	64.83	0.805	0.738	0.702	0.315	1.000	1.000	1.000	1.000
8	08-090088021-M	rs3693131	89.67	1.623	0.227	1.195	0.102	0.993	1.000	0.979	1.000

8	08-123825384-N	rs4227443	123.44	0.664	0.282	0.617	0.001	1.000	1.000	1.000	1.000
9	09-016198772-M	rs3719348	16.06	0.114	0.113	0.062	0.089	1.000	1.000	1.000	1.000
9	09-044591533-N	rs3023211	44.38	1.379	0.422	0.000	1.226	1.000	1.000	1.000	0.971
9	09-063923771-M	rs3656848	63.55	0.959	0.522	0.046	0.946	1.000	1.000	1.000	0.999
9	09-095891774-N	rs3023143	95.35	0.693	0.572	0.675	0.181	1.000	1.000	1.000	1.000
9	09-123708875-M	rs3713370	122.57	0.267	0.142	0.260	0.010	1.000	1.000	1.000	1.000
10	10-010187387-M	rs3663269	10.03	0.008	0.005	0.001	0.008	1.000	1.000	1.000	1.000
10	10-043132794-M	rs3702010	42.88	0.073	0.019	0.059	0.001	1.000	1.000	1.000	1.000
10	10-086567143-M	rs3687328	85.92	0.006	0.001	0.000	0.003	1.000	1.000	1.000	1.000
10	10-121549764-M	rs3653850	120.87	0.772	0.155	0.014	0.600	1.000	1.000	1.000	1.000
11	11-005661856-N	rs4222040	5.66	0.213	0.071	0.000	0.185	1.000	1.000	1.000	1.000
11	11-019956728-M	rs3707185	20.06	0.513	0.011	0.119	0.225	1.000	1.000	1.000	1.000
11	11-054801294-M	rs3695837	54.92	0.595	0.232	0.001	0.550	1.000	1.000	1.000	1.000
11	11-075291642-M	rs3691128	75.46	0.437	0.016	0.076	0.220	1.000	1.000	1.000	1.000
11	11-102928307-N	rs4229101	102.99	0.378	0.104	0.002	0.310	1.000	1.000	1.000	1.000
11	11-122195777-M	rs3675603	122.14	0.264	0.247	0.237	0.096	1.000	1.000	1.000	1.000
12	12-008348907-M	rs4139707	8.35	0.613	0.175	0.002	0.525	1.000	1.000	1.000	1.000
12	12-040444748-M	rs3668360	40.33	0.754	0.273	0.004	0.681	1.000	1.000	1.000	1.000
12	12-070670478-M	rs3663221	70.67	0.364	0.000	0.110	0.155	1.000	1.000	1.000	1.000
12	12-092440423-M	rs3719660	92.30	0.550	0.050	0.057	0.356	1.000	1.000	1.000	1.000
12	12-107541607-M	rs3682260	107.32	0.248	0.033	0.014	0.177	1.000	1.000	1.000	1.000
13	13-009820324-M	rs3719002	9.88	2.366	1.978	2.256	0.749	0.689	0.483	0.297	1.000
13	13-013772045-M	rs3680731	13.80	2.296	1.706	2.269	0.507	0.739	0.696	0.290	1.000
13	13-035991657-N	rs3090767	36.01	1.000	0.943	0.886	0.450	1.000	0.999	1.000	1.000
13	13-052612410-N	rs4229748	52.40	0.509	0.499	0.290	0.402	1.000	1.000	1.000	1.000
13	13-083172977-M	rs3681496	82.33	0.113	0.104	0.047	0.102	1.000	1.000	1.000	1.000
13	13-113945175-M	rs3694860	113.08	0.108	0.030	0.000	0.090	1.000	1.000	1.000	1.000
14	14-026281846-N	rs4230248	25.91	0.444	0.059	0.034	0.303	1.000	1.000	1.000	1.000
14	14-055172074-M	rs3659450	54.70	0.312	0.261	0.067	0.304	1.000	1.000	1.000	1.000
14	14-080955404-M	rs3699634	80.45	0.116	0.007	0.066	0.015	1.000	1.000	1.000	1.000
14	14-114403652-M	rs3685710	114.23	0.307	0.285	0.265	0.126	1.000	1.000	1.000	1.000
15	15-004024569-M	rs4137670	4.02	0.385	0.342	0.360	0.121	1.000	1.000	1.000	1.000
15	15-033125499-M	rs3720676	32.99	0.450	0.274	0.040	0.445	1.000	1.000	1.000	1.000
15	15-052940678-M	rs3653403	52.69	0.465	0.392	0.132	0.453	1.000	1.000	1.000	1.000
15	15-074039561-M	rs3672870	73.78	1.262	0.963	1.242	0.255	1.000	0.998	0.969	1.000
15	15-103221933-M	rs3665905	102.70	0.161	0.046	0.140	0.000	1.000	1.000	1.000	1.000

16	16-006018464-C	rs4155455	6.02	0.373	0.360	0.310	0.180	1.000	1.000	1.000	1.000
16	D16Mit103			0.361	0.012	0.185	0.065	1.000	1.000	1.000	1.000
16	16-061226828-C	rs4193065	61.31	0.409	0.068	0.304	0.016	1.000	1.000	1.000	1.000
16	16-090091107-C	rs4216475	90.03	0.011	0.001	0.008	0.001	1.000	1.000	1.000	1.000
17	17-004147924-M	rs3667161	4.18	0.828	0.008	0.329	0.264	1.000	1.000	1.000	1.000
17	17-039842179-N	rs3690398	39.09	0.465	0.398	0.159	0.418	1.000	1.000	1.000	1.000
17	17-071150883-N	rs3022791	70.53	0.310	0.216	0.310	0.031	1.000	1.000	1.000	1.000
17	17-092673068-N	rs3023460	92.03	0.374	0.208	0.028	0.368	1.000	1.000	1.000	1.000
18	18-005066417-M	rs3706767	5.18	0.384	0.238	0.380	0.033	1.000	1.000	1.000	1.000
18	18-032061772-M	rs3666799	32.17	0.879	0.391	0.011	0.838	1.000	1.000	1.000	1.000
18	18-056903229-M	rs3721446	56.99	0.340	0.330	0.175	0.269	1.000	1.000	1.000	1.000
18	D18Mit186		72.00	1.022	1.006	0.584	0.728	1.000	0.997	1.000	1.000
18	18-080151519-M	rs3656292	79.93	0.981	0.980	0.670	0.617	1.000	0.998	1.000	1.000
19	19-005218596-N	rs4232023	5.07	0.264	0.237	0.077	0.252	1.000	1.000	1.000	1.000
19	19-018239318-M	rs3691881	17.88	0.139	0.092	0.139	0.017	1.000	1.000	1.000	1.000
19	19-038092479-M	rs3695591	37.79	0.793	0.696	0.240	0.748	1.000	1.000	1.000	1.000
19	19-057152618-M	rs3703896	56.90	1.285	0.994	0.263	1.261	1.000	0.997	1.000	0.961
X	X-008280846-M	rs3653863	8.97	0.429	0.428	0.342	0.337	1.000	1.000	1.000	1.000
X	X-048681421-M	rs3695410	49.75	0.918	0.917	0.706	0.774	1.000	0.999	1.000	1.000
X	X-076336029-M	rs3685806	78.31	0.739	0.737	0.557	0.632	1.000	1.000	1.000	1.000
X	X-100282424-M	rs3683627	102.65	0.160	0.156	0.146	0.112	1.000	1.000	1.000	1.000
X	X-132656933-M	rs3705296	135.48	0.353	0.298	0.353	0.134	1.000	1.000	1.000	1.000

Supplemental Table 1: Markers ID, physical position and associated LOD scores and corrected p-values for the (AcB61 x 129S6)F2 cross. The LOD scores were obtained following one locus mapping for the survival phenotype using a parametric survival regression at the markers under a Weibull model for the survival time distribution. The significance was assessed via 10 000 bootstrapped resamples controlling the for genome wide type 1 error. The values obtained under the various models are shown.

Supplemental Table 2

Chr	Marker ID	rs#	Position (Mb)	LOD scores				Corrected p-values			
				full	add	res	dom	full	add	res	dom
1	01-009072542-M	rs3714728	9.04	0.944	0.911	0.743	0.455	1.000	1.000	1.000	1.000
1	D1Mit212		40.20	0.232	0.217	0.200	0.084	1.000	1.000	1.000	1.000
1	01-072993159-M	rs3716722	73.00	0.610	0.452	0.601	0.093	1.000	1.000	1.000	1.000
1	01-102073421-M	rs3720366	101.80	0.428	0.102	0.004	0.360	1.000	1.000	1.000	1.000
1	01-129114100-M	rs3661721	128.58	0.089	0.062	0.089	0.011	1.000	1.000	1.000	1.000
1	01-162977516-M	rs3706326	162.45	0.020	0.017	0.020	0.005	1.000	1.000	1.000	1.000
1	01-193173300-M	rs3715125	192.35	1.682	1.646	1.261	0.887	1.000	0.976	0.999	1.000
2	02-020414303-M	rs3677975	20.55	0.182	0.061	0.001	0.162	1.000	1.000	1.000	1.000
2		rs4223189	62.62	0.525	0.070	0.019	0.394	1.000	1.000	1.000	1.000
2	02-072065558-N	rs4223212	72.22	0.398	0.138	0.001	0.377	1.000	1.000	1.000	1.000
2	02-098132688-M	rs3692748	98.44	1.517	0.080	0.181	0.984	1.000	1.000	1.000	1.000
2	02-145828186-N	rs3023694	146.47	1.003	0.090	0.091	0.668	1.000	1.000	1.000	1.000
2	02-172943830-M	rs3710327	173.66	0.080	0.052	0.080	0.008	1.000	1.000	1.000	1.000
3	03-007561998-N	rs4223706	7.58	0.242	0.235	0.192	0.126	1.000	1.000	1.000	1.000
3	03-033933315-N	rs4223883	33.92	0.421	0.353	0.396	0.126	1.000	1.000	1.000	1.000
3	03-065861493-N	rs3022960	65.68	0.931	0.896	0.769	0.463	1.000	1.000	1.000	1.000
3	03-100489838-M	rs3163371	100.68	3.295	2.788	3.043	0.897	0.722	0.467	0.363	1.000
3	03-124103177-M	rs3720182	124.25	3.876	3.864	2.615	2.393	0.443	0.118	0.564	0.683
3	03-157197990-M	rs3697892	157.31	1.408	1.368	0.725	1.124	1.000	0.997	1.000	1.000
4	04-007200424-M	rs3692198	7.18	0.749	0.507	0.087	0.748	1.000	1.000	1.000	1.000
4	04-043070534-M	rs3725792	43.08	0.799	0.795	0.587	0.446	1.000	1.000	1.000	1.000
4	04-067425585-N	rs3023981	67.53	2.842	1.519	2.781	0.112	0.905	0.989	0.480	1.000
4	04-101002669-M	rs3672377	101.27	1.505	1.435	1.289	0.640	1.000	0.995	0.999	1.000
4	04-141084977-M	rs3718220	141.48	0.144	0.144	0.092	0.095	1.000	1.000	1.000	1.000
5	05-015030133-M	rs3692583	14.73	0.647	0.633	0.525	0.355	1.000	1.000	1.000	1.000
5	D5Mit294		19.70	0.436	0.396	0.402	0.169	1.000	1.000	1.000	1.000
5	05-038394376-M	rs3716546	38.10	0.233	0.188	0.053	0.229	1.000	1.000	1.000	1.000
5	05-057223972-M	rs3685257	57.07	0.803	0.339	0.005	0.757	1.000	1.000	1.000	1.000
5	05-088937905-M	rs3690045	88.60	1.656	1.378	0.418	1.591	1.000	0.997	1.000	0.983
5	05-113995253-M	rs3668084	113.26	1.325	1.087	0.337	1.282	1.000	1.000	1.000	0.999
5	05-147904991-M	rs3710365	146.77	0.282	0.039	0.010	0.189	1.000	1.000	1.000	1.000
6	06-006933444-M	rs3717555	6.93	2.806	1.063	2.644	0.003	0.916	1.000	0.551	1.000
6	06-052633670-N	rs3023069	52.29	1.622	0.962	1.604	0.106	1.000	1.000	0.982	1.000

6	06-090142535-M	rs3708822	89.83	1.008	0.815	0.990	0.212	1.000	1.000	1.000	1.000
6	06-115164803-M	rs3711196	115.06	0.584	0.258	0.538	0.005	1.000	1.000	1.000	1.000
6	06-146801412-N	rs3023100	146.44	1.208	1.006	1.184	0.309	1.000	1.000	1.000	1.000
7	07-007210802-M	rs3673010	7.04	1.331	1.175	1.237	0.465	1.000	1.000	0.999	1.000
7	07-039020313-M	rs3680765	38.02	0.006	0.001	0.004	0.000	1.000	1.000	1.000	1.000
7	07-073958894-N	rs3023147	72.66	1.411	1.004	1.351	0.241	1.000	1.000	0.998	1.000
7	07-096980068-M	rs3726275	95.65	6.071	4.344	5.803	1.128	0.030	0.061	0.008	1.000
7	07-127943093-M	rs3690034	126.85	1.445	1.443	0.829	0.998	1.000	0.994	1.000	1.000
8	08-026008244-M	rs3688321	26.02	0.037	0.005	0.028	0.002	1.000	1.000	1.000	1.000
8	08-065245444-M	rs3694940	64.83	0.017	0.000	0.006	0.006	1.000	1.000	1.000	1.000
8	08-090088021-M	rs3693131	89.67	0.180	0.053	0.153	0.000	1.000	1.000	1.000	1.000
8	08-123825384-N	rs4227443	123.44	0.128	0.018	0.007	0.093	1.000	1.000	1.000	1.000
9	D9Mit204		26.70	0.574	0.148	0.000	0.463	1.000	1.000	1.000	1.000
9	09-041967507-M	rs4140117	41.76	0.093	0.001	0.039	0.023	1.000	1.000	1.000	1.000
9	09-063923771-M	rs3656848	63.55	2.489	0.515	1.952	0.022	0.979	1.000	0.902	1.000
9	09-087760212-M	rs3691725	87.21	0.551	0.142	0.463	0.003	1.000	1.000	1.000	1.000
9	09-123708875-M	rs3713370	122.57	0.385	0.248	0.385	0.038	1.000	1.000	1.000	1.000
10	10-010187387-M	rs3663269	10.03	2.993	2.185	2.971	0.420	0.857	0.798	0.394	1.000
10	10-028554348-N	rs3023233	28.32	2.820	2.308	2.709	0.645	0.912	0.733	0.518	1.000
10		rs3023241	86.99	1.036	0.136	0.758	0.064	1.000	1.000	1.000	1.000
10	10-121549764-M	rs3653850	120.87	0.641	0.640	0.440	0.393	1.000	1.000	1.000	1.000
11	11-005661856-N	rs4222040	5.66	3.037	1.780	3.028	0.242	0.840	0.946	0.370	1.000
11	11-019956728-M	rs3707185	20.06	1.009	0.975	0.474	0.869	1.000	1.000	1.000	1.000
11		rs6239937	34.81	0.032	0.012	0.030	0.000	1.000	1.000	1.000	1.000
11	11-054801294-M	rs3695837	54.92	2.493	0.304	0.225	1.691	0.979	1.000	1.000	0.971
11	D11Mit313		55.90	2.525	1.327	0.083	2.496	0.975	0.998	1.000	0.626
11	11-075291642-M	rs3691128	75.46	1.669	1.526	0.563	1.485	1.000	0.989	1.000	0.992
11	11-102928307-N	rs4229101	102.99	1.459	1.357	1.250	0.647	1.000	0.997	0.999	1.000
11	11-121181118-M	rs3726373	121.13	0.784	0.491	0.784	0.096	1.000	1.000	1.000	1.000
12	12-008348907-M	rs4139707	8.35	2.928	2.862	2.258	1.532	0.879	0.433	0.765	0.990
12	12-040444748-M	rs3668360	40.33	2.280	2.280	1.525	1.544	0.994	0.750	0.990	0.989
12	12-070670478-M	rs3663221	70.67	0.677	0.576	0.173	0.650	1.000	1.000	1.000	1.000
12	D12Mit231		96.00	1.676	1.212	1.638	0.287	1.000	1.000	0.977	1.000
12	12-107541607-M	rs3682260	107.32	4.217	2.159	4.179	0.148	0.306	0.809	0.076	1.000
13	13-004607731-N	rs3023640	4.58	0.939	0.075	0.072	0.586	1.000	1.000	1.000	1.000
13	13-035991657-N	rs3090767	36.01	1.196	0.855	0.209	1.181	1.000	1.000	1.000	1.000

13	13-052612410-N	rs4229748	52.40	0.100	0.100	0.066	0.065	1.000	1.000	1.000	1.000
13	13-083172977-M	rs3681496	82.33	0.223	0.093	0.001	0.207	1.000	1.000	1.000	1.000
13	13-113945175-M	rs3694860	113.08	0.844	0.813	0.389	0.719	1.000	1.000	1.000	1.000
14	14-026281846-N	rs4230248	25.91	1.313	0.813	1.313	0.137	1.000	1.000	0.998	1.000
14	14-055172074-M	rs3659450	54.72	5.459	4.973	4.812	2.184	0.064	0.025	0.029	0.799
14	14-080955404-M	rs3699634	80.45	4.630	4.426	3.784	2.136	0.188	0.054	0.136	0.819
14	14-114403652-M	rs3685710	114.23	3.061	2.794	2.818	1.110	0.831	0.464	0.463	1.000
15	15-004024569-M	rs4137670	4.02	0.843	0.083	0.077	0.569	1.000	1.000	1.000	1.000
15	15-033125499-M	rs3720676	32.99	0.187	0.019	0.015	0.126	1.000	1.000	1.000	1.000
15	15-052940678-M	rs3653403	52.69	0.497	0.007	0.112	0.218	1.000	1.000	1.000	1.000
15	15-074039561-M	rs3672870	73.78	0.673	0.205	0.596	0.002	1.000	1.000	1.000	1.000
15	15-096231715-M	rs3676400	95.69	5.390	3.114	5.351	0.221	0.071	0.315	0.013	1.000
16	16-006018464-C	rs4155455	6.02	0.459	0.232	0.450	0.010	1.000	1.000	1.000	1.000
16	16-027122263-C	rs4165510	27.12	0.023	0.020	0.023	0.008	1.000	1.000	1.000	1.000
16		rs3656776	72.83	1.530	1.221	0.346	1.457	1.000	1.000	1.000	0.993
16	16-090091107-C	rs4216475	90.03	0.606	0.242	0.004	0.579	1.000	1.000	1.000	1.000
17	17-013493244-M	rs3664721	12.88	0.121	0.116	0.062	0.099	1.000	1.000	1.000	1.000
17	17-039842179-N	rs3690398	39.09	1.119	0.358	0.001	1.024	1.000	1.000	1.000	1.000
17	17-071150883-N	rs3022791	70.53	0.707	0.018	0.322	0.162	1.000	1.000	1.000	1.000
17	17-092673068-N	rs3023460	92.03	2.200	2.130	1.777	1.066	0.996	0.824	0.952	1.000
18	18-011819268-M	rs3710602	11.95	1.389	0.244	0.025	0.956	1.000	1.000	1.000	1.000
18	18-056903229-M	rs3721446	56.99	0.317	0.315	0.230	0.195	1.000	1.000	1.000	1.000
18	18-080151519-M	rs3656292	79.93	0.040	0.040	0.030	0.024	1.000	1.000	1.000	1.000
19	19-005218596-N	rs4232023	5.07	3.850	3.663	1.748	3.192	0.455	0.157	0.958	0.296
19	19-023050081-M	rs3674514	22.69	3.036	3.033	1.957	2.126	0.840	0.350	0.900	0.825
19	19-038092479-M	rs3695591	37.79	4.701	4.362	3.981	1.964	0.171	0.060	0.102	0.894
19	19-057152618-M	rs3703896	56.90	0.780	0.757	0.607	0.395	1.000	1.000	1.000	1.000
X	DXMit55		5.00	0.373	0.368	0.260	0.324	1.000	1.000	1.000	1.000
X		rs13483712	7.79	0.189	0.156	0.070	0.187	1.000	1.000	1.000	1.000
X		rs13483723	19.30	0.042	0.030	0.042	0.009	1.000	1.000	1.000	1.000
X	X-048681421-M	rs3695410	49.75	0.255	0.255	0.195	0.202	1.000	1.000	1.000	1.000
X	X-076336029-M	rs3685806	78.31	1.307	1.267	1.174	0.814	1.000	0.999	1.000	1.000
X		rs13483929	101.42	0.744	0.744	0.574	0.590	1.000	1.000	1.000	1.000
X	X-142258812-M	rs3715531	145.08	1.171	0.246	0.000	0.752	1.000	1.000	1.000	1.000

Supplemental Table 2: Markers ID, physical position and associated LOD scores and corrected p-values for the (AcB64 x DBA/2J)F2 cross. The LOD scores were obtained following one locus mapping for the survival phenotype using a parametric survival regression at the markers under a Weibull model for the survival time distribution. The significance was assessed via 10 000 bootstrapped resamples controlling for the genome wide type 1 error. The values obtained under the various models are shown.

FIGURES

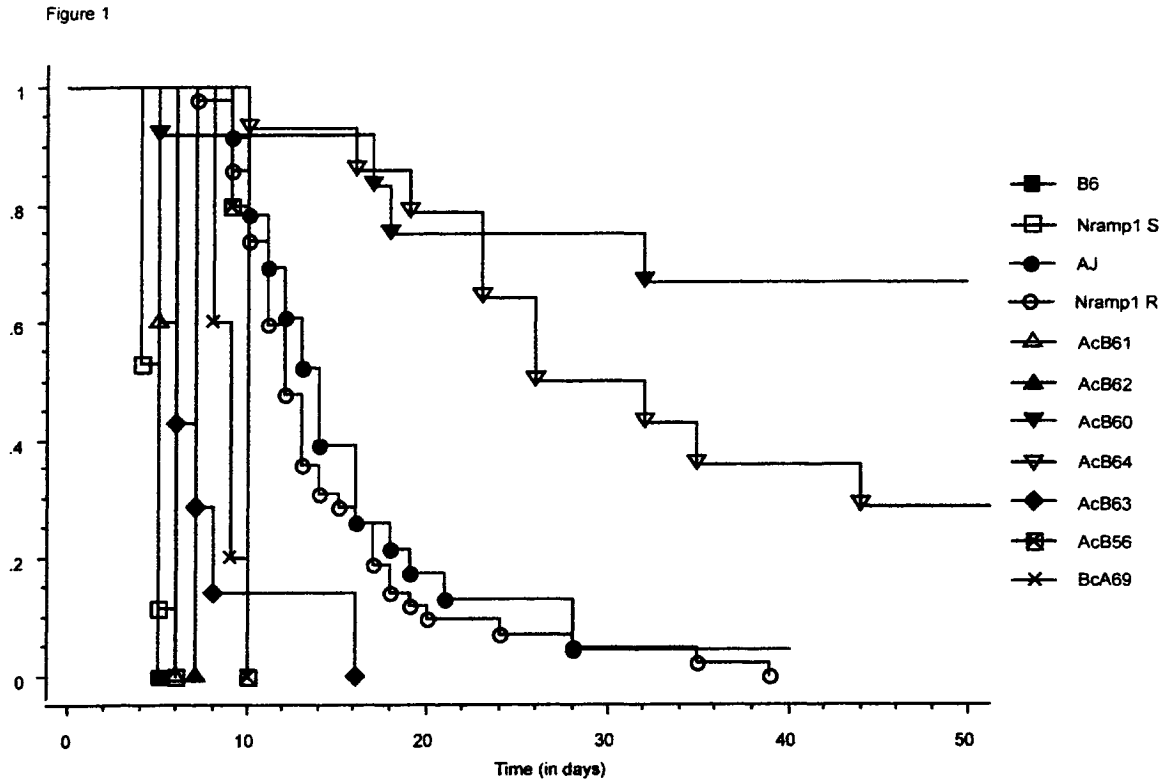


Figure 1: Cumulative survival in the AcB/BcA RCS following intravenous infection with 10^3 CFUs of *Salmonella* Typhimurium. Most of the RCS showed a phenotype that correlated with their *Nramp1* genotype with all of the *Nramp1* susceptible RCS (Nramp1 S) behaving similarly to the C57BL/6J parental strain and most of the *Nramp1* resistant RCS (Nramp1 R) behaving similarly to the A/J parental strain. However, some *Nramp1* resistant RCS showed a deviant phenotype. The AcB61 and AcB62 RCS were almost as susceptible as C57BL/6J despite carrying a resistant allele at *Nramp1*. The AcB60 and AcB64 were more resistant than their resistant parent A/J. The AcB56, AcB63 and BcA69 showed an intermediate phenotype between that of the B6 and A/J.

Figure 2

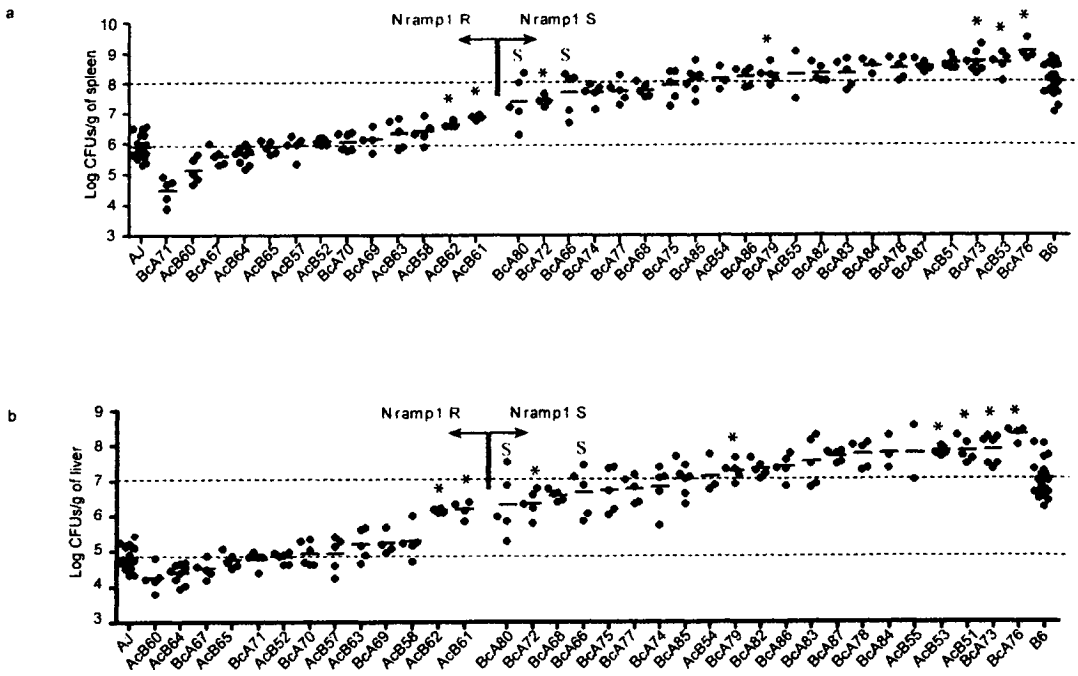


Figure 2: Bacterial load in the spleen (a) and liver (b) of the RCS at day 4 following intravenous infection with 10^3 CFUs of *Salmonella* Typhimurium. The log of CFUs per gram of spleen or liver is shown for each mouse tested and the horizontal lines represent the mean CFUs for each group. The RCS can be divided into two groups according to their known genotype at *Nramp1*. The *Nramp1* susceptible RCS (Nramp1 S) show bacterial loads that are similar to their *Nramp1* susceptible parent C57BL/6J but much higher than A/J. The *Nramp1* resistant RCS (Nramp1 R) have lower bacterial loads, similar to the A/J parental strain. Within each group however, we observe some strain-to-strain variation. Of interest, the AcB61 and AcB62 strains, which carry a resistant allele at *Nramp1*, show bacterial loads that are intermediate between that of C57BL/6J and A/J. * Indicates significantly different ($p < 0.05$) from the reference strain (A/J for the Nramp1 R group and C57BL/6J for the Nramp1 S group - ANOVA with Fisher's protected least significance difference performed in Stat View 5.0). "S" indicates a sex difference with the males having lower CFUs compared to female mice. The statistical analyses were performed on each individual infection.

Figure 3

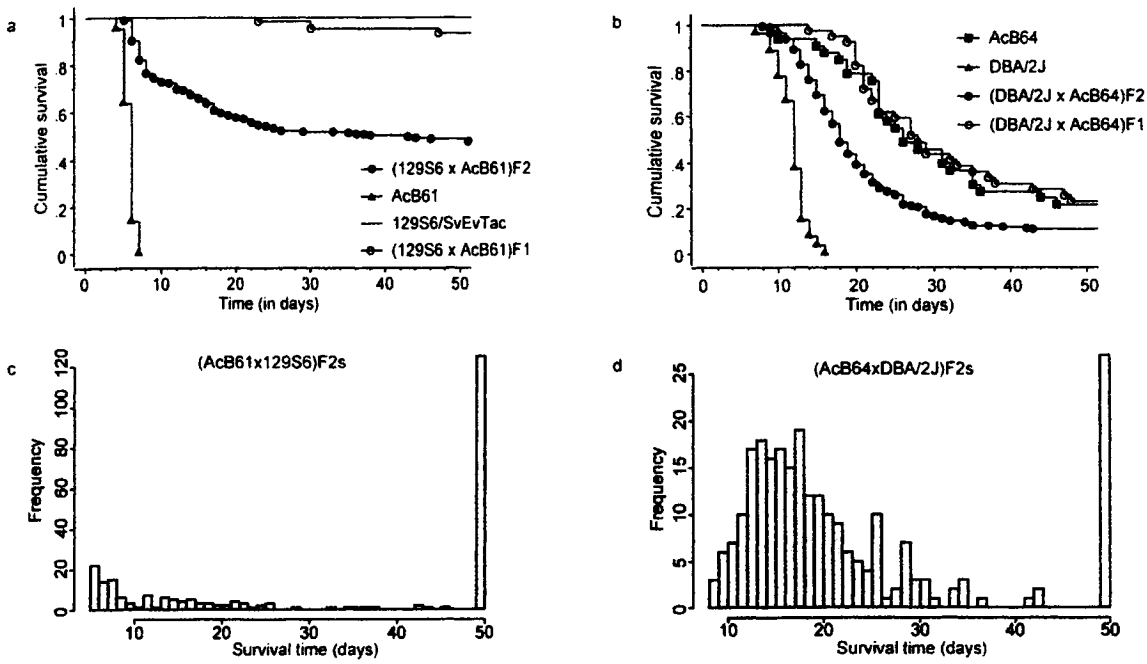


Figure 3: Cumulative survival in the F1s, F2s and the parental strains for both crosses following intravenous infection with 10^3 CFUs of *Salmonella* Typhimurium. a) (AcB61 x 129S6)F2 cross. 247 F2 animals were infected and survival was recorded. The F1s behaved as the resistant parent 129S6 while the F2 population appears to segregate more or less into three groups. Approximately 25% of the F2 mice behave similarly to the AcB61 parent and succumb early following infection. An additional 25% of the F2 mice survive for a longer period of time while 50% of the F2 are as resistant as the 129S6 parent and survive the infection. b) (AcB64 x DBA/2J)F2 cross. 249 F2 animals were infected and survival was recorded. The F1 mice behaved similarly to the resistant parent AcB64 while the F2 mice showed a continuous distribution in survival, intermediate between the two parental strains. c) Examination of the frequency distribution of the survival in the (AcB61 x 129S6)F2 mice reveals that the phenotype is not normally distributed and presents a major spike in the distribution at day 50 (censored data). d) Examination of the frequency distribution of the survival in the (AcB64 x DBA/2J)F2 mice reveals a spike in the distribution at day 50 (censored data). However, among the non-surviving mice, the phenotype is more normally distributed with a slight skew to the right.

Figure 4

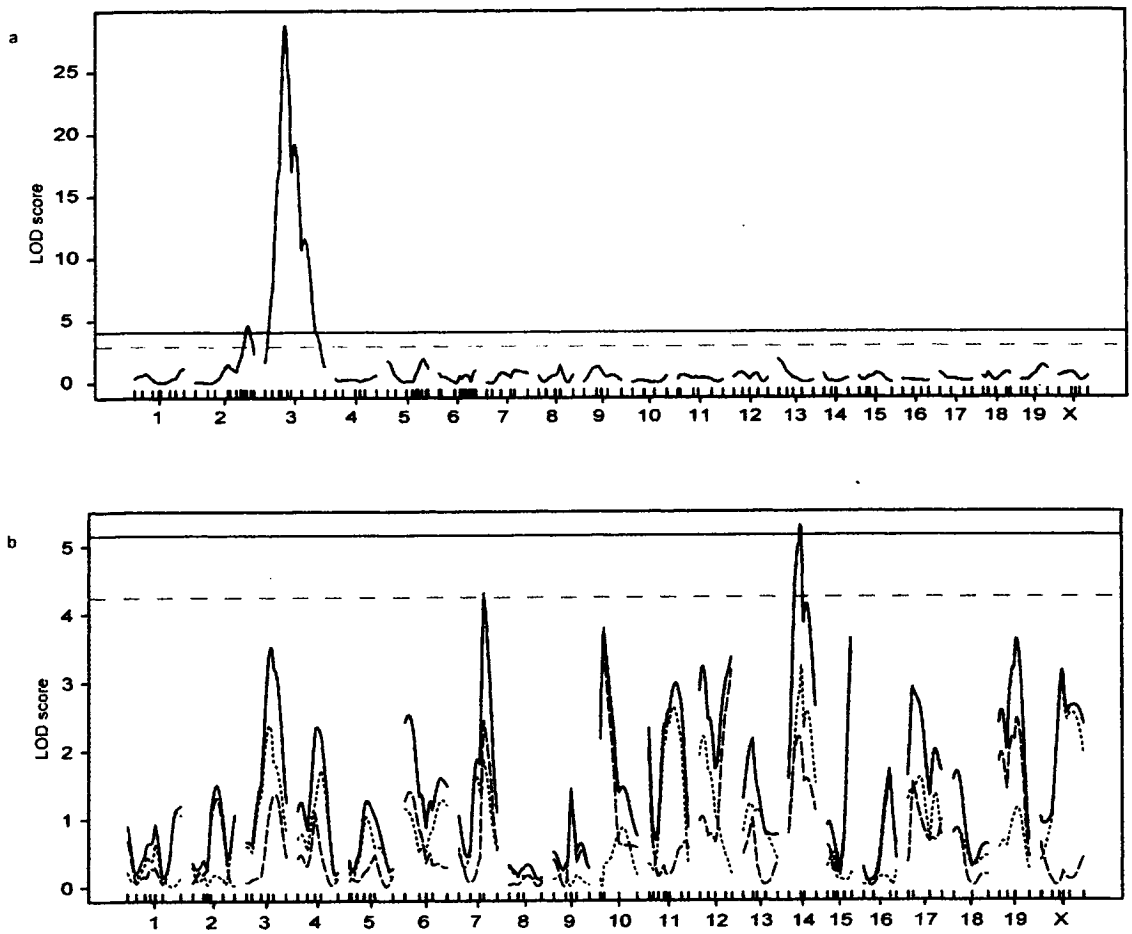


Figure 4: One locus interval mapping using a non-parametric model for the survival phenotype in the (AcB61 x 129S6) F2 mice (a) and the two-part model in (AcB64 x DBA/2J) F2 mice (b). The LOD scores are shown for each position across the genomes. In (b) the dotted line represents the LOD trace for the analysis of the survival phenotype, conditional on a non-censored phenotype (mice that did not survive the infection), the dashed line represents the LOD trace for the analysis of the binary trait (survive versus did not survive) and the continuous line is the sum of the two separate analysis. The horizontal lines represent the genome wide significance threshold after 1000 permutations (full line, p = 0.01; long dash, p = 0.05; small dash, p = 0.1). The LOD thresholds are 3.0, 3.4 and 4.2 for p = 0.1, p = 0.05 and p = 0.01 respectively for (a) and 3.9, 4.2 and 5.2 for p = 0.1, p = 0.05 and p = 0.01 respectively for (b).

Figure 5

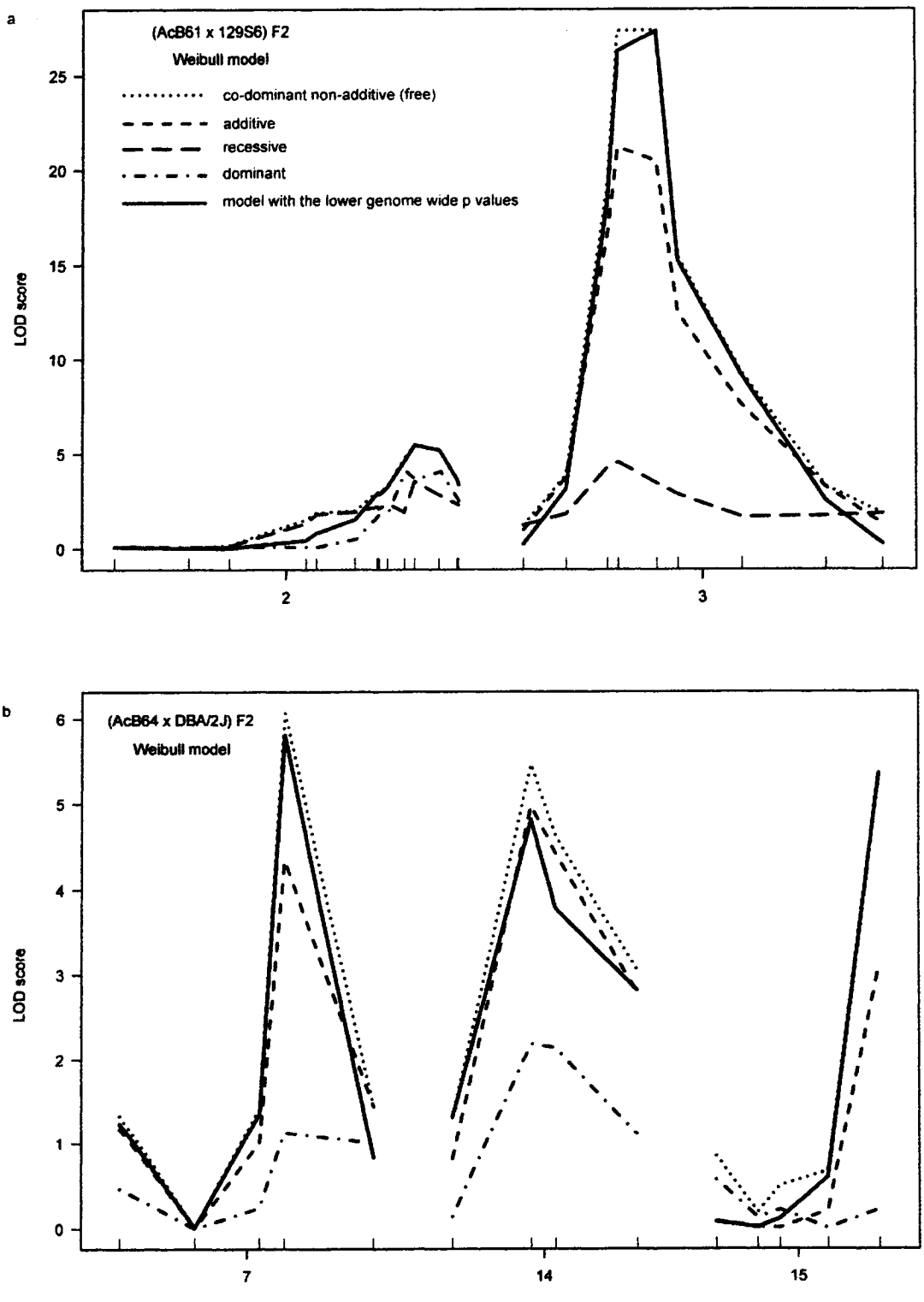

a
(AcB61 x 129S6) F2
Weibull model
co-dominant non-additive (free)
additive
recessive
dominant
model with the lower genome wide p values
LOD score
0
5
10
15
20
25
2
3
b
(AcB64 x DBA/2J) F2
Weibull model
LOD score
0
1
2
3
4
5
6
7
14
15

Figure 5 (previous page): Parametric survival regression on the markers under the Weibull distribution for the (AcB61 x 129S6) (a) and the (AcB64 x DBA/2J) (b) F2 mice. The results are shown only for the chromosomes presenting significant peaks. The lod scores obtained under the different mode of inheritance are shown. Two significant associations are found for the (AcB61 x 129S6)F2 cross (*Ity4* and *Ity5*) and three significant associations are detected for the (AcB64 x DBA/2J)F2 cross (*Ity6-Ity8*). The model with lower genome wide p values (solid line) overlays the dominant model of inheritance for chromosome 3 (a), the additive model for chromosome 2 (a) and the recessive model for chromosomes 7, 14 and 15 (b). The positions of the peaks are similar to the ones obtained with the interval mapping approach (Figure 5).

Figure 6

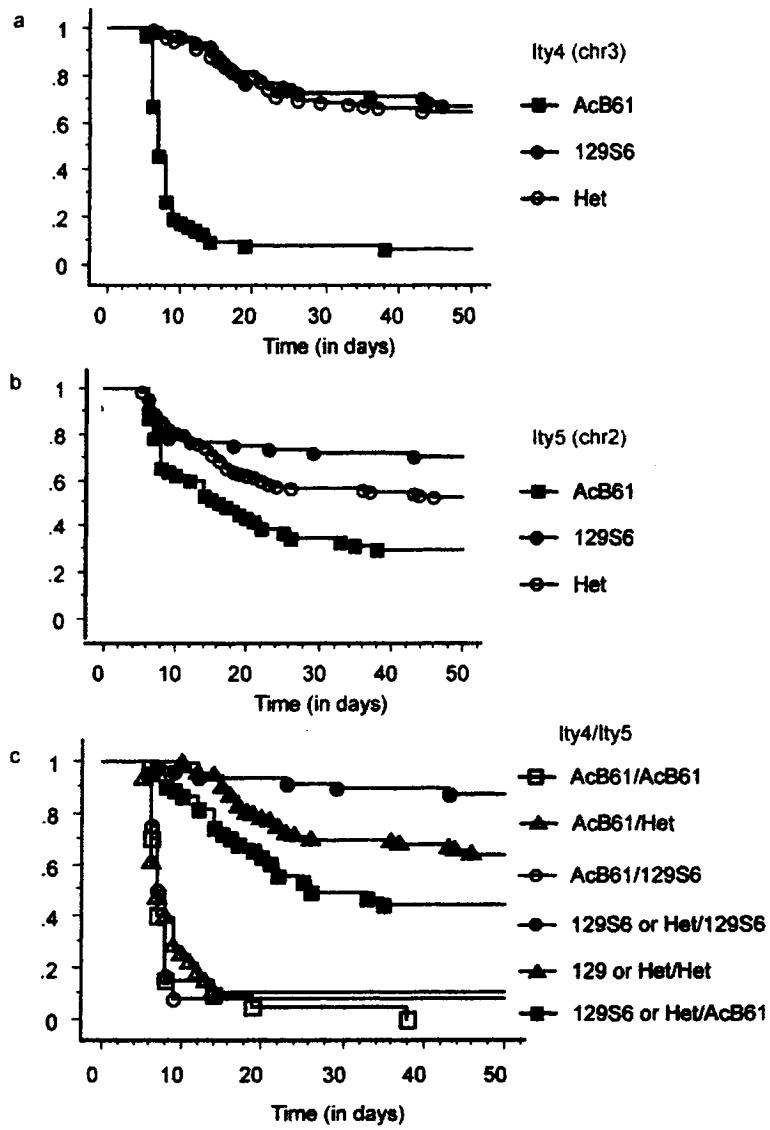


Figure 6: Cumulative survival following *Salmonella* Typhimurium infection in (AcB61 x 129S6)F2 mice according to their genotypes at *Ity4* and *Ity5*. Mice were grouped according to their genotype at the typed marker most closely associated with the peak LOD score on: a) chromosome 3 (03-098415492-M) and b) chromosome 2 (02-172943830-M). c) The mice were grouped according to their genotype at both markers. "AcB61" represents mice homozygous for the AcB61 allele. "129S6" represents mice homozygous for the 129S6 allele. "Het" represents mice heterozygous at the typed marker.

Figure 7

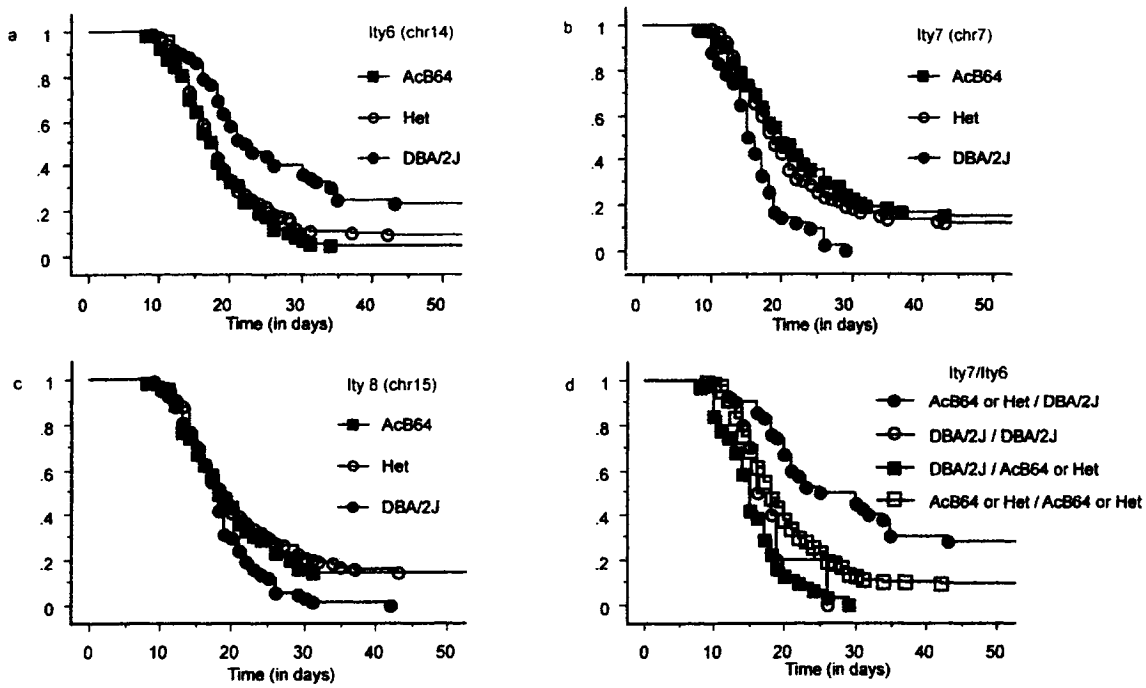
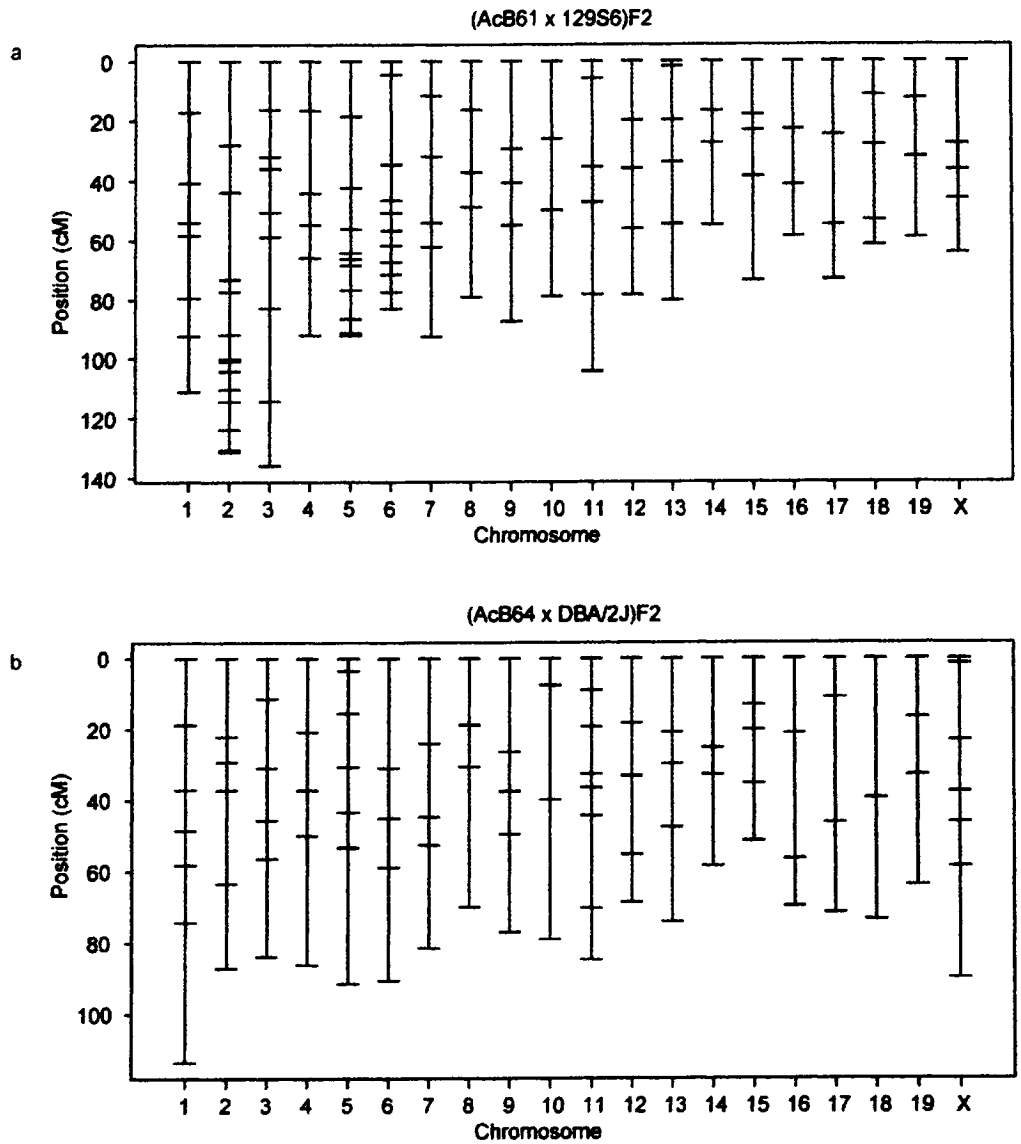


Figure 7: Cumulative survival following *Salmonella* Typhimurium infection in (AcB64 x DBA/2J)F2 mice according to their genotypes at *Ity6*, *Ity7* and *Ity8*. Mice were grouped according to their genotype at the typed marker most closely associated with the peak LOD score on: a) chromosome 14 (14-055172074-M); b) chromosome 7 (07-096980068-M); c) chromosome 15 (15-096231715-M); d) combined effect of the chromosome 7 and 14 loci. "AcB64" represents mice homozygous for the AcB64 allele. "DBA/2J" represents mice homozygous for the DBA/2J allele. "Het" represents mice heterozygous at the typed marker.

Supplemental Figure 1



Supplemental Figure 1: Genetic map of the (AcB61 x 129S6)F2 (a) and (AcB64 x DBA/2J)F2 (b) crosses. a) 247 (AcB61 x 129S6)F2 mice were genotyped with a total of 127 SNP or microsatellite markers. Among these 247 mice, 63 were genotyped only for selected chromosomes (2, 3, 5, 6 and 13). b) 249 (AcB64 x DBA/2J)F2 mice were genotyped with a total of 103 SNP or microsatellite markers. The list and physical position of the markers are available on Supplemental Tables 1 and 2.

CHAPTER IV: Pyruvate kinase deficiency confers susceptibility to *Salmonella* Typhimurium infection in mice.

PROLOGUE

The genetic analysis of the survival phenotype following *Salmonella* Typhimurium infection in (AcB61 x 129S6)F2 mice allowed identification of two novel QTL, *Ity4* and *Ity5*. *Ity4* was found to exert a major effect on the mouse response to *Salmonella*, explaining 42% of the phenotypic variance associated with survival time in this cross. The physical location of *Ity4* on mouse chromosome 3 correlates with a previously identified malaria resistance gene, the liver and red blood cell pyruvate kinase gene, *Pklr*. AcB61 is known to carry a point mutation in *Pklr*, rendering the pyruvate kinase (PK) protein nonfunctional. PK catalyses the transformation of phosphoenolpyruvate to pyruvate during glycolysis and is essential for the generation of ATP in RBCs. As a consequence of the PK deficiency, the AcB61 mice present a constitutive hemolytic anemia with reticulocytosis.

In previous experiments, we have shown that *Salmonella* infection in C57BL/10 mice leads to the development of severe anemia. Knowing that the AcB61 mice are constitutively anemic, we hypothesized that the added anemia-inducing stimuli of *Salmonella* infection would precipitate the anemia to critically low level in these mice, thereby contributing to their early demise. It was therefore conceivable that *Pklr* was indeed the gene underlying *Ity4*. In the present study, we present strong evidences in support of the candidacy of *Pklr* as the gene underlying *Ity4*. We show that PK deficient mice are more susceptible to *Salmonella* infection because of their constitutive hemolytic anemia and resultant increased body iron load.

Pyruvate kinase deficiency confers susceptibility to *Salmonella* Typhimurium infection in mice.

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Running title: Pyruvate kinase deficiency and resistance to *Salmonella*

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ABSTRACT

The mouse response to acute *Salmonella* Typhimurium infection is complex and under the influence of several genes in addition to environmental factors. In a previous study, we have identified two novel *Salmonella* susceptibility loci, *Ity4* and *Ity5*, in a (AcB61 x 129S6)F2 cross. *Ity4* maps to mouse chromosome 3 with a LOD score of 28.8 and explains 42% of the phenotypic variance. Its protective effect is associated with the 129S6 allele, which behaves in a dominant fashion. The peak LOD score associated with *Ity4* maps to the region of the liver and red blood cell specific pyruvate kinase (*Pklr*) gene, previously shown to be mutated in AcB61. During *Plasmodium chabaudi* infection, the pyruvate kinase (PK) deficiency associated with the *Pklr* mutation protects the mice against this parasite as indicated by improved survival and lower peak parasitemia. Given that red blood cell defects have previously been associated with both increased resistance to malaria and increased susceptibility to *Salmonella* we investigated the candidacy of *Pklr* as the gene underlying *Ity4*. The data presented here strongly support the candidacy of *Pklr* as a susceptibility gene to acute *Salmonella* Typhimurium infection in mice through its effect on red blood cell turnover and iron metabolism.

Key words: *Salmonella*, host resistance, anemia, iron, pyruvate kinase, infection.

Pyruvate kinase deficiency confers susceptibility to *Salmonella* Typhimurium infection in mice.

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INTRODUCTION

Infectious diseases remain a major cause of death worldwide, especially in children and young adults, with most of the burden falling on the populations of the poorest countries (www.who.int/infectious-disease-report). With emerging and re-emerging pathogens, the globalization of exchanges between countries, and the constant threat of antimicrobial resistance,[2] it becomes increasingly clear that a better understanding of the host and pathogen interactions *in vivo* is needed. One approach to this problem is the study of the genetic determinants of the host response to infection, which frequently leads to a better understanding of disease pathogenesis. The outcome of an encounter between hosts and pathogens results from the battle of two genomes in addition to environmental factors and the fitness of the two protagonists. While microbial organisms have and continue to acquire virulence factors enabling them to thrive in a particular niche,[295,296] the host genomes have accumulated polymorphisms, which at time, confer resistance or susceptibility to specific pathogens.[297-299] The importance of the host genetic makeup in the response to infection was illustrated in a seminal study by Sorenson *et al*[111] where it was shown that the relative risk of death from infectious diseases for an adoptee was significantly increased when their biological parent had died of infectious disease before age 50. Additional evidence for a major role of genetic factors comes from twin studies,[291] linkage and association studies[300,301] and numerous studies of specific gene defect conferring susceptibility or resistance to individual pathogens.[298,302]

The host response to *Salmonella* infection is also controlled by genetic factors. In humans, patients with mutations leading to sickle cell anemia,[116] chronic granulomatous disease[117] or the syndrome of Mendelian susceptibility to mycobacterial disease[119] are more susceptible to infection with non-host specific *Salmonellae*. Additionally, particular MHC haplotypes are associated with increased risk of typhoid fever (caused by the

human specific serovars Typhi and Paratyphi).[120] There are most likely several additional polymorphisms that impacts on the host response to *Salmonella* infection but their identification in human populations is hindered by the difficulties and complexities associated with human-based studies. In this context, the mouse model of typhoid fever is used to identify additional genetic factors that control the host response to Gram-negative, intracellular pathogens, factors that can later be studied in humans or relevant veterinary species.

Infection of mice with *Salmonella enterica* serovar Typhimurium (*Salmonella* Typhimurium), either orally or parenterally, results in the localization and replication of the bacteria in the spleen and liver, thereby mimicking human typhoid fever.[66] Using this model, several genes were identified as having a strong impact on the mouse susceptibility to acute *Salmonella* Typhimurium infection,[282] including *Nramp1* (Natural resistance associated macrophage protein 1, also known as *Slc11a1*) and *Tlr4* (Toll-like receptor 4). *Nramp1* is a transmembrane protein involved in the transport of divalent cations.[155,156] Following phagocytosis, it is recruited to the membrane of the phagolysosome[153] where it is believed to deprive the bacteria from essential divalent cations, including iron. Mice carrying non-functional allele at *Nramp1* are extremely susceptible to *Salmonella* infection and succumb to infection with less than 10 organisms.[121,139,140,150] *Tlr4* is the pattern recognition receptor for LPS and is responsible for most of the mouse response following infection with *Salmonella*.[264,265] Mice carrying a non-functional *Tlr4* allele show a 1000-fold reduction in LD_{50}[167,178] while increasing the number of copies of *Tlr4* from 0 to 1, 2 and 3 copies of the gene brings an incremental protective effect from death following *Salmonella* Typhimurium infection.[293]

Aiming to expand our understanding of the genetic determinants of the host response to *Salmonella* infection using the mouse model of typhoid fever, we recently undertook a systematic screening of a set of thirty-six A/J and C57BL/6J recombinant congenic strains (AcB/BcA RCS) for their response to acute *Salmonella* infection.[303] While we knew, before hand, that the parental strains, A/J and C57BL/6J, differ in their susceptibility to *Salmonella* infection mainly because C57BL/6J carries a point mutation in *Nramp1*, we hypothesized that additional genes would influence the outcome of infection and segregate in the RCS. We showed, indeed, that *Nramp1* alone is not

sufficient to explain the phenotypic variance among the RCS and that additional genes influence the response to *Salmonella.* In particular, we have identified the strains AcB61 and AcB62 as extremely susceptible to *Salmonella* Typhimurium despite the fact that they carry a functional allele at *Nramp1.* Interval mapping performed on a (129S6/SvEvTac (or 129S6) x AcB61)F2 cross revealed a major locus (*Ity4*, Immunity to Typhimurium locus 4) influencing the survival phenotype at position 44cM on mouse chromosome 3 with a LOD score of 28.8. The strains AcB61, AcB62 and AcB55 were previously found to carry a spontaneous mutation in the liver and red blood cell (RBC) specific pyruvate kinase gene, *Pklr* (Min-Oo *et al*[287] and P. Gros, personal communication). Pklr deficient mice present a constitutive hemolytic anemia with reticulocytosis and splenomegaly,[276] and are more resistant to *Plasmodium chabaudi* infection.[287] Because the position of *Pklr* is exactly under our major *Ity4* peak on chromosome 3 we evaluated its candidacy as the gene underlying the susceptibility of the AcB61 strain to *Salmonella* and characterized the phenotypic expression of the *Pklr* deficiency during infection. Additionally, we further characterized the effect of *Salmonella* infection on the mouse erythroid response. The results presented in this paper strongly support the candidacy of *Pklr* as the gene underlying *Ity4* through its effect on the mouse erythroid response and iron metabolism.

RESULTS

Evaluation of the candidacy of Pklr as the gene underlying Ity4

In a previous study, we have mapped a *Salmonella* susceptibility loci to mouse chromosome 3 in a F2 cross between the extremely susceptible AcB61 strain and the totally resistant 129S6 strain.[303] This loci, named *Ity4*, maps to position 44cM with a LOD score of 28.8, explaining 42% of the phenotypic variance. The AcB61 strain was previously shown to carry a point mutation in the *Pklr* gene, rendering it resistant to *Plasmodium chabaudi.*[287] Since *Pklr* maps directly under our chromosome 3 peak LOD score (Figure 1a) we evaluated its candidacy as the gene underlying *Ity4.*

We first genotyped 306 (AcB61 x 129S6)F2 mice for the known *Pklr* mutation and examined their survival following *Salmonella* Typhimurium infection according to their genotype at *Pklr.* Figure 1b shows a strong correlation between the mouse genotype

at *Pklr* and survival following *Salmonella* infection. Mice homozygous for the AcB61 mutated allele have very little chance of surviving the infection while mice carrying at least one 129S6 allele are much more resistant, indicating that *Pklr* or a gene tightly linked to *Pklr* confers susceptibility to *Salmonella*. Repeating the interval mapping, this time including the *Pklr* genotypes in the analysis led to a rise of the peak LOD score from 28.8[303] to 30.3 (Figure 1a). Additionally, examination of the survival following *Salmonella* infection for the three recombinant congenic strains known to carry a mutated *Pklr* allele (AcB61, AcB62 and AcB55) (Min-Oo *et al*[287] and P. Gros, personal communication) showed a concordant phenotype with all three strains being extremely susceptible (Figure 1c) while none of the other *Nramp1* resistant RCS showed such susceptibility.[303] The AcB55 strain was even more susceptible than the C57BL/6J parent with all mice dying on day 4. We attribute this extreme susceptibility to *Salmonella* to the fact that the AcB55 strain carries mutated alleles at both *Nramp1* and *Pklr*. We also observed a lack of complementation in F1 mice derived from crosses between AcB61 and AcB62, and between AcB61 and AcB55 indicating that the same gene is responsible for their susceptibility to *Salmonella* (Figure 1d). Since these three strains are known to carry the same point mutation in *Pklr*, it is likely that this gene is responsible for their susceptibility to *Salmonella* Typhimurium infection. Finally, using a fine mapping approach targeted to the mice presenting non-resolved recombination around the *Pklr* gene, we were able to decrease the support interval from a 25.3Mb region to a region of 3.7Mb just surrounding *Pklr* (Figure 2). Taken together, these results points toward *Pklr* as the gene underlying the susceptibility of the AcB61 mice.

Salmonella-induced anemia

Having accumulated strong evidence in support of *Pklr* as the gene underlying *Ity4*, we became interested in understanding how a RBC defect confers susceptibility to *Salmonella* Typhimurium. We have previously shown[293] that mice on a C57BL/10 background develop a severe anemia during the course of *Salmonella* infection. Knowing that the AcB61 mice present a constitutive anemia, we hypothesized that the added anemia-inducing stimuli of the *Salmonella* infection would lead to the rapid development of an even more severe anemia in these mice, contributing to their early demise. To test this hypothesis and also to shed light on the pathophysiology of *Salmonella*-induced

anemia in mice, we studied the RBC parameters before and during *Salmonella* Typhimurium infection in AcB61, A/J and C57BL/6J mice. As expected, the AcB61 mice present a constitutive anemia with reticulocytosis (Figure 3a-e). During *Salmonella* infection, the anemia rapidly worsens in AcB61 mice to reach critically low levels on day 5. By comparison, the RBC parameters showed only a slight decrease in the A/J (Figure 3f-j) and C57BL/6J (Figure 3k-o) parental strains. The reticulocyte response appeared blunted by *Salmonella* infection in all 3 groups (decreasing reticulocytes, Figure 3e, i, j, n, and o), which probably contributes to the development of anemia during infection. The sustained percentage of reticulocytes (Figure 3d) in AcB61 mice despite the worsening anemia and decreasing absolute reticulocyte count may indicate increased resistance of younger erythrocytes to phagocytosis by activated macrophages during *Salmonella* infection. These results suggest that *Salmonella*-induced anemia results from a slightly decreased erythropoiesis response in the face of increased phagocytosis of aging RBCs. Additionally, these findings show that *Salmonella* infection rapidly worsens the anemia in AcB61 mice and that the severity of the anemia could contribute to the early demise of these mice. In the face of severe sepsis, the low hematocrit seen in the AcB61 (mean ± SEM = 16.6 ± 1.3%) certainly interferes with the capacity of the body to maintain oxygen delivery to vital organs.[304]

The erythropoiesis response in the bone marrow was investigated in A/J, C57BL/6J and AcB61 mice before and on day 5 following *Salmonella* infection. No noticeable difference were detected between the different groups in regards to the myeloid:erythroid ratios before or during infection suggesting that the compensatory response to the decreased RBC life span in AcB61 occurs mainly in the spleen and liver as previously described.[276] Interestingly, on day 5, *Salmonella*-induced pathologies were noted. The myeloid:erythroid ratio was increased with numerous segmented granulocytes. Additionally, variable numbers of foci characterized by fibrin deposition, more or less degenerated neutrophils and necrotic cells were found. These foci were sometime angiocentric and/or associated with thrombosed vessels. In one mouse, bacteria were visible in the center of one of these foci. These findings illustrate the attempt of the mice to produce increased numbers of myeloid cells in response to acute sepsis and indicate that *Salmonella* is capable of reaching the bone marrow.

Hepcidin expression during Salmonella infection

The development of anemia during infection, referred to as anemia of inflammation, is well described and may be explained by several mechanisms including changes in iron homeostasis, decreased proliferation of erythroid progenitors, reduced erythropoietin production and decreased RBC life span.[250] The newly described protein hepcidin is an important player in iron homeostasis and a crucial link between anemia and inflammation.[251] During infection, inflammatory cytokines such as interleukin-6 are produced, leading to the up-regulation of the acute phase protein hepcidin, which in turn leads to internalization of the iron transporter ferroportin. The end result of this cascade of events is the sequestration of iron in macrophages and the diminution of absorption of alimentary iron from the duodenum. Since iron availability is crucial for RBC synthesis, these changes contribute to the development of anemia. To investigate whether hepcidin could be implicated in the development of anemia in our model, we studied its expression in the liver of our mice before and during infection. Figure 4 shows that hepcidin expression is slightly up regulated at day 5 in all three groups with significant differences detected only for AcB61 and C57BL/6J mice, the two strains that present obvious signs of disease at day 5. These results indicate that hepcidin up regulation may contribute to the development of anemia during *Salmonella* infection in mice.

Iron homeostasis during Salmonella infection

The crucial role of iron in the regulation of RBC synthesis lead us to investigate its metabolism in our three strains of mice before and during infection with *Salmonella* Typhimurium. We first measured the level of iron within the liver of our mice. As shown in Figure 5a, the level of iron in the liver of AcB61 mice is increased approximately 6 times compared to A/J or C57BL/6J, indicating that the rapid turnover of RBC in these mice somehow leads to accumulation of iron in the liver. Iron overload in human pyruvate kinase (PK) deficiency patients has been described, although usually associated with additional risk factors such as splenectomy, repeated transfusion or mutations in the primary hereditary hemochromatosis gene *HFE*.[305-307] Intracellular iron staining of sections of liver and spleen confirmed that there is increased iron stores in both organs for AcB61 compared to B6 (not shown) and A/J (Figure 6). At the time points studied, however, we could not detect an effect of *Salmonella* infection on the level

of hepatic iron. Serum iron seemed to decrease on day 3 post-infection in all three groups (significant only in A/J) followed by a significant rise on day 5 in the two most susceptible groups C57BL/6J and AcB61 (Figure 5b). The sharp rise in serum iron shortly before death in these two strains was surprising, especially in the face of increased hepcidin expression. The significance and pathogenesis of this finding is unknown but may reflect tissue necrosis with release of iron in the circulation. The circulating transferrin increased during infection in all three groups and it was usually higher in AcB61 (Figure 5c). Finally, the levels of circulating ferritin increased during infection in AcB61 and C57BL/6J and were higher in AcB61 at all time points (Figure 5d) most likely reflecting the increased iron load in these mice.

Taken together these results indicate that *Salmonella* infection impacts on the iron metabolism as indicated by the changes in serum iron, serum transferrin and serum ferritin. Additionally, these results suggest that hepatic iron overload in *Pklr* deficient AcB61 mice, as indicated by increased liver iron, increased ferritin and increased transferrin, may contribute to their increased susceptibility to *Salmonella* by providing increased access to iron to the invading intracellular pathogen. We therefore hypothesize that the susceptibility of the AcB61 mice to acute *Salmonella* Typhimurium infection is due to the rapidly worsening anemia and the increased iron levels in the spleen and liver, two direct consequences of the Pklr deficiency.

Salmonella infection in mice rendered anemic through injection of phenylhydrazine

To investigate the impact of anemia on the mouse response to *Salmonella* infection, we induced acute hemolytic anemia in A/J mice, a strain closely related to the AcB61. Phenylhydrazine, a potent oxidizing agent that causes a transient but severe hemolytic anemia with reticulocytosis, was injected intraperitoneally two days prior to *Salmonella* infection. The sole injection of phenylhydrazine was well tolerated by the mice with 100% survival (Figure 7a) and no clinically visible adverse effects. Two days following phenylhydrazine injection, the mice were severely anemic and by day 5, they showed a vigorous reticulocytosis response with improving anemia (Figure 7b-d). Mice infected with *Salmonella* two days following phenylhydrazine injection showed a dramatic increased in susceptibility compared to the controls with all mice dying on days 3 or 4 (Figure 7a). The *Salmonella* infection appeared to compromise the capacity of the

A/J mice to recover from the phenylhydrazine-induced anemia despite a seemingly adequate reticulocyte response (Figure 7b-d versus e-g), indicating again that *Salmonella* infection most likely increases the removal of RBC from the circulation.

These findings suggest that severe anemia, as seen in AcB61 or phenylhydrazine-injected A/J mice, could contribute to susceptibility to *Salmonella* infection. However, the very rapid death of the A/J mice injected with both phenylhydrazine and *Salmonella* may be due to additional effects of the phenylhydrazine and the acute hemolytic crisis. For instance, the hemolytic crisis induced by phenylhydrazine has been associated with the release of free iron and iron overload,[308] which may have favored a more rapid growth of the *Salmonellae*. Moreover, a study done in the 1960s showed that, contrary to what is observed for phenylhydrazine or anti-mouse erythrocyte antibodies-induced hemolytic anemia, bleeding of the mice to decrease their hematocrit to 30% did not increased their susceptibility to *Salmonella*.[309] Additional experiments will be needed to investigate further the role of anemia, acute hemolytic crisis and acute iron overload on mouse resistance to *Salmonella* in A/J and AcB61 mice.

Salmonella infection in iron overloaded mice

We were then interested in testing our hypothesis that the iron overload seen in the AcB61 mice is detrimental to the host in the face of *Salmonella* infection. We therefore injected A/J mice with iron dextran three times a week for three weeks before infection with *Salmonella*. The sole injection of iron was well tolerated by the mice, which showed no adverse effect from the repeated intraperitoneal iron injections. However, following *Salmonella* infection, the iron-overloaded mice showed much increased susceptibility to *Salmonella* infection compared to control mice injected with PBS (Figure 8). These results are in agreement with previous report of increased susceptibility of mice to *Salmonella* following iron overload[257,258] and indicate that the increased susceptibility of the AcB61 mice to *Salmonella* may at least partially be explained by their increased iron load.

DISCUSSION

In the present study, we investigated the candidacy of *Pklr*, the gene encoding for the liver and RBC specific pyruvate kinase, as a candidate for *Ity4*, a *Salmonella*

Typhimurium susceptibility loci previously identified by us in a (AcB61 x 129S6)F2 cross.[303] Using a fine mapping approach, we were able to reduce the interval of *Ity4* from a 25.3Mb region to a region of 3.7Mb just surrounding *Pklr*. Although this region still contains close to one hundred genes, the phenotypic correlation between the three RCS known to carry the *Pklr* mutation strongly support its candidacy. Additionally, the lack of complementation in F1 crosses derived from these strains indicate that the same gene is responsible for their increased susceptibility to *Salmonella*. Taken together, these results, along with the functional data discussed below, strongly support *Pklr* as the gene underlying *Ity4*.

The *Pklr* mutation investigated in this study was first identified in the AcB61 and AcB55 RCS as a gene conferring resistance to *Plasmodium chabaudi* infection.[287] PK is an essential enzyme for glycolysis in RBCs. Erythrocytes deficient in PK have decreased ATP, increased glycolytic intermediates such as 2,3-diphosphoglycerate, and a shortened life span.[307] As a result, humans or animals with PK deficiency show a constitutive hemolytic anemia with reticulocytosis. The mutation found in some AcB/BcA RCS arose during the breeding of the RCS and is not present in the parental strains.[287] It is associated with an isoleucine to asparagine substitution at amino acid position 90 resulting in a loss of function of the PK. When infected with *Plasmodium chabaudi*, PK deficient mice were more resistant than wild type mice as indicated by lower peak parasitemia and increased survival.

While it may first appear unexpected for a RBC defect to confer both resistance to malaria and susceptibility to *Salmonella* infection, this association is not unheard of. In fact, several hemoglobinopathies and RBC enzymatic defects are known to confer protection to malaria in human populations[310] while at the same time conferring susceptibility to various pathogens, including *Salmonella*.[116,311] A similar observation can be made in mice where, for instance, β-thalassemia is not only associated with increased resistance to malaria[310] but also with susceptibility to *Salmonella*.[312] The reasons underlying the increased susceptibility to infection in patients with RBC defects are not completely understood but may be related to the severity of the anemia, phagocyte dysfunction or iron overload.[311] Our experiments suggest that these mechanisms may also

be implicated in the increased susceptibility of the AcB61 mice to acute *Salmonella* Typhimurium infection.

Adequate tissue oxygen delivery is essential for life. Whole body oxygenation is determined by the arterial content in oxygen (dependant in part on the hemoglobin concentration), the cardiac output and the oxygen extraction ratio at the organ level. [304] During severe sepsis, such as during acute systemic *Salmonella* infection in mice, the normal physiologic mechanisms allowing adequate delivery of oxygen are compromised as a direct consequence of the systemic inflammatory response.[313] While the AcB61 mice seem perfectly adapted to live with their anemia in a normal situation, it is conceivable that the rapid decrease of their hematocrit to critically low levels during *Salmonella* infection superimposed with the sepsis-induced compromised cardiovascular function is detrimental to their survival. In this regard, we have shown that mice of a similar genetic background rendered anemic through phenylhydrazine injection are also more susceptible to *Salmonella* infection. However, a study published in 1967 showed that moderate anemia (hematocrit = 30%) induced by acute bleeding does not increase the susceptibility of mice to *Salmonella* in contrast to hemolytic anemia,[309] suggesting that the actual hemolytic nature of the anemia may be more important in increasing the susceptibility of mice to *Salmonella* than the anemia itself. The hemolytic anemia of our models (phenylhydrazine-injected and PK-deficient mice) is mainly "extravascular" and therefore, associated with phagocytosis of altered but still intact RBC by the RES. This erythrophagocytosis may by itself be related to increased susceptibility to *Salmonella* since it has been shown that erythrophagocytosis *per se* diminishes the capacity of macrophages to kill *Salmonella in vitro*.[314,315]

In addition to the adverse effect of the anemia and erythropagocytosis, the iron overload found in the AcB61 mice (and the probable acute iron release following phenylhydrazine-induced hemolytic crisis in A/J mice) most likely also contributes to their increased susceptibility to *Salmonella*. Iron is an essential nutrient to both the host and the pathogen and, during infection, they compete for this essential metal.[256] The host iron binding proteins (transferrin, ferritin, lactoferrin and hemoproteins) will limit the availability of free iron to prevent both the growth of microorganisms and tissue damage from free iron-induced reactive oxygen intermediates.[316] The normal host is a very hostile

environment for microorganisms, which require 10^{11} to 10^{12} higher concentration of free iron than what is usually available within the host. Since pathogenic microorganisms have evolved specialized mechanisms to acquire iron during infection, it is conceivable that any situation leading to iron overload may favor the multiplication of invading organisms. Indeed, iron overload in humans has been linked to increased susceptibility to some pathogens including *Mycobacterium tuberculosis*, [317] HIV, [318] fungal organisms[319] and *Listeria monocytogenes*.[320] Here, we show that A/J mice rendered iron overloaded through repeated administration of iron are markedly impaired in their resistance to *Salmonella* infection. These results are in agreement with previous studies, which have shown a role for iron overload in susceptibility to *Salmonella* infection.[257,258] The importance of iron for the growth of *Salmonella* is also illustrated by the increased resistance of mice rendered iron-deficient through dietary restrictions.[259]

Interestingly, the role of iron in bacterial growth may not be the sole factor responsible for an association between iron balance and susceptibility to infection. In fact, several studies have shown that a proper iron balance is essential to both innate and adaptive immune responses. In mice, acute iron depletion through the administration of deferoxamine, an iron chelator capable of binding both intra and extracellular iron, increased the susceptibility to *Salmonella*.[257,260] This effect was noted in both *Nramp1* resistant and *Nramp1* susceptible strains and appeared related to an impaired capacity of macrophages to restrict the growth of *Salmonella* because of impaired NADPH-dependent respiratory burst.[260] On the other hand, iron overload was shown to alter the development of TH1 response during *Candida albicans* infection in mice.[321] Iron also appears to directly impair phagocytosis as indicated by the phagocytosis defect noted in normal polymorphonuclear cells incubated with serum of thalassemic patients, a defect corrected by the addition of deferoxamine.[322] Finally, during experimental sepsis in mice, the administration of iron was associated with increased morbidity and mortality in addition to increased oxidative damage to the heart and kidneys.[323] Increased gut epithelial and splenic apoptosis resulting from the combined effect of sepsis and iron administration has been suggested to contribute to increased mortality during sepsis.[324] Our results and the findings in the above mentioned studies indicate that a fine balance of

iron is needed for optimal host defense against *Salmonella* and other pathogens, and that both iron overload and iron depletion can adversely affect the outcome of sepsis.

During the course of our experiments, we have also investigated the mechanisms underlying the development of anemia in mice during *Salmonella* infection. We have previously found that mice infected with *Salmonella* Typhimurium develop anemia, which worsens throughout the course of infection.[293] Here, we show that the development of anemia in mice during *Salmonella* infection is associated with decreased erythropoiesis as indicated by the decreasing reticulocyte count found A/J, AcB61 and C57BL/6J. During infection, circulating pro-inflammatory cytokines are believed to contribute to a decreased erythropoiesis response by a direct inhibition of the proliferation of the progenitor cell, decreased erythropoietin secretion and decreased sensitivity to the effect of erythropoietin.[250] The decreased availability of iron, because of sequestration in the RES may also contribute to the decreased erythropoiesis. The acute phase protein hepcidin plays a central role in the regulation of iron and in the development of anemia of inflammation,[251] and it was increased in our mice at day 5. Other factors that may have contributed to the rapid development of anemia in our mice during *Salmonella* infection include the relatively short half-life of RBC in normal mice (60 days in mice compared to 120 days in human) and the increased phagocytosis of the older RBCs by the activated macrophages of the RES. Evidence for this statement comes from the observation that in AcB61 mice, the percentage of reticulocytes remains high throughout infection in the face of worsening anemia and decreasing absolute reticulocyte counts, indicating increased resistance of the reticulocytes compared to mature RBC and an overall increased destruction of the RBC. Additionally, in phenylhydrazine-treated, *Salmonella*-infected mice, the anemia persists despite a reticulocyte response that is equivalent to what is seen in phenylhydrazine treated, non-infected mice, again suggesting increased RBC destruction during *Salmonella* infection. Taken together, these findings indicate that the pathogenesis of *Salmonella* infection includes both decreased erythropoiesis and increased RBC removal from the circulation.

In conclusion, we presented strong evidences in support of the candidacy of *Pklr* as the gene underlying *Ity4*. As seen with other RBC pathologies, the PK deficiency in mice is both protective against malaria and detrimental in regards to acute infection with

Salmonella Typhimurium. The reasons underlying this increased susceptibility are probably numerous but include the severity of the anemia and the iron overload found in these mice. Finally, mouse infection with *Salmonella* Typhimurium induces anemia through decreased proliferation of RBC progenitors and increased destruction of aging erythrocytes.

MATERIALS AND METHODS

Animals used: All animal experiments were performed under conditions specified by the Canadian Council on Animal Care. AcB61, AcB62 and AcB55 were generated from A/J and C57BL/6J through reciprocal double backcrosses followed by inbreeding for several generations.[285] These mice were either purchased from Emerillon Therapeutics (Montreal, Québec, Canada) or bred by us at the Montreal General Hospital Research Institute (MGHRI) animal facility. A/J and C57BL/6J were purchased from the Jackson Laboratory (Bar Harbor, ME, USA) while the 129S6/SvEvTac (129S6) mice were purchased from Taconic (Hudson, NY, USA). (AcB61 x 129S6)F2 (described in Roy *et al*[303]), (AcB61 x AcB62)F1 and (AcB61 x AcB55)F1 mice were generated at the MGHRI animal facility.

***In vivo Salmonella* infection:** The *Salmonella* infections were performed as previously described.[293] Briefly, *Salmonella* Typhimurium strain Keller was grown in trypticase soy broth and each mouse was infected intravenously with approximately 10^3 colony forming units (CFUs) diluted in 200ul of 0.9% saline. The infectious dose was verified by plating of serial dilutions on trypticase soy agar. For survival analysis, the mice were monitored twice daily and moribund animals were sacrificed by CO_2 asphyxiation. The survival data for the (AcB61 x 129S6)F2 mice are described elsewhere.[303] All mice were aged between 2 and 6 months at the time of infection.

Genotyping: DNA was extracted from 3mm biopsy samples of mice tails using overnight digestion in lysis buffer and proteinase K, followed by a chloroform extraction. Genotyping for the *Pklr* mutation was performed using restriction enzyme digestion as previously described.[287] *Pklr* exon 2 specific primers surrounding the *Pklr* mutations were used to amplify genomic DNA. The PCR products were subjected to *Sfa*N1 restriction enzyme digestion and then resolved on ethidium bromide-stained 1.5% agarose

gel. The Pklr mutation disrupts the *Sfa*N1 restriction site and therefore *Sfa*N1 digestion can be used to correctly identify the genotype of the mice at Pklr. Fine mapping around Pklr was performed by sequencing of PCR amplified DNA fragments of 400 to 500 bp surrounding known single nucleotide polymorphisms (SNPs) polymorphic between 129S6 and A/J. The sequencing was performed at the McGill University and Genome Quebec Innovation Center (Montreal, QC, Canada).

Genetic analysis: The genetic analysis for the survival phenotype in (AcB61 x 129S6)F2 mice is reported elsewhere.[303] Here, we repeated the one locus interval mapping for the survival phenotype of the (AcB61 x 129S6)F2 mice in R/qtl[288] under a non-parametric model this time including the direct genotyping data for the *Pklr* mutation.

Hematologies and iron studies: For complete blood counts, mice were sacrificed by CO_2 asphyxiation at various time points, blood was collected by cardiac puncture and immediately transferred to pediatric 200ul EDTA tubes. Analyses were performed at the Faculté de médicine vétérinaire of the Université de Montréal (St-Hyacinthe, Québec, Canada) under the supervision and with the collaboration of Dr C. Bédard. Bone marrow sections from the femur and sternum were also evaluated at the Faculté de médicine vétérinaire of the Université de Montréal by Dr P. Hélie. For iron studies, mice were sacrificed by CO_2 asphyxiation at various time points, blood was collected by cardiac puncture and allowed to clot at room temperature for 2 hours. Serum was harvested following centrifugation and kept at -80°C for future analysis. Liver sections were harvested, snap frozen in liquid nitrogen and stored at -80°C. Additional liver sections, the spleen and the gastrointestinal tract were collected, fixed for 48 hours in 10% buffered formalin, transferred to 70% ethanol and embedded in paraffin blocks. Liver iron, serum iron, transferrin and ferritin were measured at the Institut national de la santé et de la recherche médicale (INSERM) (Paris, France) under the supervision and with the collaboration of Dr F. Canonne-Hergaux. Intracellular iron staining of sections of the spleen and liver were also performed by Dr F. Canonne-Hergaux at the INSERM.

Hepcidin 1 mRNA expression: Total RNA was extracted from the liver with Trizol Reagent (Invitrogen, Burlington, ONT, Canada) according to the manufacturer instructions. cDNAs were synthesized all at once using M-MLV reverse transcriptase

(Invitrogen). Quantitative PCR was performed on Chromo4 Real-Time PCR Detection System (Bio-Rad Laboratories, Mississauga, ONT, Canada) using Brilliant SYBR Green QPCR Master Mix (Stratagene, La Jolla, CA, USA). All samples were amplified in triplicates during the same PCR run on 96 wells plates. Two housekeeping genes were used (Polymerase (RNA) II (DNA directed) polypeptide A (*Polr2a*) and TATA box binding protein (*Tbp*). Their stability was evaluated in Bestkeeper.[277] Both were found to be stable across our groups and experimental conditions and they were incorporated into a Bestkeeper Index used for relative quantification. PCR amplification efficiencies were measured on individual amplification plots using LinReg PCR.[278] The software REST 384 (http://www.gene-quantification.info/)[279] was used to analyze the relative expression of our measured transcripts using an efficiency corrected ratio. Error estimations of the calculated ratios were obtained using a Taylor's series as implemented in REST. The significance of the difference in expression ratios across experimental time points was investigated using pair wise fixed randomization tests implemented in REST (2000 randomizations for each test). The hepcidin primers specific for hepcidin 1 have previously been described.[325]

***Phenylhydrazine-induced hemolytic anemia*:** A/J mice were injected with 3.2mg of phenylhydrazine hydrochloride diluted in 100ul of PBS intraperitoneally[326] two days prior to infection with *Salmonella.* Control mice received the same volume of PBS, intraperitonneally. The course of the hemolytic anemia was followed in non-infected and *Salmonella*-infected mice by serial measurements of RBC parameters as previously described. Survival was recorded for the following three groups: 1) *Salmonella*-infected, phenylhydrazine-injected mice; 2) *Salmonella*-infected, PBS-injected mice; and 3) phenylhydrazine-injected mice.

Iron overload: Iron overload was induced in A/J mice by intraperitoneal injection of iron dextran (Sigma, Oakville, ON, Canada) four times a week for three weeks prior to infection (for a total of 8 injections) at a dosage of 1.2mg per mouse diluted in 100ul of PBS.[321] Control mice were injected with the same volume of PBS intraperitoneally. Survival was recorded and compared between normal and iron-overloaded mice, following *Salmonella* Typhimurium infection.

ACKNOWLEDGMENT

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FIGURES

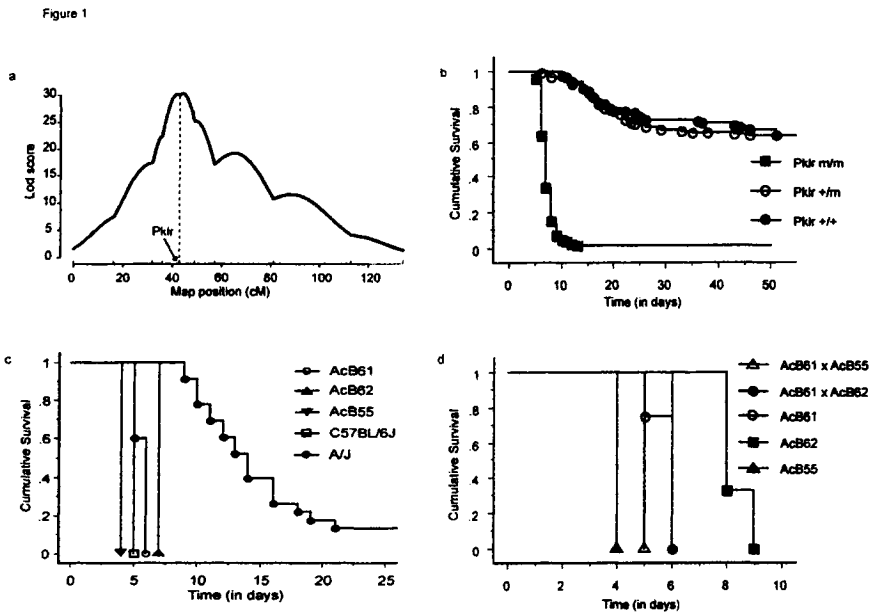

Figure 1: Candidacy of *Pklr* as the gene underlying *Ity4*. a) Interval mapping under a non-parametric model showing the LOD score trace for chromosome 3 for the survival phenotype in a 247 (AcB61x129S6)F2 mice. Adding the genotype of the mice at the *Pklr* mutation as a marker in this cross increased the peak LOD score from 28.8 to 30.3. *Pklr* maps directly under the peak LOD score as shown on the graph. The positions of the typed markers are shown as small grey vertical bars above the x axis. b) Cumulative survival in 306 F2 mice according to their genotype at the *Pklr* mutation. There is a strong correlation between the mice genotype at *Pklr* and survival. The mice that are homozygous (m/m) for the mutation have very little chance of surviving the infection while the mice that are wild type (+/+) or heterozygous (+/m) are much more resistant. c) Concordant phenotype among the three RCS known to carry a mutated *Pklr* gene. The AcB55 strain extreme susceptibility is most likely explained by the presence of mutations at both *Pklr* and *Nramp1*. (n = 5 for AcB61, AcB62 and AcB55; n = 19 for C57BL/6J; and n = 23 for A/J) d) Survival following *Salmonella* Typhimurium infection in F1 mice derived from (AcB61 x AcB62) and (AcB61 x AcB55), the RCS known to carry a mutated *Pklr* allele. The lack of complementation seen in the F1 mice indicates that their susceptibility can be attributed to the same gene. (n = 2 for AcB62, n = 3 for AcB62, n = 4 for AcB61, n = 4 for (AcB61xAcB55)F1 and n = 8 for (AcB61 x AcB62)F1)

Figure 2

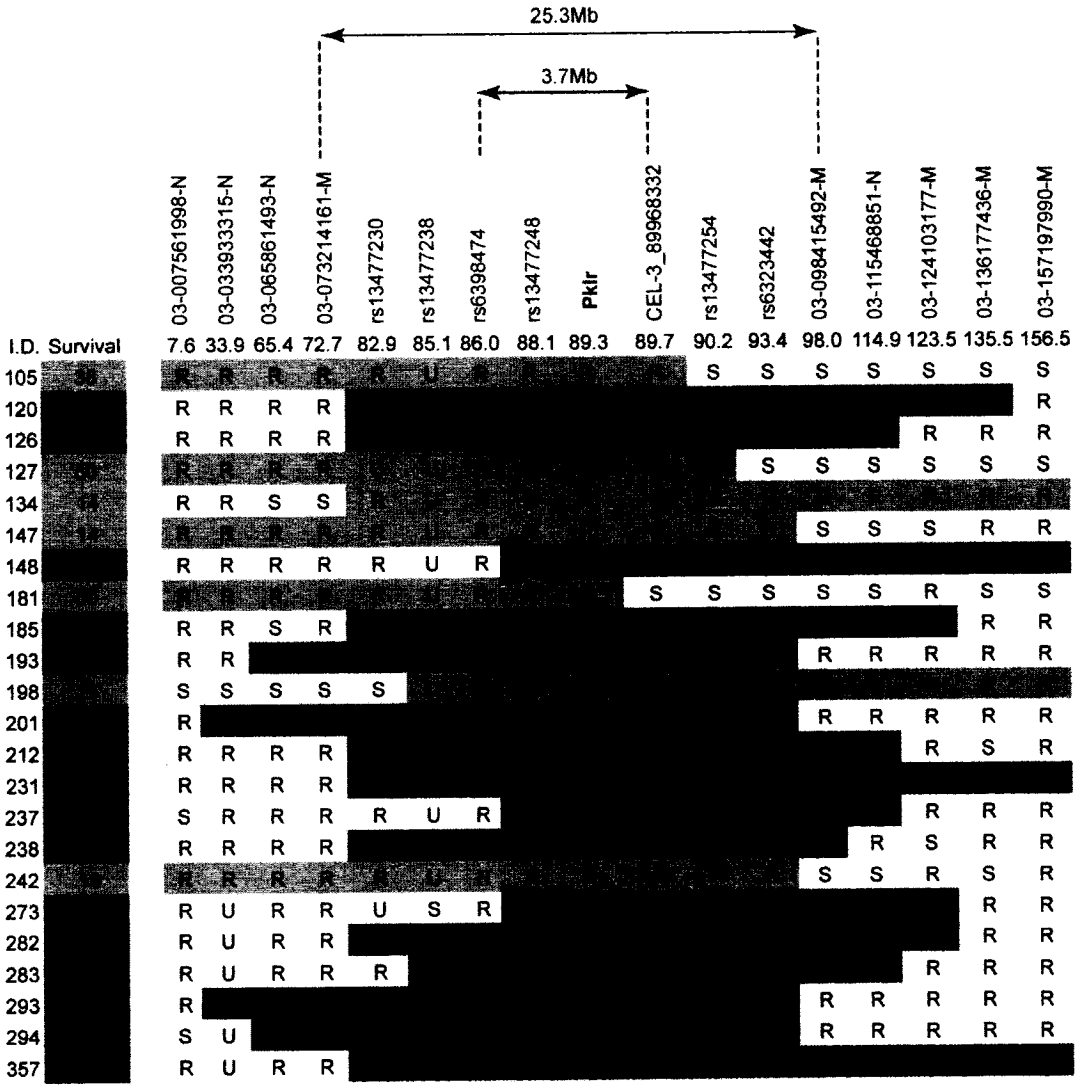

Figure 2: Fine mapping of the *Ity4* region. The *Ity4* support interval was reduced from 25.3Mb to 3.7Mb by selected genotyping of the non-resolved recombinants in the *Pklr* region. "R" represents mice carrying a resistant allele, i.e. 129S6 homozygous or heterozygous mice. "S" represents the mice carrying a susceptible allele, i.e. AcB61 homozygous. "ND" indicates that the genotype was not determined. "U" indicates an unknown genotype. The list and physical position of the markers used are shown at the top of the graph. For the Pklr marker, the mutation was directly genotyped. Light gray areas correspond to mice showing a resistant phenotype and their associated resistant haplotype around Pklr. Dark grey areas correspond to mice showing a susceptible phenotype and their associated susceptible haplotype around Pklr.

Figure 3

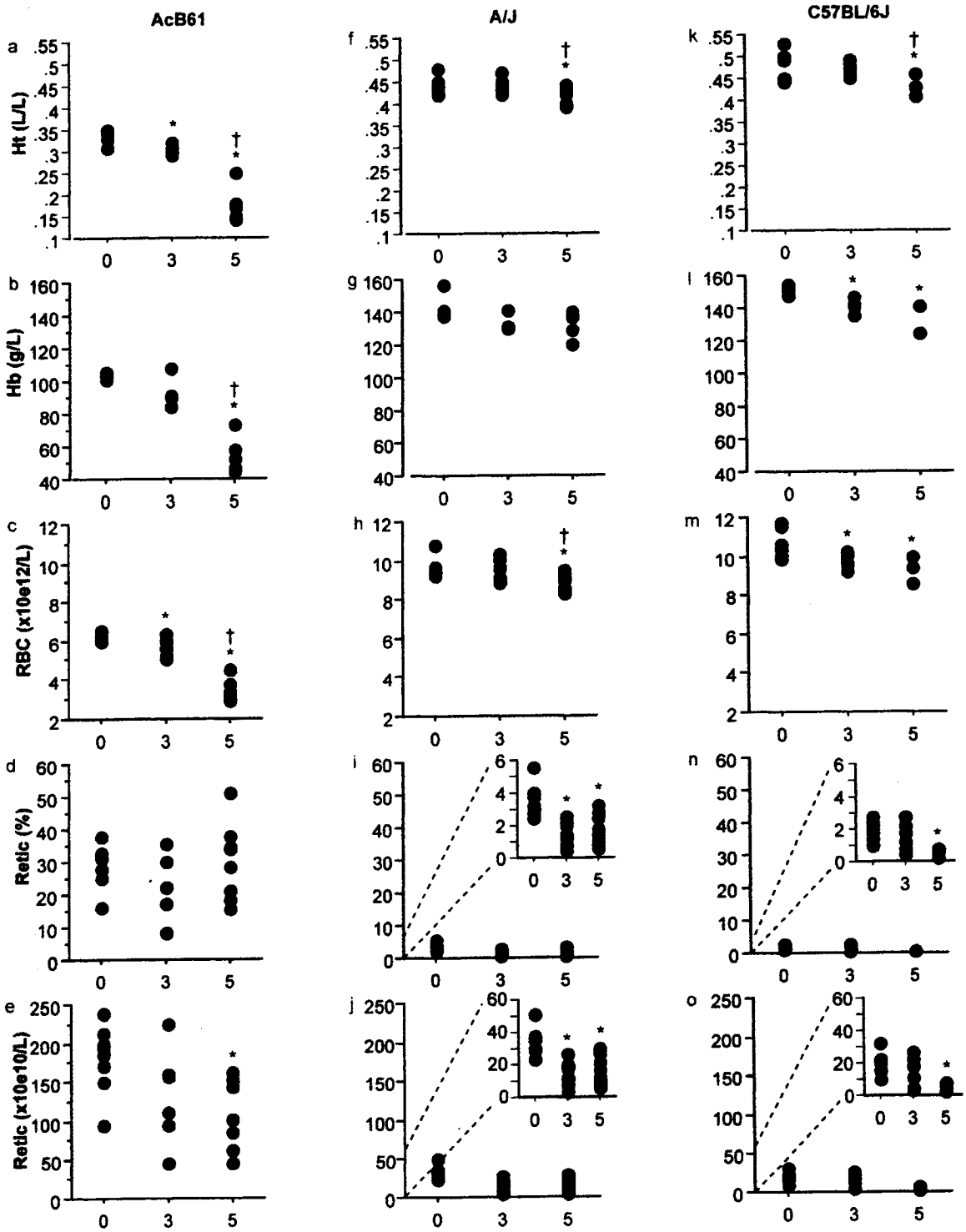
AcB61
A/J
C57BL/6J
a
b
c
d
e
f
g
h
i
j
k
l
m
n
o
Ht (L/L)
Hb (g/L)
RBC (x10e12/L)
Retic (%)
Retic (x10e10/L)
0
3
5

Figure 3 (previous page): RBC parameters in AcB61, A/J and C57BL/6J mice before (day 0) and after (days 3 and 5) infection with *Salmonella* Typhimurium. The values for each individual mouse are shown. The AcB61 mice show a constitutive anemia with reticulocytosis (a-e). During *Salmonella* infection the anemia in AcB61 mice rapidly worsens (a-c) while the parental strains, A/J and C57BL/6J, are only starting to develop a mild anemia (f-h and k-m). The reticulocyte counts are decreasing in all three strains (e, j, and o) indicating that the erythropoietic response is decreased during *Salmonella* infection. The sustained percentage of reticulocytes seen in AcB61 (d) may indicate greater resistance of the reticulocytes to the increased phagocytosis of aging RBC by activated macrophages. n = 8 for each group and each time points except for AcB61 on day 3 (n = 6), A/J on day 5 (n = 12) and C57BL/6J on day 5 (n = 3; most mice being dead early on day 5). "*" indicates significantly different ($p < 0.05$) from day 0. "†" indicates significantly different ($p < 0.05$) from day 3. (ANOVA with Fisher's protected least significant difference post-hoc test done in StatView 5.0)

Figure 4

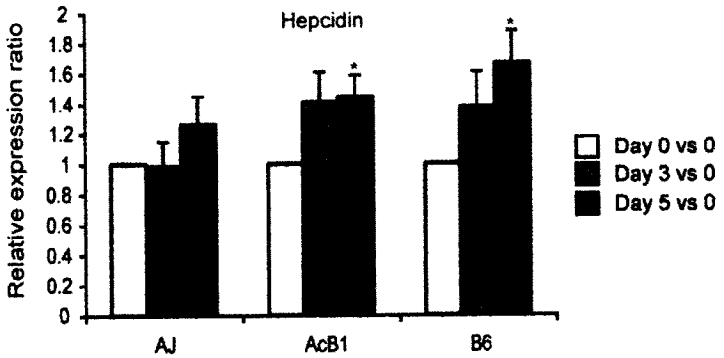

Figure 4: mRNA hepcidin expression in the liver of mice during *Salmonella* infection. Hepcidin is slightly increased during *Salmonella* infection in all strains. Significant differences ($p < 0.05$ following 2000 randomizations as implemented in REST 384) are found in AcB61 and C57BL/6J mice at day 5.

Figure 5

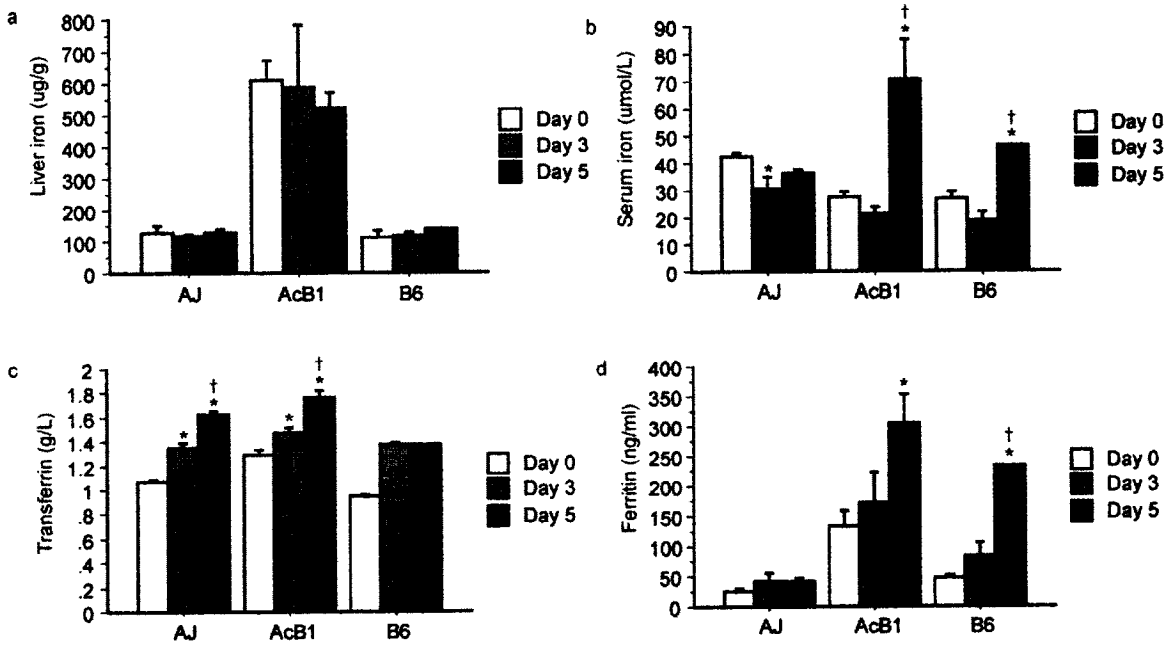

Figure 5: Iron parameters in AcB61, A/J and C57BL/6J mice before (day 0) and following (days 3 and 5) infection with *Salmonella* Typhimurium. Means +/- s.e.m. are shown. a) Liver iron is dramatically increased in AcB61 mice compared to the two parental strains. *Salmonella* infection has no detectable effect on liver iron. b) Serum iron is increased in non-infected A/J mice compared to the two other strains. During *Salmonella* infection serum iron tends to decrease on day 3 to finally increase on day 5, especially in most susceptible strains AcB61 and C57BL/6J. c) Serum transferrin is increased in AcB61 compared to the two parental strains and it increases during *Salmonella* infection in all three strains. d) Serum ferritin is increased in AcB61 compared to the two parental strains and it increases during *Salmonella* infection in the most susceptible strains AcB61 and C57BL/6J. n = 4 for each group and each time points except for the C57BL/6J mice on day 5 when only 1 mouse was still alive at the time of tissue harvest. "*" represents significantly different ($p < 0.05$) from day 0. "†" represents significantly different ($p < 0.05$) from day 3. (ANOVA with Fisher's protected least significant difference post-hoc test performed in StatView 5.0).

Figure 6

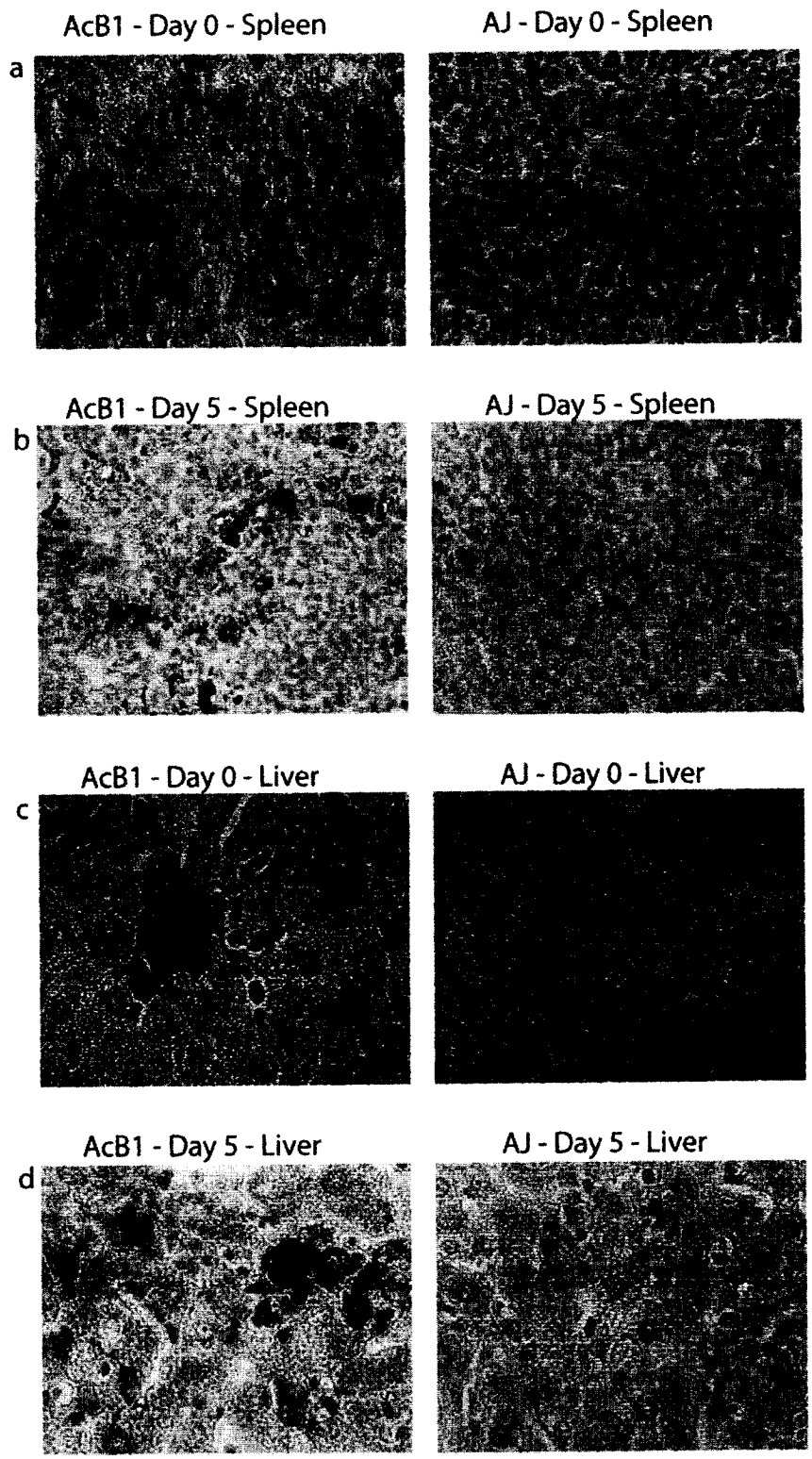
AcB1 - Day 0 - Spleen
AJ - Day 0 - Spleen
a
AcB1 - Day 5 - Spleen
AJ - Day 5 - Spleen
b
AcB1 - Day 0 - Liver
AJ - Day 0 - Liver
c
AcB1 - Day 5 - Liver
AJ - Day 5 - Liver
d

Figure 6 (previous page): Intracellular iron staining in the spleen and liver of AcB61 and A/J mice before infection and at day 5 post-infection. Objective 63x. Perls staining. a) Spleen, uninfected mice. b) Spleen, day 5 post-infection. c) Liver, uninfected mice. d) Liver, day 5 post-infection. Increased amounts of iron are detected in the AcB61 compared to A/J and C57BL/6J (data not shown) in non-infected and day 5 mice. The differences are more pronounced in the liver.

Figure 7

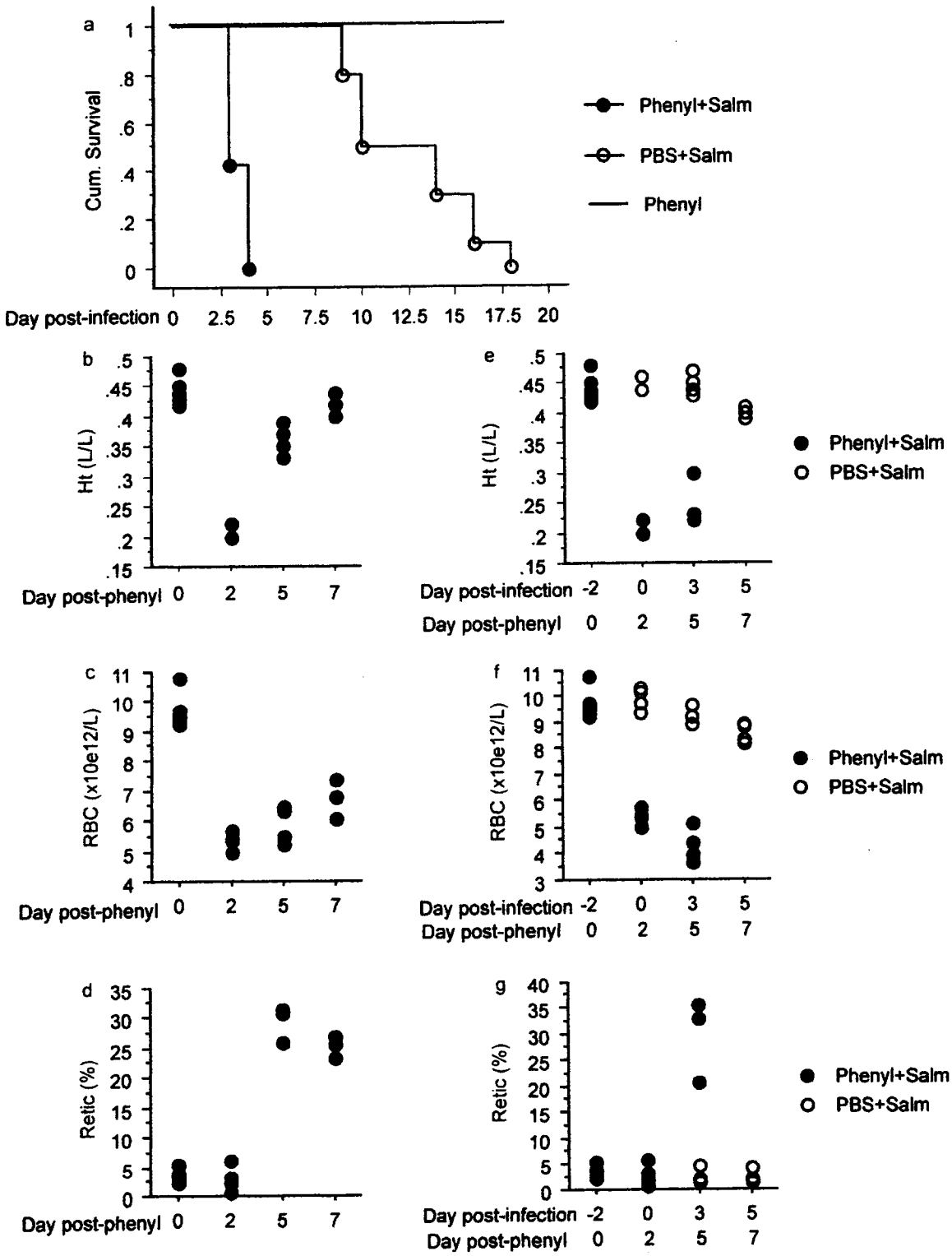
a
Cum. Survival
1
.8
.6
.4
.2
0
Day post-infection 0 2.5 5 7.5 10 12.5 15 17.5 20
Phenyl+Salm
PBS+Salm
Phenyl
b
Ht (L/L)
.5
.45
.4
.35
.3
.25
.2
.15
Day post-phenyl 0 2 5 7
e
Ht (L/L)
Day post-infection -2 0 3 5
Day post-phenyl 0 2 5 7
Phenyl+Salm
PBS+Salm
c
RBC (x10e12/L)
11
10
9
8
7
6
5
4
Day post-phenyl 0 2 5 7
f
RBC (x10e12/L)
Day post-infection -2 0 3 5
Day post-phenyl 0 2 5 7
Phenyl+Salm
PBS+Salm
d
Retic (%)
35
30
25
20
15
10
5
0
Day post-phenyl 0 2 5 7
g
Retic (%)
40
35
30
25
20
15
10
5
0
Day post-infection -2 0 3 5
Day post-phenyl 0 2 5 7
Phenyl+Salm
PBS+Salm

Figure 7 (previous page): Survival and RBC parameters in A/J mice injected with phenylhydrazine prior to *Salmonella* infection. Mice were injected intraperitonneally with phenylhydrazine or PBS two days prior to infection with 1000 CFUs of *Salmonella* Typhimurium intravenously. a) Cumulative survival in A/J mice injected with phehylhydrazine only (Phenyl), phenylhydrazine and *Salmonella* (Phenyl+Salm) or PBS and *Salmonella* (PBS+Salm). Prior injection with phenylhydrazine results in much increased susceptibility to *Salmonella.* (n = 10 for PBS+Salm, n = 14 for Phenyl+Salm and n = 4 for Phenyl) b-d) Phenylhydrazine injection in A/J mice leads to the rapid development of anemia followed by a regenerative response. (n = 8 for day 0, n = 4 for days 2 and 5 and n = 3 for day 7) e-g) Mice injected with phenylhydrazine following *Salmonella* infection showed a severe anemia at day 3 post-infection. This level of anemia is close to what is seen in AcB61 mice one or two days prior to death. Mice injected with both phenylhydrazine and *Salmonella* appear unable to recover from the phenylhydrazine-induced anemia compared to mice that received phenylhydrazine only (b and c versus e and f), despite a seemingly adequate reticulocyte response (d versus g). (n = 8 for day -2 and n = 4 for all other time points)

Figure 8

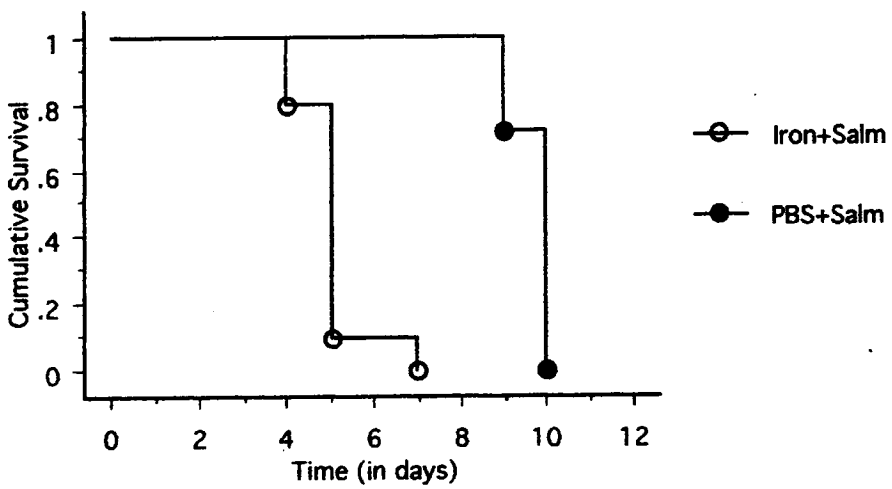

Figure 8: Survival following *Salmonella* Typhimurium in iron overloaded mice. A/J mice were injected intraperitoneally with iron dextran or PBS 3 times a week for 3 weeks (total of 8 injections) prior to infection with 1000 CFUs of *Salmonella* Typhimurium intravenously. Mice having received repeated iron injections (Iron+Salm) showed decreased survival following *Salmonella* infection compared to control mice injected with PBS (PBS+Salm). (n = 10 for Iron+Salm and n = 7 for PBS+Salm)

CHAPTER V: DISCUSSION

From a clinical point of view, it has long been recognized that the outcome of infectious diseases varies between species and between individuals of a same species. While several factors influence the outcome of a host-pathogen interaction, the genetic makeup of both the host and the pathogen plays a major role. Strictly speaking, any clinical infectious diseases may be seen as an immunodeficiency of the host in the context of this particular battle occurring between the two genomes.[1] The unraveling of the genetic components involved allows us to improve our understanding of infectious disease pathogenesis and paves the way to new prophylactic or therapeutic interventions. It is crucial for us to rapidly reach a higher level of understanding of the pathogenesis of infectious diseases and of the genomic interactions occurring during the course of infection if we want to successfully face the threat of increasing antimicrobial resistance, globalization of infectious diseases and newly emerging or the re-emerging pathogens.[2]

The ultimate goal of the genetic dissection of the host response to various pathogens is to find direct application for human or relevant veterinary species. The unraveling of complex traits, such as the susceptibility to infectious diseases in human, is complicated by the intrinsic characteristics of these traits. By definition, complex traits are complex because they lack a direct genotype-phenotype correlation. They are, instead, influenced by environmental factors in addition to genetic factors, and the latter are complicated by phenomena such as incomplete penetrance, genetic heterogeneity and phenocopy.[327] In these circumstances, the use of relevant mouse models greatly facilitates the process of mapping and cloning of the genetic components of the complex traits by allowing the control of experimental conditions, the *ad libitum* mating of phenotypically distant strains and the rapid generation of hundreds of progeny. The genes identified in such experimental crosses can later be tested in human populations or at least give a clue into the possible pathways involved in the disease of interest. Moreover, the cloning of susceptibility genes to a particular pathogen usually brings a higher understanding of the disease pathogenesis.

The phenotype underlying a complex trait is often measured as a quantitative trait (e.g. survival time, as opposed to a binary trait, survived or died) and for this reason, the

genomic region associated with these phenotypes are named QTL. QTL mapping in experimental crosses is a long and tedious process beginning with the identification of phenotypically distant strains. These strains are then crossed to generate segregating populations (generally a F2 or backcross) and each progeny is carefully studied for the phenotype(s) of interest. Each individual is then genotyped with a set of polymorphic markers and genetic analysis allows identification of the QTL influencing the measured phenotype(s). The cloning of the QTL to identify the actual quantitative trait gene (QTG) is the ultimate goal of the process but also its rate-limiting step. As of 2005, more than 2000 QTL had been described but the associated QTG had been identified for less than 1% of them.[328]

Several factors may explain the difficulties mouse geneticists have had in identifying the culprit. Among others are the mapping resolution of classical mouse crosses, the common lack of a clear coding sequence variants to account for the QTL, the small individual effect of each QTL, the presence of physically linked QTG underlying a single identified QTL and the computing difficulties associated with interaction mapping considering more than two loci at a time. Different approaches are being proposed to resolve some of these issues including new genomics resources (whole genome sequence for several strains and several thousands SNPs), new techniques (*in silico* mapping and expression QTL analysis), and new animal resources (chromosome substitution strains, the collaborative cross, heterogenous stocks).[328] One can therefore hope that some progress in QTG identification will be made in the near future.

During the course of my PhD, I have used two different approaches to gain further understanding of the genetic components underlying the complex host response to *Salmonella* Typhimurium infection in mice. First, I have used a hypothesis driven approach, often used by immunologists and named "reverse" genetics, where one starts from a specific gene and studies the phenotypic consequences of a given manipulation of this gene. In a second time, I have used the more classical "forward" genetic approach of QTL mapping described above, starting from the phenotype and, without any prior hypothesis, attempting to identify the gene(s) underlying the phenotype.

In Chapter II, I presented data pertaining to the reverse genetic analysis of the Tlr4 gene in the context of mouse typhoid. While *Tlr4* was initially identified as a gene

having a strong impact on the mouse response to LPS and *Salmonella* infection using a classical "forward" genetic approach,[178,179] it was decided to further study its function starting from the hypothesis that its level of expression would influence the host response to both LPS and *Salmonella*. Prior to my arrival to the lab, transgenic mice carrying 1, 3, 6 and >30 copies of *Tlr4* were generated and partially characterized.[266] It was found that indeed, the number of copies of *Tlr4* had a direct impact on the level of expression of the transcript and the protein, along with a strong functional impact on the *in vitro* proliferative response of splenocytes to LPS. *In vivo*, the impact of the level of expression of *Tlr4* on the response to LPS-induced septic shock was also evident while the response to systemic *Salmonella* Typhimurium was seen only in the context of a wild type allele at *Nramp1*, with a plateau effect starting at 3 copies of the gene for both phenotypes. I then took over the project and we became interested in understanding how increasing the number of copies of *Tlr4* impacts on the *in vivo* mouse response to *Salmonella*. In addition, we wanted to compare the effect of 1 and 3 copies of the transgene to the effect of two natural, non-transgenic copies of *Tlr4* in its natural genomic context. The generation of a double congenic strain, B10.Cg-*Nramp1/Tlr4*, allowed direct comparison of the effect of 0, 1, 2 or 3 copies of *Tlr4* in mice of identical genetic background. Using these mice we could show an incremental protective effect for survival following *Salmonella* Typhimurium infection in mice carrying 0, 1, 2 and 3 copies of *Tlr4*. The improved survival correlated with improved control of the bacterial growth in the spleen and the liver. Additionally, gene expression analysis using qPCR identified that the higher constitutive expression of relevant effector genes downstream of the Tlr4 signaling cascade probably influence the outcome of *Salmonella* infection in mice.

Several additional experiments could be done to further understand the real biological impact of the small increments in *Tlr4* expression. First, it would be useful to identify the main cell type involved in these Tlr4-induced gene expression differences. The gene expression analyses presented in Chapter II were done on whole spleen RNA and may not totally reflect the real changes occurring within different cell populations of the spleen. By sorting the various populations of cells prior to RNA extraction, we could identify different patterns of gene expression in the different cell types. This could give a

clearer picture of the real impact of the level of *Tlr4* expression. Second, the changes in gene expression identified in our experiments were more pronounced at days 0 and 4 compared to day 8, suggesting that events occurring early during infection are important in determining the outcome. Accordingly, it could be interesting to study gene expression at earlier time points such as days 1, 2 or 3. Third, while gene expression analysis are interesting for understanding basic biological processes, the real biological impact can only be fully evaluated by studies done at the protein level. Protein arrays and liquid bead arrays are becoming increasingly available and could be used to study the impact of *Tlr4* expression at the protein level in the spleen, liver or serum of our mice. Finally, while we have exploited our *Tlr4* transgenic and congenic mice in the context of the mouse response to *Salmonella*, these mice represent an invaluable tool that could be used to study a wide array of diseases relevant to human health. Recently, several publications have identified a role for *Tlr4* polymorphisms in different diseases including atherosclerosis, late-onset Alzheimer's disease and various infectious diseases.[192-194,198-200] Since these *Tlr4* variants have been associated with subtle functional differences, the mice we have developed could be used to study the impact of small changes in Tlr4 signaling in these diseases.

In Chapter III, I presented a different approach for the genetic dissection of the mouse response to *Salmonella*. Using a more classical "forward" genetic approach, we have screened a set of AcB/BcA RCS to identify phenotypically interesting strains that could later be used in fully informative crosses for QTL identification and cloning. The screening of the RCS was very informative. Prior knowledge of the genetics of the mouse response to *Salmonella* predicted that this screening would mainly identify *Nramp1* as a strong determinant of the outcome of infection. However, the shuffling of different small C57BL/6J or A/J congenic fragments on the opposite genetic background allowed identification of genetic effects independent of *Nramp1*. Two strains, which appeared most interesting to us, were chosen for further genetic analysis: the very susceptible AcB61 and the very resistant AcB64 strain. Fully informative crosses derived from these strains allowed identification of five novel QTL involved in the mouse response to *Salmonella* Typhimurium, thereby increasing our list of *Ity* loci from three to eight members. The strong effect associated with the *Ity4* locus and the prior knowledge

of the genetics of the RCS allowed us to identify the QTG underlying this locus (Chapter IV). However, *Ity5*, identified in the same (AcB61 x 129S6)F2 cross, probably also has a strong impact on the host response to *Salmonella* and should be amenable to positional cloning. We intend to confirm its existence in congenic strains and use subcongenics to further decrease the *Ity5* interval. The genomic region of *Ity5* is not too gene rich and the generation of subcongenics may allow us to reduce the *Ity5* genomic interval to a region containing a reasonable number of candidates.

The situation is more complex for the (AcB64 x DBA/2J)F2 cross with several QTL of small effects most likely involved, in addition to environmental factors. Increasing the number of F2 mice in our genetic analysis will allow us to get a clearer picture of the number, position and strength of the QTL involved before we undertake the confirmation of some of these QTL in congenic strains. Given the phenotypic resemblance of the AcB60 strain with AcB64, one may hypothesize that the same QTL contribute to the outcome of infection in these two strains. Genetic analysis in a (AcB60 x DBA/2J)F2 cross could confirm this hypothesis and may be helpful in the cloning of *Ity6-Ity8*. The screening of the RCS identified additional strains that may be of interest. While we have shown that the extreme susceptibility of AcB55 and AcB62 is most likely also attributable to *Pklr* (Chapter IV), the sex effect identified in BcA66 and BcA80, the intermediate susceptibility of the AcB56, AcB63 and BcA69 and the high CFUs seen in AcB53, BcA73 and BcA76 may be worth further investigation.

In Chapter IV, I presented the work done for the identification of the gene underlying *Ity4*. Our prior knowledge of a *Pklr* mutation in AcB61[287] along with the physical position of *Pklr* in relation to *Ity4* clued us into the possible involvement of this gene. The phenotypic comparison between the three RCS known to have a mutated *Pklr* along with the complementation studies further strengthened the candidacy of *Pklr*. Moreover, using a fine mapping approach we were able to decrease the *Ity4* interval from 25Mb to a region of 3.7Mb just surrounding *Pklr*. Finally, we presented biologically plausible explanations for a role of this RBC enzymatic defect in the susceptibility to *Salmonella* in mice. Although our data strongly favor the candidacy of *Pklr* as the gene underlying *Ity4*, we cannot exclude that a gene closely linked to *Pklr* is responsible or contributing to the phenotype. The formal proof for a role of *Pklr* in the mouse response

to *Salmonella* would be obtained by *Pklr* complementation in AcB61 or disruption in A/J mice.

Some observations made during the course of my PhD work have led us to become interested to the effect of anemia and iron on the host response to infection. We first observed in C57BL/10 mice that *Salmonella* infection leads to the development of severe anemia (Chapter II). To our knowledge, this finding has not been reported in the recent scientific literature. In human typhoid fever, anemia appears to be a common clinicopathological finding, however, not much has been written on the pathogenesis of this phenomenon. We, therefore, sought to understand the mechanisms underlying the development of anemia during *Salmonella* infection and the results of these experiments were also presented in Chapter IV. The preliminary investigations made indicate that *Salmonella* infection in mice decreases the erythropoietic response as indicated by decreased numbers of reticulocytes in the peripheral circulation. Intriguingly, the anemia and decreasing reticulocyte counts occur even in the face of extramedullary erythropoiesis as seen on histopathology in the spleen and liver of the C57BL/10 mice. The rapid development of the anemia in our mice cannot, therefore, be explained solely by the decreased output of RBC. Some of our observations and the current understanding of the pathogenesis of anemia of inflammation[251] rather suggest that the destruction of the RBC by the RES is probably accelerated during infection. We can thus conclude that anemia develops in mice during *Salmonella* infection because of failure of the erythropoiesis response to compensate for the increased destruction of the RBCs as is usually the case in anemia of inflammation. Additionally, the rapidity with which the anemia develops in mice is probably favored by the shorter half-life of the RBC in this species compared to human.

Several of our findings indicate that the *Salmonella*-induced anemia in mice somehow fits the definition of anemia of inflammation including the decreased erythropoiesis, the increased RBC destruction, the increased ferritin and increased hepcidin expression. However, although the serum iron seemed to decrease on day 3 in our mice, it was markedly increased on day 5 in AcB61 and C57BL/6J; in contrast, anemia of inflammation is usually characterized by hypoferremia. Furthermore, the transferrin was increased during infection in the three strains studied while it is usually

decreased or normal in anemia of inflammation. These discrepancies are intriguing and may be related to the acute and severe nature of the infection in our mice, which may have a direct effect on these iron parameters. Additionally, these findings may be specific of the *Salmonella*-induced anemia in mice. It would be interesting to see if a different model of sepsis having a similar time course in mice would also result in the development of anemia or if this is strictly a feature of systemic *Salmonella* infection.

In Chapter IV, I also presented the results of various experiments aimed at understanding the biological basis of the susceptibililty of the PK deficient AcB61 strain to *Salmonella*. Given our knowledge of the constitutive anemia in AcB61 and our prior observations of the *Salmonella*-induced anemia, we hypothesized that these mice may be overly susceptible to *Salmonella* because of development of a fatal anemia during infection. Examination of the RBC parameters during infection showed that, as predicted, the anemia in AcB61 rapidly worsens during *Salmonella* infection to reach critically low and potentially harmful levels just prior to death. To confirm the role of anemia in the susceptibility of the AcB61 mice, we used a model of acute hemolytic anemia induced by intraperitoneal administration of phenylhydrazine in a closely related strain, A/J. Although we have shown that phenylhydrazine-injected mice are more susceptible to *Salmonella* compared to control mice, it does not allow us to conclude that anemia was the sole factor responsible for this increased susceptibility. The rapid release of iron following phenylhydrazine injection[308] could have contributed to the increased susceptibility of our mice because of free iron-induced tissue damage,[316] compromised innate immunity or increased bacterial growth.[256] Additionally, a study published in the 1960s indicated that, contrary to what is seen during phenylhydrazine-induced hemolytic anemia, acute bleeding of the mice to create anemia of similar amplitude does not increase the susceptibility of mice to *Salmonella*.[309] This finding suggest that the hemolytic nature of the anemia (implying phagocytosis of RBC by the RES) rather that the anemia itself is detrimental to the host during *Salmonella* infection. Indeed, it has been shown that erythopagocytosis *per se* impairs the bactericidal function of *Salmonella*-infected macrophages.[314,315]

During our investigations of the nature of the *Salmonella*-induced anemia, we unexpectedly noted that the iron load in AcB61 was much increased compared to A/J and

C57BL/6J. This observation raised the possibility that iron overload could also be implicated in the susceptibility of the AcB61 mice to *Salmonella*. Indeed, previous studies have showed that iron overload is detrimental to the host during systemic *Salmonella* infection.[257,258] To confirm that this was also true in our experimental model of *Salmonella* Typhimurium infection, we induced iron overload in A/J mice through repeated iron injections and showed that iron overloaded mice were in fact more susceptible to *Salmonella* compared to control mice.

Thus, it seems that the reasons underlying the susceptibility of PK deficient mice are numerous but probably involve the severity of anemia, its hemolytic nature and the iron overload. Additional experiments will be needed to clarify the respective role of these mechanisms and we can envision a few here. First, we could measure the bacterial load at day 3 in phenylhydrazine-treated, *Salmonella*-infected mice and compared it to control mice (*Salmonella* and PBS). Second, we could repeat the phenylhydrazine experiment but this time, infecting with *Salmonella* on day 4 after the phenylhydrazine injection rather than on day 2. This delay in infecting the mice with *Salmonella* may allow us to avoid the acute hemolytic crisis with its associated release of iron while still having anemic mice with a marked reticulocytosis, a situation that reflects what is seen in AcB61 mice. Third, we could perform *Salmonella* infection in A/J mice following acute bleeding to decrease the hematocrit to various levels, and observe the effect these manipulations on survival. Fourth, we could try iron chelation or iron deficient-diet in AcB61 mice prior to infection to see if we can improve their resistance by decreasing the iron load. In such experiments, however, one would need to carefully titrate the iron load to avoid completely depleting the cells of iron given the importance of this metal in the generation of ROI for host defense.[260] Fifth, it would be interesting to measure the iron parameters in the AcB55 and AcB62 mice, which have a similar phenotype compared to the AcB61 mice and are also known to carry the *Pklr* mutation. Finally, following the iron load in Pk deficient mice at various ages and correlating it with the susceptibility to *Salmonella* could also be informative.

In conclusion, the work I have done throughout my PhD has increased our understanding of the pathogenesis of acute systemic *Salmonella* Typhimurium infection in mice. We have showed that a fine regulation of an important innate immune pathway

such as Tlr4 signaling, early in infection influences the course of the disease. Additionally, we have showed that, in a model apparently explained by a simple Mendelian trait such as the mouse response to *Salmonella* Typhimurium in C57BL/6J and A/J mice, deeper investigations reveal additional complexity that was hidden by the strong genetic effect of *Nramp1*. Building on these findings, we have identified five novel QTL, *Ity4-Ity8*, thereby contributing greatly to the knowledge of the genomic regions involved susceptibility in mouse typhoid. Finally, we have showed that *Pklr*, a gene previously shown to increase mouse resistance to *Plasmodium chabaudi* infection in mice is also involved in the mouse susceptibility to *Salmonella* thereby revealing the molecular nature of *Ity4*. The impact of the *Pklr* mutation on the mouse response to *Salmonella* is explained by its effect on the RBC turnover and iron metabolism. As a whole, the work presented here lays the foundation to numerous additional studies that could be undertaken to further define the genetic and pathophysiologic basis of acute *Salmonella* Typhimurium infection in mice.

STATEMENT OF ORIGINALITY

The work presented in this thesis contributes significantly to our knowledge of the genetics and pathophysiology of the mouse response to acute *Salmonella* Typhimurium infection.

Chapter II: Incremental expression of Tlr4 correlates with mouse resistance to *Salmonella* infection and fine regulation of relevant immune genes.

-Generation of C57BL/10ScNCr.Cg-*Nramp1/Tlr4*.

-Identification of an incremental protective effect from 0 to 1, 2 and 3 copies of *Tlr4* on mouse survival following acute *Salmonella* Typhimurium infection.

-Identification of a *Tlr4* expression level effect for the control of the bacterial proliferation in the spleen and liver.

-Identification of a *Tlr4* expression level-induced fine regulation of relevant downstream immune genes.

-Identification of a *Salmonella*-induced anemia in mice.

Chapter III: Complexity in the host response to *Salmonella* Typhimurium infection in AcB and BcA recombinant congenic strains.

-Screening of the 36 A/J and C57BL/6J RCS for their susceptibility to *Salmonella* Typhimurium through monitoring of survival time and bacterial load in the spleen and liver.

-Identification of *Nramp1*-independent genetic control for the host response to *Salmonella* infection in A/J and C57BL/6J.

-Generation of two novel F2 crosses: (AcB61 x 129S6) and (AcB64 x DBA/2J) and phenotyping of these F2 mice for their susceptibility to *Salmonella* Typhimmurium.

-Identification of five novel *Salmonella* susceptibility loci, *Ity4-Ity8*.

Chapter IV: Pyruvate kinase deficiency confers susceptibility to *Salmonella* Typhimurium infection in mice.

-Identification of *Pklr* as a susceptibility gene in the mouse response to *Salmonella*.

-Investigation and description of the nature of the *Salmonella*-induced anemia in mice.

-Identification of iron overload in AcB61 mice.

-Description of the biological basis for a role of *Pklr* on the mouse response to *Salmonella* infection.

-*In vivo* expression of hepcidin at days 0, 3 and 5 following *Salmonella* infection in A/J, C57BL/6J and AcB61 mice.

-Combined phenylhydrazine and *Salmonella* infection in mice with phenotyping for survival and RBC parameters.

-Investigation of the iron metabolism (iron, ferritin and transferrin dosages and Perls staining for iron in the spleen and liver) during *Salmonella* infection in A/J, C57BL/6J and AcB61 mice.

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APPENDIX 1: Review Paper: Genetic Regulation of Host Responses to Salmonella Infection in mice.

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REVIEW

Genetic regulation of host responses to Salmonella infection in mice

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Salmonella spp are Gram-negative bacteria capable of infecting a wide range of host species, including humans, domesticated and wild mammals, reptiles, birds and insects. The outcome of an encounter between Salmonella and its host is dependent upon multiple factors including the host genetic background. To facilitate the study of the genetic factors involved in resistance to this pathogen, mouse models of Salmonella infection have been developed and studied for years, allowing identification of several genes and pathways that may influence the disease outcome. In this review, we will cover some of the genes involved in mouse resistance to Salmonella that were identified through the study of congenic mouse strains, cloning of spontaneous mouse mutations, use of site-directed mutagenesis or quantitative trait loci analysis. In parallel, the relevant information pertaining to genes involved in resistance to Salmonella in humans will be discussed.
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Introduction

Salmonella are facultative intracellular Gram-negative bacteria that are found ubiquitously in nature with 2463 serotypes currently recognized. DNA–DNA hybridization has shown that almost all *Salmonella* serotypes belong to the same species now designated *Salmonella enterica*.[1] Some *Salmonella* serotypes are host adapted (Typhi and Paratyphi in humans, Dublin in cattle, Gallinarum in birds) while others (Typhimurium and Enteritidis) have the ability to infect a wide range of hosts including domesticated and wild mammals, reptiles, birds and insects. These serotypes are considered zoonotic organisms because they have the capacity to cause disease in animals and humans. The principal clinical manifestations associated with *Salmonella* infection in humans are enteric fever (typhoid and paratyphoid) and a self-limiting gastroenteritis (salmonellosis). Additionally, silent carriage of this bacterium is frequent and contributes to disease dissemination. Enteric fever, caused by *Salmonella* Typhi and *Salmonella* Paratyphi, still represents a major public health problem in many developing countries with over 16 million cases reported annually worldwide and 600 000 deaths associated with untreated infection.[2] On the other hand, industrialized countries experience an increased incidence of non-typhoidal *Salmonella* infections with most cases tracing back to food contamination. Approximately 1.4 million cases (sporadic form or outbreaks) per year of salmonellosis occur in the United States alone.[2] In veterinary medicine, *Salmonella* spp are also significant pathogens associated with economic and productivity losses, food contamination,[3,4] and outbreaks among horses hospitalized in large animal clinics[5] or on breeding farm facilities.[6]

The contribution of the host genetic background to the risk of infection and disease severity has been evidenced in humans by studies documenting racial differences in disease susceptibility, a higher concordance in monozygotic *vs* dizygotic twins and, more recently, using large-scale family-based genome scans and association studies.[7–9] It is clear from these genetic analyses that the molecular mechanisms of resistance and susceptibility to infectious diseases are extremely complex and multifactorial with microbial virulence determinants and geographical environment factors modifying the expression of specific host susceptibility loci.

In humans, the risk and outcome of *Salmonella* infection are also influenced by multiple factors. For instance, the use of antimicrobials may lead to disruption of the host endogenous flora and subsequently favor proliferation of pathogenic bacteria such as *Salmonella* spp.[10] Similarly, loss of the gastric acid barrier (because of achlorhydria or use of acid suppressor drugs) may increase host susceptibility to enteric pathogens.[11] Several specific human populations are also at increased risk of *Salmonella* infection including patients with sickle cell anemia,[12] chronic granulomatous disease (CGD),[13]

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pathogenic mutations within cytokine signaling pathways[14,15] or HIV infection.[16] Indeed, AIDS patients are not only at increased risk for non-typhoidal *Salmonella* infection but they also experience higher incidence of bloodstream invasion and focal suppurative complications.[17] Finally, clustering of reactive *Salmonella*-triggered arthritis within families suggests that genetic factors may also play a role in the development of post-infectious sequelae.[18]

The understanding of the complex host response to *Salmonella* infection in humans and other animal species has advanced considerably through the use of mouse models of infection. The laboratory mouse is well known to have a broad range of host susceptibility to human pathogens. Early work with classical inbred strains of mice has shown differential host response to infection with *Salmonella*. The development of genomic technologies (large-scale cloning and sequencing, gene targeting, etc) and mouse genome databases (http://www.informatics.jax.org) in the late 1980s and early 1990s combined with classical genetics contributed to the successful identification of several *Salmonella* resistance genes (*Ity, Lps, xid*, etc) in laboratory mice. More recently, the development of novel models of infection together with quantitative trait mapping has identified additional host susceptibility loci.[19,20] This review will highlight key studies that led to the discovery of major *Salmonella* resistance genes in mice using different approaches including the generation of congenic mouse strains, positional cloning of spontaneous mouse mutations associated with susceptibility to *Salmonella* infection, targeted disruption of candidate genes and quantitative trait loci (QTL) mapping (Figure 1).

Mouse model of *Salmonella* Typhimurium infection

Salmonella Typhimurium infection in mice induces a systemic disease similar to human typhoid fever. Systemic infection with a sublethal inoculum of *Salmonella* Typhimurium in the mice is characterized by four distinct phases of infection (reviewed in Ref. [21]). The first phase involves rapid clearance of the organisms from the bloodstream (within 2 h), followed by the localization of approximately 10% of the inoculum within macrophages and polymorphonuclear cells of the spleen and liver where the bacteria have the ability to survive and replicate.[22,23] The second phase of infection takes place over the following week, with an exponential growth of the organisms within the reticuloendothelial system (RES). The macrophages exert an important regulatory function during this phase since administration of silica (a macrophage poison) results in a major increase in bacterial load and a substantial decrease in the LD_{50} by a factor of 100 times.[24] The host innate system, through the

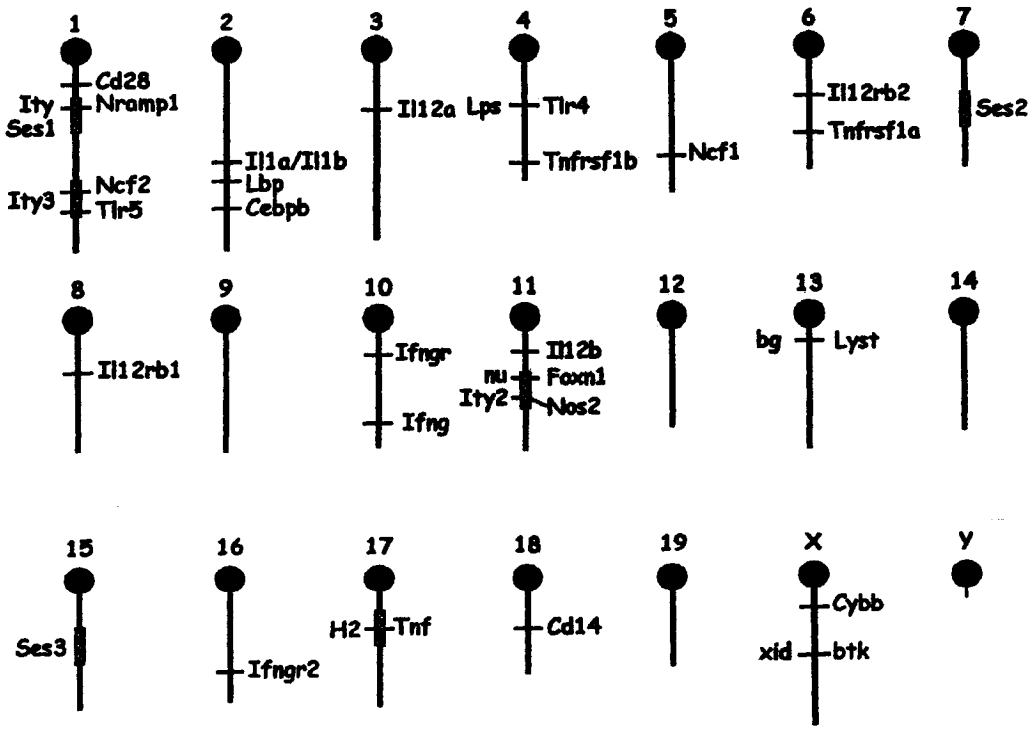

Figure 1 *Salmonella* host resistance/susceptibility loci mapped in the mouse genome. The positions of the loci were taken from the Mouse Genome Informatics database of the Jackson Laboratory (http://www.informatics.jax.org/). The genes whose functions were uncovered by the study of spontaneous mouse mutants are shown in red with the corresponding locus shown in black when applicable. Genes studied by targeted mutation are shown in green. Finally, the blue boxes represent locis involved in susceptibility/resistance to *Salmonella* that were mapped by QTL analysis. The corresponding identified genes or the loci name are also shown in blue.

recognition of *Salmonella* or *Salmonella*-conserved motifs such as lipopolysaccharide (LPS), regulates adaptive immunity. Recognition of pathogen-associated molecular patterns (PAMPs) by the host cell pattern recognition receptors (PRRs) triggers an innate immune inflammatory response characterized by the production of several cytokines including tumor necrosis factor (TNF) and interferon-γ (INFγ) and by an abundant mononuclear infiltration of the RES aimed at the elimination of the pathogen. The activation of the innate immune system to stop bacterial growth results in the establishment of a plateau (third) phase. The final (fourth phase) resolution of infection is clearly a function of activation of the acquired immune system and depends on T and B cells activation.[25–28]

It was recognized over 30 years ago that the susceptibility of inbred mice to infection with virulent *Salmonella* Typhimurium varied from strain to strain and that these differences were genetically controlled.[29,30] In general, classical inbred strains of mice can be classified into three distinct categories in regard to their susceptibility to *Salmonella*.[20,30] 129S6/SvEvTac mice are extremely resistant to infection with *Salmonella* Typhimurium compared to A/J mice that present an intermediate susceptibility phenotype and show increased survival time with decreasing infectious dose but cannot survive the infection (Figure 2). Other strains such as C57BL/6J, BALB/cJ and C3H/HeJ are extremely susceptible to infection and all succumb within the first week independent of the inoculum size. Differential susceptibility to *Salmonella* is also recognized among the wild-derived mice with CAST/Ei being very resistant in comparison to MOLF/Ei or SPRET/Ei mice.[20]

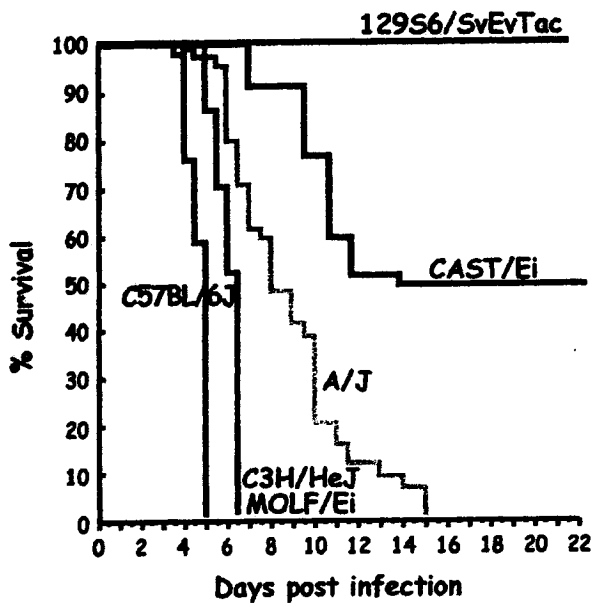

Figure 2 Survival curves following intravenous challenge with 10^4 CFU of *Salmonella* Typhimurium strain Keller. 129S6/SvEvTac are the most resistant mice followed by CAST/Ei and A/J. C57BL/6J, MOLF/Ei and C3H/HeJ are extremely susceptible to *Salmonella* Typhimurium with no individual surviving beyond 7 days post-infection.

Salmonella resistance loci identified using congenic mice

Genes of the major histocompatibility complex (H2)

The mouse histocompatibility complex (H2 complex) on MMU17 is a large genomic region encoding dense clusters of immune loci defining more than 120 genes (reviewed in Ref.[31]). The mouse H2 was first identified during the course of transplantation and serological studies, and was later shown to influence the outcome of several immune diseases including resistance to infection with *Salmonella* Typhimurium.[25,32] The H2 complex is divided into three major regions: class I, class II and class III. Mouse H2 haplotypes are defined by alleles at polymorphic class I and class II genes. The class I region contains highly polymorphic Ia genes which present peptides to αβ T cells and less polymorphic Ib genes which engage NK cells and γδ T cells. The number of class Ia and class Ib genes carried by different strains of mice varies considerably: mice with $H2^b$ and $H2^k$ haplotype possess only one H2-D locus compared to mice with $H2^d$, $H2^b$ and $H2^q$ haplotypes which have five H2-D loci. The class II region is highly conserved between the mouse and man both at the level of coding sequences and gene structure. The class II region contains the polymorphic class IIα and class IIβ genes as well as proteins involved in class I restricted antigen presentation (Abcd2/Tap1 and Psmb9/Lmp2). The class III region is the most conserved region in mammals and contains several genes involved in innate and adaptive immunity including the complement components *C4* and *C2*, the pro-inflammatory cytokines *Tnf* and *Lta* and the chaperone *Hsp70-1*.

The effect of the H2 complex on mouse susceptibility to *Salmonella* Typhimurium infection was evaluated in C57BL/10 congenic lines (all *Ity*^{s}—see below for a description of *Ity*).[25] Mice carrying $H2^b$ and $H2^d$ haplotypes were more susceptible (LD_{50} $<10^3$ CFU) to a strain of *Salmonella* Typhimurium of intermediate virulence than those carrying $H2^a$, $H2^k$ and $H2^f$ haplotypes (LD_{50} $\geq 10^4$ CFU). F1 hybrids between $H2^b$ (susceptible) and $H2^f$ (resistant) showed an intermediate phenotype suggesting a codominant mode of inheritance. Susceptibility of $H2^b$ mice was apparent 4 weeks following the infectious challenge and reached a maximum at 7–8 weeks post-inoculation. The bacterial load was 10- to 100-fold higher in the spleen and liver after infection of susceptible $H2^b$ congenic strains compared to resistant $H2^f$. Using congenic mouse strains carrying recombinant H2 haplotypes, *Salmonella* susceptibility was mapped to the MHC class II I-Eα subregion. The major role of MHC class-II-dependent immune mechanisms in the elimination of *Salmonella* Typhimurium during the late course of infection was later highlighted using mice lacking MHC class II molecules (ie lacking H2-I-Aβ chain) and mature CD4+ TCRαβ cells.[33,34]

In another series of experiments using *Salmonella* Typhimurium C5TS (a temperature-sensitive mutant), three categories of susceptibility were defined by comparing late bacterial clearance among H2 congenic mice on a C57BL/10 genetic background.[35] The lowest rate of bacterial clearance was observed in $H2^b$, intermediate clearance in $H2^d$, $H2^f$, $H2^k$, $H2^p$, $H2^r$, $H2^a$ and $H2^v$, and high clearance in $H2^j$, $H2^q$ and $H2^u$. The influence of the H2 haplotype on bacterial clearance was

influenced by different genetic backgrounds. Further analysis using H2 recombinant congenic mice on a C57BL/10 genetic background suggested that at least two additional regions of the H2 complex, H2-D and H2-K, are involved in determining the late-clearance phenotype.[32] A role for class-I restricted T cells in the immune response to *Salmonella* Typhimurium infection was later shown using mice lacking β2-microglobulin (β2 m).[36] β2 m is a 12 kDa protein known to associate with class I (H2-K, H2-D, H2-Q and H2-T) molecules that promotes activation of CD8+ T cells that specifically recognize cells infected with *Salmonella*.

In humans, recent studies support the contribution of the MHC to the host immune response to infection with *Salmonella*. Class II MHC was shown to be associated with susceptibility to *Salmonella* Typhi in Vietnam where typhoid fever is endemic,[37] and MHC class Ib molecules were associated with the development of autoimmune reactive arthritis following *Salmonella* infection.[38,39] The inherent complexity and polymorphism of the MHC complex and the linkage disequilibrium between loci will continue to pose a significant challenge to the identification of specific *Salmonella* susceptibility genes located in this area of the genome.

Salmonella resistance genes identified by positional cloning

Nramp1

The first description of *Ity* (immunity to Typhimurium) in the mouse appeared in 1976.[29] In this early paper, the authors showed that eight strains of inbred mice fell into two sharply defined groups with respect to resistance to infection. Four strains (CBA, A/J, C3H/He and DBA/2) were resistant ($LD_{50} > 10^5$) to *Salmonella* Typhimurium C5 and the other four strains (BALB/c, C57BL, B10.D2 and DBA/1) were susceptible ($LD_{50} < 10$). These strain differences in disease resistance were shown to behave as a simple Mendelian trait with a dominant mode of inheritance. Three years later, *Ity* was located to mouse chromosome 1 using distinguishable phenotypes as chromosome markers.[40] Around the same time, two other groups identified host resistance loci for two unrelated pathogens, *Mycobacterium bovis* (*Bcg*) and *Leishmania donovani* (*Lsh*).[41,42] These two loci were also located on mouse chromosome 1 to the same genomic subregion as *Ity*.[43,44] At that time, it was not clear if *Bcg*, *Ity* and *Lsh* were either the same or very closely related genes. The identification of a unique gene underlying *Ity/Bcg/Lsh* came almost 20 years after its first description.[45,46]

Two allelic forms were recognized for *Ity*: a resistance allele (*Ity*^{r}) and a susceptibility allele (*Ity*^{s}).[40] The resistance allele is dominant and influences the rate of bacterial growth during the exponential phase of multiplication in the RES and the LD_{50} after lethal challenge with high doses of *Salmonella* Typhimurium.[29] *Ity* is expressed by macrophages and *Ity*^{r} confers an increased bactericidal capacity to this cell type in comparison to *Ity*^{s}.[47] Studies involving an experimental mouse model of infection with *Mycobacteria* and *L. donovani* showed that *Bcg*^{r} and *Lsh*^{r} behaved similarly, being expressed in macrophages and conferring resistance to bacterial multiplication of the intracellular parasites during the early phase of infection.[41,48,49] A positional cloning approach was undertaken to identify the gene underlying the *Bcg* phenotype.[50,51] *Bcg* was mapped by segregation analysis to the proximal portion of mouse chromosome 1 closely linked to the villin (*Vil*) gene.[52] High-resolution linkage and physical maps were generated and allowed to narrow the chromosomal segment encompassing the *Bcg* locus to a size amenable for positional cloning.[53,54] Subsequently, using exon amplification, a candidate gene for the *Bcg* locus was found and named natural resistance-associated macrophage protein gene or *Nramp1*.[45] *Nramp1* was recently renamed *Slc11a1* because of its membership to a family of solute carriers.[55] Predicted protein sequence analysis of *Nramp1* between *Ity*^{r} and *Ity*^{s} strains revealed a single mutation resulting in glycine to aspartic acid substitution at position 169.[56] This mutation results in a complete lack of function of Nramp1 in susceptible mice.[57] The identity of *Nramp1*, *Bcg*, *Ity* and *Lsh* was confirmed by targeted disruption of *Nramp1* in mice and phenotypic comparison among different genotypes of mice during experimental infections with *Salmonella* Typhimurium, *M. bovis* and *L. donovani*.[46] Additionally, susceptible mice were rendered resistant to BCG and *Salmonella* Typhimurium by transfer of the resistance allele, further confirming the identity of *Nramp1* with the phenotypic resistance to *Salmonella* Typhimurium.[58]

The identification of *Nramp1* and its function opens a whole new field in the area of host resistance to intracellular pathogens. *Nramp1* encodes for a highly hydrophobic 56 kDa protein, which possesses 12 transmembrane (TM) domains and a glycosylated extracytoplasmic loop. It is expressed in the membrane fraction of macrophages and neutrophils as a phosphoglycoprotein of 90–100 kDa (reviewed in Ref.[55]). During *Salmonella* infection, phagocytes ingest the bacteria into a phagosome, which matures by sequential fusions with a series of endosomal and lysosomal compartments and results in the formation of phagolysosomes that possess antimicrobial properties. To survive into the host cells, *Salmonella* evade this process by affecting the maturation process of the phagosome. *Salmonella* generate a unique compartment termed *Salmonella*-containing vacuole (SCV) (reviewed in Ref. [59]). During phagocytosis, Nramp1 is recruited to the membrane of the phagosome and remains associated with this structure during its maturation to phagolysosome.[60] Nramp1 appears to have an impact on SCV maturation: SCVs formed in Nramp1-deficient macrophages fail to acquire M6PR (mannose 6 phosphate receptor), a protein known to regulate the delivery of a subset of lysosomal enzymes from the trans-golgi network to the pre-lysosomal compartment, thereby facilitating bacterial killing.[61]

These findings support the hypothesis that Nramp1 controls the replication of intracellular parasites by altering the intravacuolar environment of the phagosome. In fact, it was recently shown that Nramp1 functions as a pH-dependent manganese transporter.[62] Divalent cations like manganese are likely to be important for the survival of pathogens, and removal of these from the phagosome probably results in enhanced bacteriostatic or bactericidal activity and hence in increased resistance to intracellular pathogens. Nramp orthologues have been identified in *Salmonella* (MntH)

and other bacteria. They are also proton-dependent manganese transporters and appear to function as an import system for the acquisition of divalent metals from the extracellular environment.[63] *Salmonella* MntH and mammalian Nramp1 proteins might influence the outcome of bacterial infection through competition for the same essential substrates within the microenvironment of the phagosome.[55]

Because of the critical role of Nramp1 in the mouse model of typhoid fever, the human homologue of mouse *NRAMP1* was investigated in resistance to typhoid fever in humans. Despite the fact that *NRAMP1* contributes clearly to the risk and the progression of mycobacterial infections in humans,[7,64–66] no allelic association was identified between *NRAMP1* and typhoid fever susceptibility in humans in southern Vietnam.[67] However, these studies do not preclude a role for *NRAMP1* in susceptibility to human *Salmonella* infections because of the known effect of the ethnic genetic background on the expression of disease susceptibility. The role of allelic variation at *NRAMP1* was also examined in a chicken model of *Salmonella* infection.[68] Salmonellosis in young chickens is a major systemic disease resulting in economic losses for the poultry industry. In adult chickens, *Salmonella* infection does not cause significant clinical signs or mortality, therefore constituting an insidious risk for human health. In chickens, resistance to infection with *Salmonella* Typhimurium is inherited as a complex trait.[68,69] Using crosses between resistant (W1) and susceptible (C) chickens, *NRAMP1* was shown to account for 18% of the early differential resistance to infection.[68] A genome scan performed more recently on the same animal panel clearly showed that the region surrounding *NRAMP1* has a major impact on the susceptibility of chickens to *Salmonella* infection and proves the utility of comparative genomics in studying host resistance to infection (V Forgetta and D Malo, unpublished data).

Tlr4

Toll-like receptor 4 (Tlr4; first described as the *Lps* locus) is another critical gene that regulates innate resistance to infection with *Salmonella* Typhimurium and the host response to LPS in mice. Bacterial LPS is a major constituent of the outer membrane of Gram-negative bacteria and is essential to the structure and survival of these bacteria (reviewed in Ref. [70]). Through evolution, the immune system of eucaryotes has learned to recognize LPS as an indicator of Gram-negative bacterial infection. Indeed, very small amounts of LPS are able to initiate a robust inflammatory response in the host. LPS molecules are PAMP that are naturally recognized through receptors of the innate immune system known as PRRs (reviewed in Ref. [71]). The Toll-like receptor (TLR) family is a good example of PRRs and, among them, Tlr4 was identified as an important component of the signal transduction initiated by LPS.

The discovery of Tlr4 was rendered possible because of earlier identification of LPS hyporesponsiveness of C3H/HeJ mice.[72] C3H/HeJ mice can withstand 20–38 times the LD_{50} for other inbred strains when challenged intravenously with LPS and show an altered intraperitoneal inflammatory response with a reduced ratio of neutrophils to mononuclear cells following local injection of LPS.[72,73] However, if challenged with Gram-negative bacteria such as *Salmonella* Typhimurium, C3H/HeJ mice present a markedly increased susceptibility to this pathogen. The LD_{50} for LPS-responsive mice to *Salmonella* Typhimurium infection is $\geqslant 2 \times 10^3$, while LPS hyporesponsive strains succumb to infection with less than two organisms.[74] LPS hyporesponsiveness was also recognized in other strains including C57BL/10ScCr,[75] its progenitor C57BL/10ScN[76] and more recently in C57BL/6.KB2-mnd.[77] The failure of (C3H/HeJ × C57BL/10ScCr)F_1 and (C3H/HeJ × C57BL/6.KB2-mnd)F1 to respond to LPS suggests that these three strains carry mutations at the same gene.[77,78] Segregation analysis in backcrosses between responsive and hyporesponsive strains revealed that this phenotype was inherited as a simple Mendelian trait.[79,80] The locus was named *Lps*, and two alleles were defined: *Lps*^{n} and *Lps*^{d} for normal and defective response to LPS respectively.[81] The mode of inheritance varied with the phenotype studied and the strain combination used: heterozygote mice issued from C3H/HeJ and C57BL/6J present an intermediary LPS response consistent with a codominant mode of inheritance,[80] whereas crosses made between C57BL/10ScCr and *Lps*^{n} mice show a fully dominant wild-type allele.[78] The response to *Salmonella* infection was inherited as a single dominant trait in all crosses performed between *Lps*^{d} and *Lps*^{n} mice.[74,82]

Early linkage analysis studies revealed that *Lps* cosegregated with the major urinary protein locus (*Mup-1*) and the polysyndactyly (*Ps*) mutation indicating that *Lps* is located on mouse chromosome 4.[80,81] High-resolution genetic, physical and transcriptional maps of the area were thereafter generated[82,83] and led to the identification of *Tlr4* as a candidate for *Lps*.[83,84] Three different *Tlr4* mutant alleles were identified: C3H/HeJ mice present a single missense mutation resulting in a proline for histidine substitution at codon 712 within the signaling domain;[84,85] in C57BL/10ScCr mice, there were no *Tlr4* transcripts detected[84,85] as a consequence of a 75 kb chromosomal deletion encompassing the whole Tlr4 gene;[86] the mutation identified in C57BL/6.KB2-*mnd Tlr4* consists in a complete deletion of exon II. This mutation leads to a frameshift resulting in the appearance of a stop codon just downstream of the exon junction. The putative Tlr4 mutant protein is equivalent to the first 31 N-terminal residues of its wild-type counterpart (835 residues).[77] Confirmation of the role of *Tlr4* in LPS hyporesponsiveness was obtained through examination of mice that had been rendered deficient for *Tlr4*.[87]

The TLR family (reviewed in Refs. [71] and [88]) is composed of type 1 TM receptors characterized by an extracellular leucine-rich repeat domain and an intracellular domain similar to the intracellular domain of the interleukin (IL)-1 receptor called the TIR (Toll/IL-1 receptor) domain. The first identified member of this family, *Drosophila Toll*, functions in a pathway that controls the dorso-ventral axis formation of the fly (reviewed in Ref. [89]). In adults, mutations in *Drosophila Toll* lead to increased susceptibility to fungal infection because of failure to induce the antifungal peptide Drosomysin, thereby linking this gene to innate immunity. In mammals, at least 10 TLRs have been identified and some of them have been shown to be essential for defense against different pathogens by sensing specific PAMPs. TLR4 appears to interact directly with LPS with

the cooperation of LBP (LPS-binding protein) and co-receptors CD14 and MD-2.[90–92] Ultimately, LPS sensing by TLR4 leads to the activation of the transcription factor NF-κB and the MAP kinases, JNK and p38, through the activation of two known signaling pathways: a common TLR signaling MyD88-TOLLIP-IRAK-TRAF6 pathway (reviewed in Ref.[71]) and an MyD88-independent pathway involving the adapter protein TIRAP.[93,94] Activation of TLR4 by LPS leads to the induction of various host defense genes including pro-inflammatory cytokines such as IL1, IL6, IL8 and IL12, chemokines, costimulatory molecules (CD80 and CD86), MHC class II and NOS2 by APC cells.[95–97] Induction of CD80/CD86 and IL-12 by TLRs contributes to the initiation of adaptive immunity and the induction of TH1 effector responses.[97]

btk

The role of B lymphocytes in immunity to *Salmonella* Typhimurium was studied initially in CBA/N inbred mice, a strain that has impaired humoral immunity because of a peripheral defect in B-cell activation and function (reviewed in Ref. [98]). This defective phenotype was mapped to chromosome X and named *xid* for x-linked immunodeficiency. The B-cell defect of CBA/N mice is characterized by an impaired maturation of B cells, diminished immunoglobulin production (CBA/N mice have low serum IgM and IgG_3 levels) and compromised T-independent immune response. Because of their immunodeficiency, CBA/N mice present a late susceptibility to infection with pathogens such as *Salmonella* Typhimurium.[28] Susceptibility of *xid* mice to *Salmonella* Typhimurium is recessive; hemizygous males and homozygous females present the susceptibility phenotype while heterozygous females are resistant[28] due to preferential inactivation of the X chromosome carrying the defective *xid* allele.[99] Passive transfer of immune serum restores resistance in affected males, an effect attributed to the presence of specific anti-*Salmonella* antibodies.[100] These results clearly indicated a role for circulating antibodies in resistance to *Salmonella* Typhimurium during the late (>10 days) phase of infection.

The *xid* mutation was localized to a region of mouse chromosome X showing conserved homology with a region of the human genome carrying the gene involved in X-linked agammaglobulinemia (XLA),[101] a disease (reviewed in Ref. [102]) that resembles the phenotype expressed in *xid* mice. Male patients with XLA have a severe B-cell immunodeficiency characterized by reduced numbers of mature circulating B cells, diminished serum Ig levels and disrupted secondary lymphoid architecture. Intestinal infections with *Salmonella* spp have been described in XLA patients; however, bacterial infections involving the respiratory tract caused by *Streptococcus pneumoniae, Haemophilus influenzae, Staphylococcus aureus* and *Pseudomonas* spp are the most frequent infections.

In 1993, mutations in the gene encoding Bruton's tyrosine kinase (btk) were reported to cause *XLA* in humans[103,104] and *xid* in mice.[101,105] btk belongs to the Tec family of non-receptor protein-tyrosine kinases, known to be highly expressed in hematopoietic cells (reviewed in Ref. [106]). *btk* is expressed at all stages of B-cell development from pro- to mature B cells, but is down-regulated in plasma B cells. Its expression occurs also in erythroid precursors, myeloid cells, mast cells and megacaryocytes but not in T or NK cells. btk participates in intracellular signal transduction in a number of cell activation pathways such as those mediated by the B-cell antigen receptor (BCR) and the Toll-like receptor RP105.[107] *btk* encodes a tyrosine kinase that possesses pleckstrin-homology (PH) and Tec-homology (TH) domains in addition to src homology (SH) domains (SH1, SH2 and SH3).[108] btk, as a major component of the BCR signalosome, plays a critical role in the regulation of pre-B and mature BCR signaling.[109–111] Recruitment of btk to the cellular membrane and its subsequent activation triggers the mobilization of intracellular calcium and the activation of PKC, resulting in the degradation of the NF-κB inhibitory protein I-κBα and the translocation of NF-κB to the nucleus.[112–114] In humans, more than 175 different mutations involving all domains of the btk gene have been identified in XLA patients.[102] In *xid* mice, a missense mutation at a conserved arginine residue (R28C) within the PH domain of btk impairs its ability to translocate to the plasma membrane and trigger signaling cascades that regulate B-cell survival and growth,[101,105,111] consequently affecting resistance to infection with *Salmonella*.[28]

Salmonella resistance loci identified using gene-deficient mice

The use of gene targeting has been very successful for investigating the role of several *Salmonella* resistance genes in mice. Candidate genes have been selected based on the biological understanding of the disease phenotype or based on the *in vitro* response to the pathogen. In the following sections, we will discuss selected critical genes whose role in mouse resistance to *Salmonella* infection was uncovered by engineering knockout mice.

Lbp and Cd14

Lbp and Cd14, like Tlr4, are known to bind LPS and have been involved in innate defense against *Salmonella* Typhimurium. Lbp is an acute-phase protein found in the serum that accelerates the binding of LPS to Cd14 and initiates signals through membrane-bound Tlr4 in monocytes and myeloid cells (reviewed in Ref. [115]). Work with Lbp-deficient mice has shown that Lbp is essential for the induction of a rapid inflammatory response and for survival following intraperitoneal infection with *Salmonella* Typhimurium.[116] The critical function of Lbp in resistance to infection was confirmed by the rescue of the susceptible $Lbp^{-/-}$ mice with recombinant mouse Lbp supplementation.[117]

Cd14 is a glycosylphosphatidylinositol-anchored molecule that is expressed on monocytes and neutrophils and acts as a high-affinity receptor for LPS. Cd14-deficient mice were found to be extremely resistant to the effect of LPS, with 100% survival and almost no detectable clinical signs following challenge with 10 times the LD_{100} for control mice. This increased resistance correlated with markedly decreased expression of cytokines such as TNFα and IL6.[118] Interestingly, Cd14-deficient mice were also more resistant to Gram-negative (*Escherichia coli*) bacterial challenge with a decreased level of bacteremia, suggesting a role for Cd14 in bacterial dissemination.[120] However, in the case of the Gram-negative intracellular pathogen *Salmonella* Typhimurium,

Cd14, as for Tlr4, is essential to improve survival to infection.[119]

NADPH oxidase and Nos2

Following phagocytosis of virulent *Salmonella* Typhimurium, two major enzyme systems come into play to inactivate the pathogen within the phagosome: the phagocytic nicotinamide dinucleotide phosphate (NADPH) oxidase and the inducible nitric oxide synthase (Nos2). NADPH oxidase and Nos2 participate in the generation of reactive oxygen intermediates (ROI) and reactive nitrogen intermediates (RNI) respectively (reviewed in Refs. 121 and 122).

The importance of NADPH oxidase in resistance to *Salmonella* infection was originally uncovered in humans suffering from CGD, a group of inherited disorders characterized by recurrent infections and chronic inflammation. The disease results from mutations in any one of four subunits of the NADPH oxidase. The majority of patients with CGD present X-linked deficiencies of $gp91^{phox}$ (renamed CYBB); most autosomal recessive CGD are associated with mutations within $p47^{phox}$ (*NCF1*) or $p67^{phox}$ (*NCF2*); a rare autosomal recessive form is associated with mutations within $p22^{phox}$ (CYBA) (reviewed in Ref. [123]). Mice deficient in $gp91^{phox}$ were engineered as a model for CGD and found to be extremely susceptible to infection with *Salmonella* Typhimurium.[124,125] Early death of $gp91^{phox}$-deficient mice after *Salmonella* infection was associated with spleen and liver bacterial load exceeding by at least 3 log the wild-type controls.[124]

Studies with Nos2-deficient mice support a dual role for NO during virulent *Salmonella* infection *in vivo*. Enhanced production of NO provides increased host defense against pathogens but also contributes to inflammation, tissue damage and even endotoxic shock.[124,126,127] Although *Nos2* knockout mice are able to control the early replication of *Salmonella* in the RES organs, they are unable to suppress bacterial growth later during infection and eventually die.[124] This observation contrasts with the $gp91^{phox}$-deficient mice which are extremely susceptible to *Salmonella* Typhimurium early during the course of infection even with a very low inoculum.[124]

It is clear from these studies that intracellular killing of *Salmonella* Typhimurium is dependent on both ROI and RNI systems; however these reactive intermediates appear to act at different stages of infection (NADPH oxidase being more critical early after infection and Nos2 later during infection). Double mutant mice ($gp91^{phox-/-}$/$Nos2^{-/-}$) exhibited spontaneous infections caused by organisms of the normal flora, resulting in the formation of large internal abscesses.[125] This phenotype was not exhibited by mice deficient in only one of the enzyme activities, indicating that in spontaneous infections with indigenous flora, $gp91^{phox}$ and Nos2 can compensate for each other and that no other pathway could compensate for their simultaneous absence.

The critical role of phagocyte NADPH oxidase and Nos2 was exemplified by recent work showing how *Salmonella* have evolved strategies to circumvent the action of the bactericidal activities of RNI and ROI. In macrophages, *Salmonella* inhibit the fusion of SCV with endosomes and lysosomes[128] and prevent the localization of NADPH oxidase[129,130] and Nos2[131] with SCVs, thereby protecting the bacteria from the microbicidal effect of ROI and RNI.

Cytokines: Tnf, Ifng and Il12

The release of pro-inflammatory cytokines from activated T cells, NK cells and macrophages is pivotal in controlling the primary immune response to *Salmonella*. TNFα (reviewed in Ref. [132]) is a pleiotropic pro-inflammatory cytokine produced mainly by macrophages but also by activated NK cells and TH1 lymphocytes. It is encoded by the *Tnf* gene and exerts its effects through two types of receptors: TNFRp55 (encoded by *Tnfrsf1a* for TNF receptor superfamily 1a gene) and TNFRp75 (*Tnfrsf1b*). This cytokine plays a key role in host defense against pathogens through several mechanisms including activation of neutrophils and platelets, enhancement of killing activity of macrophages and NK cells, and activation of the immune system. Mice carrying a targeted disruption of *Tnfrsf1a* were found to be more susceptible to challenge with virulent *Salmonella* Typhimurium and to attenuated *purE*, *aroA* and *sseB* mutants.[133,134] The early susceptibility of $Tnfrsf1a^{-/-}$ mice to *Salmonella* was attributed to a defect in the early bactericidal capacity of $Tnfrsf1a^{-/-}$ macrophages.[134] Although this early phase of bacterial killing within the macrophages is associated with activation of the NADPH oxidase system, comparable levels of superoxide were detected within the infected macrophages of normal and knockout mice. Further analysis, using fluorescence microscopy and transmission electron microscopy, showed that TNFRp55 is necessary for targeting of NADPH phagocyte oxidase-harboring vesicles to SCVs.[134]

INFγ (reviewed in Ref. 15) is produced by activated T cells and NK cells following IL12 stimulation, and plays a key role in TH1 responses. INFγ is responsible for activating macrophages and influences also the antibody class switching. The growth of attenuated *Salmonella* Typhimurium $aroA^{-}$ is contained in wild-type mice; however INFγ-deficient mice succumb to infection due to unrestricted bacterial proliferation.[33,135] These experiments point out the central role that INFγ plays in mice against bacterial strains of poor virulence. Deficiencies in the IFNγ axis are not only associated with higher susceptibility to infection with *Salmonella* in mice but also with increased susceptibility to other intracellular pathogens (reviewed in Ref.[136]).

IL12 (reviewed in Refs. 14 and 15) is a heterodimeric cytokine composed of two subunits, IL12p35 (encoded by *Il12a*) and IL12p40 (encoded by *Il12b*), linked by two disulfide bonds. IL12 is produced and secreted mainly by antigen-presenting cells (dendritic cells and macrophages). The IL12 receptor (composed of two subunits IL12Rβ1 and IL12Rβ2) is found predominantly on T and NK cells. The principal known function of IL12 is the induction of IFNγ and consequently the development of TH1 responses. Administration of monoclonal antibodies directed against IL12 exacerbates the mild disease usually caused by *Salmonella* Typhimurium $aroA^{-}$ in BALB/c mice, eventually leading to death.[137] The administration of anti-IL12 antibodies resulted in decreased local and systemic IFNγ concentration, lower tissue Nos2 activity and increased serum IL10 levels in infected mice. The role of IL12 in resistance to *Salmonella* infection was also studied in mice carrying targeted

disruptions of genes encoding for either subunits of IL12, *Il12a* or *Il12b*.[138] In a model of systemic infection with *Salmonella* Enteritidis, *Il12a*$^{-/-}$ and *Il12b*$^{-/-}$ mice were more susceptible to infection than wild-type mice. However, the increased susceptibility was more pronounced in *Il12b*$^{-/-}$ mice. Lack of *Il12b* resulted in a TH2 response, which was inadequate for immunity against the intracellular pathogen *Salmonella* Enteritidis.

Pathogenic mutations in genes coding for the IFNγR1, IFNγR2, IL12p40 and IL12Rβ1 were reported in the syndrome of Mendelian susceptibility to mycobacterial disease (reviewed in Refs, [14] and [15]). These pediatric patients were first identified because of their susceptibility to poorly virulent mycobacterial species such as BCG. The patients rarely develop other infectious diseases with the exception of *Salmonella* infections that are found in almost half of the cases. The clinical manifestations of the disease are heterogenous and range from abdominal abscesses and adenitis to severe sepsis. Several *Salmonella* serotypes have been identified and include *Salmonella* Paratyphi[139,140] or non-typhoid *Salmonella* serotypes such as *Salmonella* Typhimurium,[140] *Salmonella* Enteritidis,[139,141–143] *Salmonella* group B[139,140] or untyped *Salmonella*.[139,144,145]

Host resistance loci identified using QTL analysis

Other loci influencing resistance or susceptibility to *Salmonella* infection in mice have been identified using QTL analyses.[19,20] The wild-derived inbred mice, *Mus musculus molossinus* (MOLF/Ei), were initially found to be extremely susceptible to *Salmonella* Typhimurium with survival time comparable to the survival time of C57BL/6J (*Nramp1*^{s}). Subsequently, linkage analysis using 252(C57BL/6J × MOLF/Ei)F2 allowed the mapping of two QTLs, which significantly affect survival time following lethal infection with *Salmonella* Typhimurium.[20,146] A *Salmonella*-resistant phenotype (*Ity2*) was linked to a region on mouse chromosome 11 (LOD score of 7.0 at *D11Mit5)* and contributed 10% to the variance. Several candidate genes were detected in the surrounding region including granulocyte/macrophage colony-stimulating factor (*Csfgm*), interleukin 3 (*Il3*), inducible nitric oxide synthase (*Nos2*) and myeloperoxydase (*Mpo*). The candidacy of *Nos2* was evaluated by measuring *Nos2* mRNA levels and nitrite production in MOLF/Ei mice during infection. MOLF/Ei mice showed a decreased capacity to induce *Nos2* mRNA and to produce NO.[147] As mentioned earlier in this review, studies with *Nos2*-deficient mice support a dual role for NO during virulent *Salmonella* infection *in vivo*. Enhanced production of NO provides increased host defense against pathogens but also contributes to inflammation, tissue damage and even endotoxic shock (reviewed in Ref.[148]). Although *Nos2* knockout mice are able to control the early replication of *Salmonella* in the RES organs, they were unable to suppress bacterial growth and eventually die from infection.[124] In this model, low *Nos2* mRNA levels correlate with a reduced NO production and a decreased inflammatory response, a finding that may explain the protective effect of *Ity2* on chromosome 11.

A second QTL (*Ity3*) conferring recessive susceptibility was located on mouse chromosome 1, approximately 25 cM distal to *Nramp1* (LOD score of 4.8 at *D1Mit100*). The effect of this locus was identified only after adjustment for the effects of *Nramp1*, and contributed to 7% of the variance in survival time. The chromosomal region harboring *Ity3* is also rich in candidate genes including several genes involved in complement activation (*C4bp*, *Cfh* and *Daf1*), inducible prostaglandin synthase 2 gene (*Ptgs2*) and a member of the Toll-like receptor family, *Tlr5*. The candidacy of *Tlr5* as the gene underlying *Ity3* was assessed by mapping, expression and sequencing analyses.[146] *Tlr5* is expressed predominantly in the liver, a main site for the proliferation of *Salmonella* in MOLF/Ei. Analysis of *Tlr5* mRNA expression during infection of mice with *Salmonella* Typhimurium shows that *Tlr5* mRNA levels in the liver are consistently lower in MOLF/Ei than in classical inbred mouse strains including C57BL/6J, 129S6/SvEvTac, C3H/HeJ and C57BL/10J. There is at least a 50% reduction in the amount of *Tlr5* mRNA expressed in MOLF/Ei mice throughout infection. Finally, *Tlr5* sequence analysis in MOLF/Ei and 47 other inbred strains shows various sequence variants that define a unique *Tlr5* haplotype in MOLF/Ei mice associated with a lower level of *Tlr5* mRNA expression.[146] It is possible to envisage that low levels of *Tlr5* expression may lead to the inability of MOLF/Ei mice to regulate a proper immune response to *Salmonella* Typhimurium. Most importantly, *Tlr5* has been shown to mediate the innate immune response to bacterial flagellin from *Salmonella* Typhimurium.[149,150] Flagellin is a structural component of bacterial flagella and a virulence factor recognized by the host innate immune system. Activation of *Tlr5* by flagellin mobilizes the nuclear factor NFκβ and stimulates TNFα and IL6 production.

In another study, a chronic model of infection with *Salmonella* Enteritidis was used to identify loci implicated in the late bacterial clearance of the organism from the spleen.[19] Two strains, C57BL/6J (*Nramp*^{s}) and 129S6/SvEvTac (*Nramp1*^{r}), were characterized according to their ability to clear *Salmonella* Enteritidis from the spleen. 129S6/SvEvTac mice were found to have markedly impaired ability to clear the parasite when compared to C57BL/6J. A genome scan performed on 302 (C57BL/6J × 129S6/SvEvTac) F2 progeny identified three dominant loci associated with the phenotype. The loci, designated *Ses1*, *Ses2* and *Ses3* (*Salmonella* Enteritidis susceptibility), were located on chromosome 1 (*D1Mcg5*), 7 (*D7Mit62*) and 15 (*D15Mit29*) respectively. *Ses1* showed the strongest LOD score (9.9), contributed to 14% of the phenotypic variance and mapped in the area of *Nramp1*. *Ses2* and *Ses3* had smaller effects with respective LOD scores of 4 and 3.2. The effect of all three loci on bacterial clearance was greater in females than in males. In this model of chronic infection with *Salmonella* Enteritidis, functional polymorphism at *Nramp1* was associated with increased bacterial clearance during the late phase of infection. In addition to its specific role as a cation transporter,[62] Nramp1 has been associated with regulation of macrophage activation as measured by the production of nitric oxide, IL1β, INFγ and MHC class II expression[151] and TH1/TH2 differentiation.[152] In humans, a dual role for *Nramp1* has also been reported in acute and chronic infection with the intracellular pathogen *Mycobacterium leprae*.[64,66]

Conclusion

Resistance to *Salmonella* infection is a complex biological trait controlled by several host genes along with bacterial virulence factors. Mouse models of *Salmonella* infection have contributed substantially to the identification of *Salmonella* resistance genes and to our understanding of the complex interaction between the host and the microbe genomes *in vivo*. For the past 50 years, identification of *Salmonella* resistance genes was facilitated by the availability of unique mouse mutants that present *Salmonella* susceptible phenotypes inherited as simple Mendelian traits. Now the future of mouse genetics in the study of host resistance to *Salmonella* and other infections is moving toward the identification of inbred mouse strains that show a complex mode of inheritance to specific pathogens for QTL gene identification.[19,20,153–160] Dissection of the complex host response to *Salmonella* infection combined with the complete mouse genome sequence will contribute further to our understanding of the genetic control of host immunity.

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APPENDIX 2: Publications and Presentations

Publications

Roy MF, Riendeau N, Bédard C, Hélie P, Canonne-Hergaux F, Gros P and Malo, D. Pyruvate kinase deficiency confers susceptibility to *Salmonella* Typhimurium infection in mice. (Manuscript in preparation)

Roy MF, Riendeau N, Loredo-Osti JC, Malo D. Complexity in the host response to *Salmonella* Typhimurium infection in AcB and BcA recombinant congenic strains. (Accepted for publication)

Roy MF, Lariviere L, Wilkinson R, Tam M, Stevenson MM, Malo D. Incremental expression of Tlr4 correlates with mouse resistance to Salmonella infection and fine regulation of relevant immune genes. *Genes Immun* 2006; **7**: 372-383.

Desrochers AM, Dolente BA, **Roy MF**, Boston R, Carlisle S. Efficacy of Saccharomyces boulardii for treatment of horses with acute enterocolitis. *J Am Vet Med Assoc* 2005; **227**: 954-959.

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Roy MF, Parente EJ, Donaldson MT, Habecker P, Axon J. Successful treatment of a colonic adenocarcinoma in a horse. *Equine Vet J* 2002; **34**: 102-104.

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Léguillette R, Lavoie JP, **Roy MF**. Obstruction oesophagienne causée par un clou traitée par oesophagostomie et nutrition entérale chez un cheval. *Médecin vétérinaire du Québec* 1998; **28**: 87-90.

Presentation (oral and poster)

Roy MF, Wilkinson R, Larivière L, Laroche L, Gros P, Malo D. Dissection génétique de la réponse de l'hôte à l'infection à Salmonella Typhimurium chez les souris recombinantes congéniques A/J et C57BL/6J. ACFAS. Montréal, Québec (2006).

Roy MF, Riendeau N, Laroche L, Wilkinson R, Larivière L, Gros P, *et al.* Complexity in the host response to *Salmonella* Typhimurium infection revealed through the study of AcB and BcA recombinant congenic strains. Complex Trait Consortium. Chapel Hill, NC (2006).

Roy MF, Loredo-Osti JC, Bédard C, Wilkinson R, Larivière L, Laroche L, *et al.* Genetic dissection of the mouse response to *Salmonella* Typhimurium using recombinant congenic strains. 19th International Mouse Genome Conference. Strasbourg, France (2005).

Roy MF, Wilkinson R, Larivière L, Laroche L, Tam MF, Stevenson M, *et al.* Tlr4 expression and the host response to *Salmonella* Typhimurium infection in mice. Canadian Bacterial Disease Network Annual Meeting. Banff, Alberta (2005).

Roy MF, Wilkinson R, Larivière L, Laroche L, Tam MF, Stevenson M, *et al.* Tlr4 expression and the host response to *Salmonella* Typhimurium infection in mice. 12th Internaltional Congress of Immunology and 4th Annual Conference of FOCIS. Montreal, Quebec, Canada. (2004).

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Dear Dr. Malo,

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