The Genetic Dissection of the Host Immune Response to *Salmonella* Typhimurium Infection in the Wild-derived Mouse MOLF/Ei

Maria Vanessa Sancho Shimizu

Department of Human Genetics

McGill University, Montreal, Canada

August 2006

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

Your file Votre référence
ISBN: 978-0-494-32381-6
Our file Notre référence
ISBN: 978-0-494-32381-6

ABSTRACT

Salmonella infections remain a global health concern exacerbated by the emergence of multidrug resistant strains. Host genetics have been demonstrated to influence the immune response to *Salmonella* infections. Adopting the susceptible wild-derived MOLF/Ei mice as a model of systemic *Salmonella* Typhimurium infection has previously identified a resistance locus *Ity2* and a susceptibility locus *Ity3*.

Both the *Ity2* and *Ity3* loci were validated through the creation of congenic mice and through linkage confirmation in a new segregating cross. Refinement of the critical intervals was achieved using subcongenic mice. Transcriptional profiling of the congenic mice identified differentially regulated candidate genes within the two loci that can be examined for potential relevance to *Salmonella* infection in MOLF/Ei mice.

Previous studies proposed *Tlr5* as a candidate for *Ity3* based on the sequence variants and reduced expression in MOLF/Ei liver. *Tlr5* function was characterized *in vitro* and *in vivo* using the *Ity3* congenic strains. Both analyses pointed towards the exclusion of this gene as a candidate for *Ity3* due to discordant functional responses between MOLF/Ei and the *Ity3* congenics.

Ncf2 was pursued as a candidate for the *Ity3* locus based on its map position and its antimicrobial role in *Salmonella* infection. Sequencing of *Ncf2* led to the identification of one non-conservative amino acid in a conserved domain of the protein. Functional analysis indicated a reduced response attributed to the MOLF/Ei allele, suggesting a potential involvement of *Ncf2* in *Salmonella* pathogenesis of *Ity3* congenic mice.

Taken together, we have been able to narrow down the critical intervals and confirm the contribution of *Ity2* and *Ity3* to *Salmonella* infection. We have eliminated *Tlr5* but highlighted the potential involvement of *Ncf2* for the *Ity3* locus. The genetic dissection of the host response in MOLF/Ei mice has captured the inherent complexity of the immune response towards *Salmonella* infection. Our findings have also revealed the genetic and phenotypic diversity of the wild-derived MOLF/Ei mice. Furthermore, a complete genome scan of the additional informative cross as well as the validation of candidates identified here, should resolve of the intricacies of the susceptibility of MOLF/Ei mice and contribute to our understanding of *Salmonella* pathogenesis.

RÉSUMÉ

Les infections à *Salmonella* constituent un problème de santé public mondial, exacerbé par l'émergence de souches multirésistantes aux antibiotiques. Il a été démontré que le fond génétique de l'hôte influence sa réponse aux infections à *Salmonella*. Le locus de résistance, *Ity2,* et le locus de susceptibilité, *Ity3*, ont été antérieurement identifiés chez la souris dérivée de lignée sauvage et susceptible MOLF/Ei, dans un modèle d'infection systémique à *Salmonella* Typhimurium.

L'existence des loci *Ity2* et *Ity3* a été validée par la création de souris congéniques et par la confirmation d'une liaison génétique au moyen d'un nouveau croisement ségrégué. Le raffinement des intervalles critiques a été réalisé en utilisant des souris sous-congéniques. Le profil transcriptionnel des souris congéniques a permis d'identifier des gènes candidats régulés de façon différentielle au sein des deux loci, lesquels seront étudiés pour leur implication potentielle au cours d'une infection à *Salmonella* chez la souris MOLF/Ei.

Des études antérieures ont proposé *Tlr5* comme gène candidat pour *Ity3* en s'appuyant sur les variants séquentiels et une expression réduite dans le foie des souris MOLF/Ei. La fonction de *Tlr5* a été caractérisée *in vitro* et *in vivo* en utilisant les lignées congéniques *Ity3*. Ces deux analyses tendent à exclure ce gène comme candidat pour *Ity3*, en raison de réponses fonctionnelles discordantes qui ont été observées entre les souris MOLF/Ei et les souris congéniques *Ity3*.

Ncf2 a été étudié comme gène candidat pour le locus *Ity3*, étant donné sa position sur la carte physique et son rôle antimicrobien lors d'infections à *Salmonella*. Le séquençage de *Ncf2* a mené à l'identification d'une mutation de l'acide R394Q dans un domaine hautement conservé de la protéine. L'analyse fonctionnelle de l'allèle MOLF/Ei

par utilisation de souris congéniques *Ity3* a montré une réponse moindre, suggérant une implication possible de ce gène dans la pathogénèse de *Salmonella* chez les souris congéniques *Ity3*.

Nous avons réduit les intervalles critiques et confirmé la contribution de ces loci à l'infection à *Salmonella* par l'utilisation de souris congéniques et à travers un autre croisement. Nous avons éliminé *Tlr5*, mais souligné le rôle potentiel de *Ncf2* pour le locus *Ity3*. La dissection génétique de la réponse de l'hôte à l'infection à *Salmonella* chez MOLF/Ei souligne la complexité inhérente de la réponse immunitaire. Nos résultats ont également révélé la diversité génétique et phénotypique des souris dérivées de la lignée sauvage MOLF/Ei. En outre, le criblage complet du génome d'un croisement informatif additionnel ainsi que la validation des candidats identifiés devraient aider à expliquer la susceptibilité des souris MOLF/Ei à *Salmonella*.

ACKNOWLEDGEMENTS

First and foremost, I would like to thank my supervisor Dr Danielle Malo, for giving me this opportunity to work with her in the lab. I appreciate the endless encouragement, support, mentorship, guidance and faith in my project.

I'd like to thank all the present and past members of the lab, Giovanna, Judith, Gary, Vince, Laurent, Isabelle, Marie France, Etienne, Mayss, Line Larivière, Line Laroche, Rosalie, Noémie, Beatrice, Jacinthe, Amanda, and Geneviève, for making life fun in the lab, and for their technical assistance.

Serge, Caroline, Line, Inge and Scott for the editing and final preparations of the thesis.

My friends going through this with me especially Inge, Caroline, Emily, and others at the MGH - Scott, Loic, Liz, for their friendship and support.

To my supervisory committee members, Dr Tonin, Dr Schurr and Dr Morgan for their advice and guidance.

To Laura, Kandace and Fran at the Human Genetics Department for their invaluable assistance.

Everyone at the animal facility at the MGH, for putting up with my "crazy" mice.

The McGill Univiersity Health Center Research Institute Fellowship, McGill Univeristy Graduate Student Fellowship, Faculty of Medicine Internal Studentship, A. and R. Pietrangeli Memorial Travel Award, and the International Mouse Genome Conference Scholarship Award for financial support during my PhD.

Serge for your love, support, and inspiration to continue along the paths we have chosen.

Last but not least, my family, Mom, Dad, David and Mikel, for their continued and dedicated support from miles away, without which I could have never made it to where I am today. No words are enough to thank you.

TABLE OF CONTENTS

CONTRIBUTION OF AUTHORS

This dissertation is comprised of five chapters, Chapter one representing the General Introduction, Chapters 2-4 constitute full length manuscripts, followed by a Discussion in Chapter 5. Chapters 3 and 4 represent published manuscripts in peer-reviewed journals.

The second Chapter entitled "Molecular genetic analysis of two loci (*Ity2* and *Ity3*) involved in the host response to infection with *Salmonella* Typhimurium using congenic mice and expression profiling" represents a manuscript in preparation. Most aspects involving *Ity3* microarray experiments, ranging from the printing of the arrays, RNA labeling, array hybridizations, to data analysis for the *Ity3* oligonucleotide array were carried out in collaboration with Serge Mostowy, under the supervision of Dr Marcel Behr. Line Lariviere and Noemie Riendeau assisted in genotyping of mice. The primary author was responsible for work including the creation, and maintenance of congenic mouse strains as well as the generation of F2 progeny, coordination of infections, tissue collection, RNA extraction, bacterial load determination, real time PCR validation, sequencing, and in the writing and preparation of the manuscript, under the supervision of Dr Danielle Malo.

Chapter 3 entitled "*Tlr5* is not primarily associated with susceptibility to *Salmonella* Typhimurium infection in MOLF/Ei mice" was published in Mammalian Genome 17(5): 385-397 (Sancho-Shimizu et al. 2006). Isabelle Angers was in charge of the construction of plasmids, transfections and luciferase assays involved in the *in vitro* testing of the *Tlr5* alleles. Dr Andrew Gewirtz contributed purified flagellin and the IL-8 CAT vector used in the study. Dr Albert Descoteaux assisted in the coordination of the *in*

vitro experiments. I was in charge of the *in vivo* testing of the *Tlr5* alleles including generation and maintenance of all mouse strains used in the experiment, as well as in the tissue collection during *Salmonella* infection, RNA extraction, real time PCR of *Tlr5* expression, and injection of mice with flagellin. Manuscript preparation was shared with Isabelle Angers, however I was the primary editor of the manuscript, under the supervision of Dr Danielle Malo.

The work in Chapter 4, "Sequence, expression and functional analyses support the candidacy of *Ncf2* in susceptibility to *Salmonella* Typhimurium in wild-derived mice", was published in the Journal of Immunology 176(11): 6954-6961 (Sancho-Shimizu and Malo 2006). All experiments presented in this paper in addition to the preparation of the manuscript were carried out by the primary author under the supervision of Dr Danielle Malo.

LIST OF ABBREVIATIONS

The gene nomenclature used in this dissertation is in accordance with the rules and guidelines provided by the Human Gene Nomenclature Committee (HGNC) (http://www.gene.ucl.ac.uk/nomenclature/guidelines.html) and the International committee on Standardized Genetic Nomenclature for Mice (http://www.informatics.jax.org/mgihome/nomen/index.shtml last updated January, 2005) (Wain et al. 2002).

Ankrd43	ankyrin repeat domain 43
129S6	129S6/SvEvTac
18srRNA	18S ribosomal RNA
AP-1	activating protein-1
APAF	apoptotic peptidase activating factor 1
ASC	apoptosis-associated speck-like protein containing a CARD
ATCC	American type culture collection
B6	C57BL/6J
BCG	Bacille Calmette-Guerin
Bcl2	B-cell leukemia/lymphoma 2
bp	base pairs
BSA	bovine serum albumin
Btk	Bruton's tyrosine kinase
Butr1	butyrophilin related 1
C/EBPβ	CCAAT enhancer binding protein
CARD	caspase activation and recruitment domain
Casp1	caspase-1
CAT	chloramphenicol acetyltransferase
Ccdc16	coiled-coil domain containing 16
CDC	Centers for disease control and prevention
Cdc73	Vcell division cycle 73, Paf1/RNA polymerase II complex component, homolo; cerevisiae)
cDNA	complementary DNA
CFU	colony forming units
CGD	chronic granulomatous disease

Chi3l1	chitinase 3 like 1
CHO	Chinese hamster ovary cells
cM	centimorgan
CNS	central nervous system
CR	complement receptor
CREB	cyclic AMP-response element binding protein
Crlf3	cytokine receptor-like factor 3
CSF	colony stimulating factor
CXCL1	chemokine (CXC motif) ligand 1
CYBA	cytochrome b-245, alpha polypeptide
CYBB	cytochrome b-245, beta polypeptide
Cyfip2	cytoplasmic FMR1 interacting protein 2
DC	dendritic cells
DMEM	Dulbecco's modified Eagle's medium
DNA	deoxyribonucleic acid
dsRNA	double stranded RNA
dUTP	deoxyuridine triphosphate
EEA1	early endosomal antigen 1
ELISA	Enzyme-Linked Immunosorbent Assay
EST	expressed sequence tag
F2	filial generation 2
FBS	fetal bovine serum
Fcamr	FcαμR or Fc receptor, IgA, IgM, high affinity
FcR	Fc receptor
Foxn1	forkhead box N1 or *Whn*
Gapdh	glyceraldehyde-3-phosphate dehydrogenase
GATA1	GATA binding protein 1 (globin transcription factor1)
GST	glutathione S-transferase
H_2O_2	hydrogen peroxide
Havcr2	hepatitis A virus cellular receptor 2
Hist3h2ba	histone 3, H2ba
HLA	human leukocyte antigen
Hmmr	hyaluronan mediated motility receptor
HPLC	high-pressure liquid chromatography
Hprt	hypoxanthine guanine phosphoribosyl transferase
ICAM-1	intercellular adhesion molecule-1
ICE	IL-1β converting enzyme
IFNγ	interferon gamma
Ig	immunoglobulin
Ikbke	inhibitor of kappaB kinase epsilon
IKK	IκB kinase

IL	interleukin
IL-1Ra	interleukin 1 receptor agonist
IP	intraperitoneal
Ipaf	ICE protease activating factor
IRF	interferon response factors
Itk	IL-2 inducible T-cell kinase
Ity	Immunity to Typhimurium
IV	intravenous
IκBα	inhibitor of κBα
JNK	c-Jun N-terminal kinase
kb	kilobase
LAMP-1	lysosome-associated membrane glycoprotein-1
Lax1	lymphocyte transmembrane adaptor 1
LD50	lethal dose 50
Lgtn	ligatin
LOD	logarithm of odds
LPS	lipopolysacchride
LRR	leucine rich repeat
LRS	likelihood ratio statistic
LTA	lipoteichoic acid
MAPK	mitogen activated kinase
Mb	megabase
MHC	major histocompatiblity
MOLF	MOLF/Ei
Mpo	myeloperoxidase
mRNA	messenger RNA
MST	mean survival time
MyD88	myeloid differentiation primary response gene 88
N.D.	non-detectable
N5	Backcross generation 5
NADPH oxidase	nicotinamide adenine dinucleotide phosphate-oxidase
NAIP	neuronal apoptosis inhibitory protein
NALP	NACHT, leucine rich repeat and PYD containing 1
NaOH	sodium hydroxide
NCF1	neutrophil cytosolic factor 1
Ncf2	neutrophil cytosolic factor 2
NFAT	nuclear factor of activated T cells
NF-κB	nuclear factor -κB
NK	natural killer cells
NOD-LRR	nucleotide binding oligomerization domain – leucine rich repeat proteins
Nos2	nitric oxide synthase 2

Nox4	NADPH oxidase 4
Nramp1	Natural resistance associated macrophage protein 1
nu	nude locus
OD	optical density
PAMP	pathogen associated molecular pattern
PB1	phox and Bem1
PBS	phosphate buffered saline
PCR	polymerase chain reaction
phox	phagocytic NADPH oxidase
PMA	phorbol 12-myristate 13-acetate
PMNs	polymophonuclear cells
poly-IC	polyinosine-polycytidylic acid
PRR	pathogen recognition receptor
Ptgs2	prostaglandin-endoperoxide synthase 2
QPCR	quantitative polymerase chain reaction
QTL	quantitative trait locus
Rbbp5	Retinoblastoma binding protein 5
RES	reticuloendothelial system
RMA	robust multi-array analysis
RNA	ribonucleic acid
RNI	reactive nitrogen intermediates
RNS	reactive nitrogen species
ROI	reactive oxygen intermediates
ROS	reactive oxygen species
RPMI	Rosswell Park Memorial Institute (type of complete media)
SCID	severe combined immunodeficiency disease
SCV	*Salmonella* containing vacuole
SD	standard deviation
SDS	Sodium dodecyl sulphate
SEM	standard error of the mean
Sft2d2	SFT2 domain containing 2
SH3	SRC-homology 3 domain
SIF	*Salmonella*-induced filaments
Slc11a1	Solute carrier family 11 member 1
Slfn	Schlafen
SNP	single nucleotide polymorphism
SPI	*Salmonella* pathogenicity island
Sqstm1	Sequestosome 1
SSCP	single strand conformational polymorphism
SSLP	simple sequence length polymorphism
ssRNA	single stranded RNA

STAT1	signal transducer and activator of transcription 1
subsp.	subspecies
Tbp	TATA binding protein
TCR	T cell receptor
Tgtp	T-cell specific GTPase
T_H1	T helper 1
T_H2	T helper 2
TIR	Toll/IL-1 receptor like
Tlr	Toll like receptor
TNF	tumor necrosis factor
Tnfrsf1a	tumor necrosis factor receptor superfamily member 1a
TPR	tricopeptide repeat domain
TRAF6	Tnf receptor-associated factor 6
tRNA	transfer RNA
TTSS	type 3 secretion system
Ublcp1	ubiquitin-like domain containing CTD phosphatase 1
UTR	untranslated region
UV	ultraviolet
xid	x-linked immunodeficiency
XLA	X-linked agammaglobulinemia
Zfp62	zinc finger protein 62

CHAPTER 1:

GENERAL INTRODUCTION

SECTION 1: *SALMONELLA*

1.1 Introduction

In spite of all the advances in medicine, including the advent of antibiotics and the development of vaccines, infectious diseases remain a leading cause of mortality and morbidity worldwide (Edelman and Levine 1986; Crump et al. 2004; Mastroeni 2006). The genetic dimension of the host defense system in pathogenic infections has been established and described in mouse model systems and observed in human populations. From an epidemiological perspective, there are observed genetic differences in the susceptibility to infections, exemplified by the protection against malaria in people with mutations leading to sickle cell hemoglobin, and the high concordance among monozygotic twins in the occurrence of tuberculosis (Kallmann 1942; Allison 1954; Malo and Skamene 1994; Schroder and Schumann 2005). Genetically occurring variation in susceptibility to bacterial, viral and parasitic infection among inbred strains of laboratory mice has been extensively studied during the past 50 years. One of the best-characterized infectious models for the identification of host resistance genes in the mouse is infection with the Gram-negative bacteria, *Salmonella enterica* subsp. *enterica* serovar Typhimurium (*Salmonella* Typhimurium). The following general introduction will provide an overview of the pathogen, disease, host resonse to infection and the rationale for the use of a wild-derived inbred mouse model of infection.

1.2 Description

The genus *Salmonella* is comprised of a large group of highly related Gram negative bacteria. *Salmonella* are found ubiquitously in nature, often in the gastrointestinal tracts of mammals, reptiles, birds, and even insects (Mastroeni 2002). These facultative intracellular bacteria are characterized by low peptidoglycan and high lipopolysacchrides (LPS) in their outer membranes. LPS is comprised of three structural components, the O antigen consisting of polysaccharides, the core, and lipid A consisting of fatty acids and phosphates bound to a central glucosamine dimer. The lipid A component is found on the outer membrane of the lipid bilayer and therefore exposed to the immune system (Ernst et al. 2001). There are over 2463 *Salmonella* serotypes that have been identified, exhibiting different host specificities through their cell wall and flagellar antigen composition, O and H antigens respectively (Edelman and Levine 1986; Brenner et al. 2000).

1.3 Classification

Though different serovars were initially considered to be separate individual species, DNA-DNA hybridizations studies revealed the high degree of relatedness among the serovars, suggesting they should be considered as one species, *Salmonella enterica* (Crosa et al. 1973; Le Minor 1987). This classification system has become widely accepted by many organizations, and to date, in addition to *Salmonella enterica*, two other species have been identified, *Salmonella bongori* and *Salmonella subterranea* (Reeves et al. 1989; Brenner et al. 2000; Shelobolina et al. 2004; Heyndrickx et al. 2005). Within the *Salmonella enterica* species, there are six subspecies: *Salmonella enterica enterica, Salmonella enterica houtenae, Salmonella enterica arizonae, Salmonella*

enterica diarizonae, Salmonella enterica indica, and *Salmonella enterica salamae.* Approximately 60% of all known serovars belong to the *Salmonella enterica enterica* subspecies, are known to reside in mammalian hosts, and account for the overwhelming majority of the disease-causing *Salmonella* species. All other subspecies are usually found in cold-blooded animals or in the environment (Brenner et al. 2000; Heyndrickx et al. 2005). There are various *Salmonella enterica* subsp. *enterica* serovars that exclusively infect humans such as *Salmonella enterica* subsp. *enterica* serovar Paratyphi A, Paratyphi B, Paratyphi C, and Typhi, (*Salmonella* Paratyphi A, *Salmonella* Paratyphi B, *Salmonella* Paratyphi C and *Salmonella* Typhi) as well as those that infect both humans and mice such as *Salmonella enterica* subsp. *enterica* serovar Typhimurium (*Salmonella* Typhimurium) (Santos et al. 2001).

SECTION 2: HUMAN DISEASE AND EPIDEMIOLOGY

In humans, *Salmonella* infection occurs in two major forms, a systemic disease known as typhoid fever and a gastrointestinal infection known as salmonellosis. Other manifestations of the disease include bacteremia, vascular infection, life threatening sepsis, and a chronic carrier state (Bhan et al. 2005).

2.1 Salmonellosis

In contrast to *Salmonella* Typhi the causative agent of typhoid fever, non typhoidal *Salmonella* species, such as *Salmonella* Typhimurium and *Salmonella enterica* subsp. *enterica* serovar Enteritidis (*Salmonella* Enteritidis) that cause gastroenteritis are commonly encountered in the industrialized world. These microbes are known food

borne pathogens transmitted by the consumption of infected agricultural products causing a self-limiting gastroenteritis in 95% of all cases, and rarely bacteremia (5%) and focal infections (Hohmann 2001). The Center for Disease Control (CDC) has estimated that there are approximately 45,000 salmonellosis cases yearly leading to 400-600 deaths annually in the United States alone. However, due to under-reporting, the true estimate is probably closer to 1-3 million cases per year. Furthermore, non-typhoidal *Salmonella* presents a significant problem to the agricultural community as it accounts for approximately 30% of all food borne deaths in the U.S., and is the second most frequently isolated bacteria from diarrheal samples after *Campylobacter* (Mead et al. 1999). Of these isolated *Salmonella* species, *Salmonella* Typhimurium and Enteritidis represent 50% of all isolates from salmonellosis patients. Risk factors for salmonellosis include: diabetes, cancer, HIV infection, rheumatological disease, age, antibiotic therapy affecting the natural microbiota of the gut, use of antacids, and any immunosuppressive therapies (Hohmann 2001).

Salmonellosis cases occur via the oral route leading to a range of symptoms including fever, diarrhea, and abdominal cramping with varying severity. Cultures from the stool of salmonellosis patients show significant neutrophil infiltrates (Harris et al. 1972; Day et al. 1978; McGovern and Slavutin 1979). The onset of symptoms is rapid, following an incubation period of 6-72hrs, and lasts for approximately 10 days. The bacteria target the intestine and mesenteric lymph nodes, the infection is subsequently cleared by the adaptive immune response however fecal shedding can continue for 4 weeks in adults and up to 7 weeks in children under the age of 5 (Buchwald and Blaser 1984; Hohmann 2001). In fewer than 5% of all salmonellosis cases, the disease will progress to bacteremia leading to fatal consequences (Hohmann 2001).

2.2 Typhoid Fever

In many countries, typhoid fever caused by *Salmonella* Typhi has been eradicated due to increased sanitation practices, proper waste disposal systems, and access to clean water supplies. Despite this, typhoid fever is a serious health concern for many developing regions in the world where it is endemic, such as Africa, South America, India, and Southeast Asia, especially with the emergence of multidrug resistant strains of *Salmonella* (Fica et al. 1996; Shanahan et al. 1998; Ling et al. 2000; Parry et al. 2002; Threlfall et al. 2003; Kariuki et al. 2004) (Figure 1). The global burden of typhoid in the year 2000 was 21.6 million cases and 220,000 deaths (Crump et al. 2004). Although rare, cases of typhoid fever in the industrialized world do occur, mostly due to returning travelers from endemic regions (Threlfall et al. 2003). Despite the fact that *Salmonella* Typhi is a human-adapted serovar, it is capable of surviving for days in contaminated water, and for months in contaminated food such as oysters (Nishio et al. 1981; Cho and Kim 1999).

Salmonella Paratyphi A, B and C are also human-adapted serovars that can cause enteric fever similar to that caused by serovar Typhi but infection results in less severe disease. There were an estimated 5.4 million cases of paratyphoid in 2000 (Crump et al. 2004).

Salmonella Typhi is usually ingested through contaminated food or water, and the oral infectious dose is anywhere between 1,000 and 1 million colony forming units (CFUs) (Hornick et al. 1970). The bacteria enter the digestive tract and colonize the intestine and mesenteric lymph nodes, leading to primary bacteremia as a result of its dissemination into the general circulation. The bacteria take up residence in the reticuloendothelial system (RES) of the spleen and liver as well as the bone marrow

within 24 hours of infection. This leads to hepatosplenomegaly, enlargement of the mesenteric lymph nodes, and to the development of granulomatous lesions. The symptoms of clinical illness coincide with secondary bacteremia as bacteria are shed back into the general circulation. Typhoid fever is characterized by the onset of fever, headache, malaise, myalgia, cough, anorexia and nausea. The incubation period is 10 to 14 days, and symptoms including fever, can persist up to 4 weeks if untreated and lethargy can last for months (Bhan et al. 2005). One third of all typhoid fever patients develop diarrhea, but this is distinct from salmonellosis in that they have a largely mononuclear infiltrate in the stool as opposed to neutrophils (Harris et al. 1972; Kraus et al. 1999). Severe disease can occur in 10-15% of typhoid patients, presenting with gastrointestinal bleeding or typhoid encephalopathy, and complications including cardiovascular, neuropsychiatric, and respiratory problems may develop (Parry et al. 2002). The fatality rate in treated patients is about 1%, however if left untreated can be as high as 30-50% in certain populations (Rogerson et al. 1991). Fecal shedding of *Salmonella* Typhi can last for 3 months in untreated patients. Of those infected, 1-5% become chronic carriers with detectable amounts of bacteria in the urine or stool that persists for more than one year. The majority of the carriers are asymptomatic and 25% of them have never had any signs of disease (Levine et al. 1982).

2.3 Treatment and Vaccine

The majority of typhoid patients are treated with oral antimicrobials on an outpatient basis. Treatment of typhoid is typically accomplished by the prescription of fluoroquinolones, which have a cure rate of approximately 98%, resulting in reduction of

fever in 4 days (Parry et al. 2002). The combination of bedrest, hydration, proper nutrition, antipyretics, and antibiotics is most commonly sufficient for recovery (Bhan et al. 2005). Available vaccines for typhoid fever include one live oral vaccine, and two based on the Vi antigen of *Salmonella* Typhi (Vi polysaccharide and Vi conjugate) (Acharya et al. 1987; Levine et al. 1987; Mai et al. 2003). Of the three available, the Vi-conjugate vaccine has shown efficacy of 89% with protection up to approximately 4 years, and is licensed for use in children over the age of 2. Typhoid vaccines are effective against serovar Typhi but not Paratyphi A (Arya and Sharma 1995). As a preventative measure vaccinations are indicated for people traveling to endemic areas, for children living in endemic areas, and to prevent or control epidemic outbreaks.

SECTION 3: *SALMONELLA* PATHOGENESIS AND VIRULENCE FACTORS: THE PATHOGEN PERSPECTIVE

Salmonella establish infection due to numerous virulence mechanisms, enabling them to invade, survive and replicate in host cells. The versatility and adaptability of *Salmonella* to changing environments is reflected in its capacity to move from extracellular to intracellular life, which is required for virulence as *Salmonella* is an intracellular pathogen. This remarkable capacity is carried out in part by their two-component regulatory system PhoPQ. PhoPQ exerts pleiotropic effects on *Salmonella* by responding to extracellular signals such as magnesium concentration, however it also plays an important role in virulence as demonstrated by the attenuated virulence in *PhoP* and *PhoQ* mutants (Groisman 2001). *Sod* genes are also essential for intracellular survival through their ability to scavenge highly toxic reactive oxygen intermediates (ROI) produced by the host cell (De Groote et al. 1997). In addition to the genes mentioned

above, other genes necessary for virulence are found on *Salmonella* pathogenicity islands (SPI), or chromosomal regions that harbor a cluster of functionally related genes (Mills et al. 1995; Hansen-Wester and Hensel 2001). There are two SPIs, SPI-1 and SPI-2, that encode two distinct types of type III secretion systems (TTSS), which function as molecular syringes that are assembled by the bacteria to translocate various effectors into the host cell (Hueck 1998). SPI's typically encode regulators and structural genes that make up the TTSS, as well as translocated effectors and chaperones (Hueck 1998; Kuhle and Hensel 2004). SPI-1 TTSS are essential for the invasion of non-phagocytic cells and are upregulated by the intestinal environment of the host (Patel and Galan 2005). SPI-2 encoded genes, on the other hand, are required for intracellular survival (Kuhle and Hensel 2004). The description of *Salmonella* pathogenesis that follows is mainly based on observations made using the mouse model of systemic infection with *Salmonella* Typhimurium as well as the bovine model of gastroenteritis. A brief overview of a selection of virulence factors will be discussed here (Figure 2).

3.1 Invasion (SPI-1 mediated events)

In most natural infections, both *Salmonella* Typhimurium and *Salmonella* Typhi are ingested through the oral route and end up in the terminal ileum and colon after having resisted the low pH of the stomach. *Salmonella* Typhi adhere to the epithelial cells of the intestinal lumen through interactions with the cystic fibrosis transmembrane conductance regulator protein (Lyczak et al. 2001). Subsequent to attachment, studies using *Salmonella* Typhimurium infection in mice demonstrate that the bacteria enter the enterocytes and M cells lining the intestinal lumen through a process of invasion using the

genes encoded by SPI-1, and SPI-1 translocated effectors. These include SopE and SopE2, two guanidine nucleotide exchange factors for the Rho family of GTPases, ultimately affecting cell invasion; SipA (also known as SspA) an actin binding protein involved in the internalization of bacteria into host cells; SipB, C, and D (also known as Ssp proteins) required for intestinal invasion and thought to act as translocator; SopB (also known as SigD) an inositol phosphatase; and SptP a tyrosine phosphatase involved in cytoskeleton rearrangements (Hardt et al. 1998; Norris et al. 1998; Fu and Galan 1999; Zhou et al. 1999; Stender et al. 2000; Mirold et al. 2001; Zhou et al. 2001). *Salmonella* invasion induces membrane ruffling and substantial actin rearrangements by the SPI-1 encoded TTSS (Patel and Galan 2005). Following their successful entry during systemic *Salmonella* infection, the bacteria will cross over to the lamina propria where they reside within neutrophils and monocytes (Zhou et al. 1999; Santos et al. 2002).

In localized *Salmonella* infection resulting in gastroenteritis, the infected epithelial cells are shed into the intestinal lumen resulting in the loss of villi and loss of absorption leading to diarrhea (Wallis and Galyov 2000). SopB has been shown to promote the development of diarrhea by increasing electrolyte loss and fluid secretion (Wood et al. 1998). Concurrent to the actions of SopB, phagocytes recognize the presence of bacteria through their various pathogen recognition receptors (PRRs), including Toll-like receptors (TLR) 1, 2, 4, 5, and 6 on the surface of their membranes (Nau et al. 2003). Recognition via PRRs eventually induces the secretion of pro-inflammatory cytokines including interleukin-8 (IL-8) and chemokines which will eventually lead to the massive recruitment of neutrophils typically seen in Typhimurium infected human patient stool samples (Raffatellu et al. 2005).

Interestingly, the enteric fever causing serovar Typhi differs from Typhimurium in that the invasion of the intestinal mucosa does not trigger this robust host response leading to neutrophil influx. Instead, monocytes are recruited to the site of infection (Raffatellu et al. 2005). This mechanism is thought to be mediated by a virulence factor that is present only in serovar Typhi, the capsular Vi antigen. Numerous studies have demonstrated the ability of Vi to evade immunity by downregulating pro-inflammatory cytokines such as TNF (tumor necrosis factor) (Hirose et al. 1997).

3.2 Survival and Replication (SPI-2 mediated events)

In systemic *Salmonella* infection, after entry through the intestinal epithelia, the bacteria drain into the mesenteric lymph nodes, thoracic duct, and eventually spread to the spleen and liver. Within these organs bacteria survive and replicate preferentially within macrophages by inducing many of the 31 known SPI-2 encoded TTSS genes (Ochman et al. 1996; Kuhle and Hensel 2004). The importance of SPI-2 mediated activities has been demonstrated by the observation that SPI-2 mutants are highly attenuated in mouse models of systemic infection, do not succeed in replicating in the spleen or liver, and have reduced survival in primary macrophages (Shea et al. 1996; Hensel et al. 1998; Shea et al. 1999). SPI-2 has a wide range of effects on host-pathogen interactions, including the alteration of host microbicidal function, disruption of the intracellular transport process, modification of the cytoskeleton, as well as the induction of cell death (Kuhle and Hensel 2004). *SseB, SseC* and *SseD* encode the structural proteins for the translocator, while SseA acts as a chaperone. Some examples of effectors translocated by SPI-2 TTSS include SpiC (also known as SsaB) known to interfere with

vesicular traffic and induce interleukin-10 (IL-10), SspH1 involved in repression of nuclear factor κB (NF-κB) related genes, SspH2 participating in the inhibition of actin polymerization and SseF, SseG, SifA which are all involved in the production of *Salmonella*-induced filaments (SIFs, described below). However, mutations in these effector genes have little impact on *Salmonella* survival, suggesting redundancy of their roles (Kuhle and Hensel 2004). For an exhaustive list of SPI-2 mediated events, please refer to a recent review by Kuhle and Hensel.

3.3 *Salmonella* Containing Vacuole

Once inside a host cell, *Salmonella* reside in a unique compartment called the *Salmonella* containing vacuole (SCV) which escapes phagosome maturation. The SCV follows a distinct fate from that of the normal endocytic pathway which would result in the destruction of the bacteria. The SCV provides a safe niche for the bacteria to survive and replicate. The SCV matures initially like other endosomes by acquiring early endosomal markers such as the early endosomal antigen 1 (EEA1) and the transferrin receptor, followed by late endosomal markers Rab-7, LAMP-1, LAMP-2, LAMP-3/LIMP-1, and the vacuolar ATPase (Holden 2002; Knodler and Steele-Mortimer 2003). The SCV does not acquire the mannose-6-phosphate receptor, cathepsin D, and cathepsin L, which distinguishes it from the destructive phagosomal pathway. *Salmonella* eventually form SIF's (*Salmonella*-induced filaments), which are tubular extensions from the SCV (Garcia-del Portillo et al. 1993). Although SPI-2 TTSS effectors are necessary for its formation suggesting its importance in intracellular survival, the biological

relevance of the SIF's remain unknown to date. In this protected environment, *Salmonella* are able to evade host effectors and replicate.

SECTION 4: MOUSE MODEL OF SYSTEMIC SALMONELLA INFECTION: THE HOST PERSPECTIVE

4.1 Overview of the Course of Infection

Experimental infection of inbred mouse strains with *Salmonella* Typhimurium has been used extensively as a model of human typhoid fever (Santos et al. 2001). The distribution of bacteria and formation of lesions in the mouse resemble those of patients with typhoid fever, and the fact that *Salmonella* Typhimurium is a natural pathogen for rodents, makes it an excellent model to study the etiology of typhoid fever (Edwards 1943; Santos et al. 2001). Other artificial experimental mouse models, such as *Salmonella* Typhi infection of mice pre-treated with iron exist, however *Salmonella* Typhi is not a natural rodent pathogen and is incapable of replicating in the mouse, thus making it a less relevant model of infection (O'Brien 1982). A brief overview of the course of systemic *Salmonella* Typhimurium infection in mice is described in the following section.

Intravenous sublethal infection of mice with *Salmonella* Typhimurium can be modeled into 4 distinct phases of infection: I) bacterial clearance from the blood compartment, II) exponential bacterial growth in the RES, III) suppression of bacterial replication leading to a plateau phase, and finally IV) the resolution of the infection (O'Brien 1982; Mastroeni 2002).

The pathogen first enters the blood stream and is cleared from the blood compartment via the action of the complement cascade and macrophages within the first 2 hrs of infection (Biozzi et al. 1960). A small fraction (approximately 10%) of the initial inoculum survives and is localized in macrophages, PMNs, and dendritic cells of the RES, mainly in the liver and spleen (Dunlap et al. 1991; Richter-Dahlfors et al. 1997; Salcedo et al. 2001; Yrlid et al. 2001).

Although a portion of the bacteria is killed within the first 6hrs of infection by the phagocytes, during the second phase of infection the bacteria continue to divide exponentially (5-15 fold per day) primarily within neutrophils and macrophages for the first week of infection (Hormaeche 1980; Dunlap et al. 1991; Richter-Dahlfors et al. 1997). In the initial stages of infection, lesions appear in the liver along with a sudden proliferation of neutrophils. Bacterial killing by phagocytes is crucial at this stage to control bacterial growth. This is executed mainly through the production of reactive oxygen intermediates (ROI) and to a lesser extent through reactive nitrogen intermediates (RNI) via NADPH oxidase and *Nos2* (nitric oxide synthase 2) respectively, and also through the action of lysosomal enzymes and defensins (Groisman et al. 1992; De Groote et al. 1997; Shiloh et al. 1999).

In the third phase of infection, neutrophils are gradually replaced by accumulating macrophages. Bacterial growth at this stage is halted by the onset of adaptive immunity which eventually leads to the plateau phase of growth. Hepatosplenomegaly is seen in addition to the presence of granulomas rich in macrophages (Mastroeni 2002). The bacteriostatic role of RNI's is crucial in this phase along with the induction of multiple cytokines which is essential for the control of bacterial growth (Mastroeni et al. 2000). These cytokines include the pro-inflammatory TNF, interferon gamma (IFNγ),

interleukin-1 (IL-1), interleukin-6 (IL-6), interleukin-12 (IL-12), interleukin-18 (IL-18) as well as the anti-inflammatory cytokines interleukin-4 (IL-4) and IL-10 (de Jong et al. 1998; Mastroeni 2002).

In the final stage, T lymphocytes (T cells), specifically $CD4^+$ T cells, as well as the production of antibodies participate in the clearance of bacteria from the RES (Mittrucker et al. 1999; McSorley and Jenkins 2000). The major histocompatibility complex in mice (H2 complex) has also been shown to play a major role in the extent of clearance with different haplotypes conferring high, intermediate or low clearance rates (Nauciel et al. 1988).

4.2 Relevant Immune Cell Subsets in *Salmonella* Infections

Multiple immune cell types are involved in the response to *Salmonella* infection. During the early phases of infection, phagocytic cells including neutrophils, macrophages and dendritic cells (DC) are the main cells that harbor *Salmonella* (Mastroeni 2002). Macrophages are involved in the uptake of *Salmonella* either through SPI-1 mediated invasion, or phagocytosis via opsonin-independent receptors (such as scavenger receptors, macrophage mannose receptor, TLRs) or opsonin-dependent phagocytosis via Fc and complement receptors (FcR and CR). Phagocytosis can accelerate bacterial uptake as well as initiate microbicidal activity, cytokine and chemokine production and antigen processing which are central to macrophage function (Stuart and Ezekowitz 2005). In contrast, bacterial SPI-1 mediated invasion is distinct in that it does not involve signaling molecules such as tyrosine kinase, phosphatidylinositol kinase required for macrophage activation (Rosenshine et al. 1992; Ireton et al. 1996). Recent reports have focused on the

role of DCs that share many of the functions of macrophages during *Salmonella* infection including the ability to internalize and kill bacteria, and in the secretion of cytokines such as IL-12, IL-18, TNF and IFNγ (Pietila et al. 2005). However, differences in the amount and types of cytokines produced suggest that DCs play a more important role in the priming of naïve T cells towards a T_H1 adaptive response which is characterized by the activation of macrophages, natural killer cells (NK), and cell mediated immunity (Romagnani 1996; Pietila et al. 2005). On the other hand, macrophages have a more prominent role in promoting inflammation via cytokine secretion (Pietila et al. 2005). Finally, T and B cells are required for the clearance of infection and for protection from subsequent *Salmonella* infections (Mastroeni 2002).

4.3 Host Factors in *Salmonella* Infection

The following section will focus on the specific host effectors affecting the different phases of *Salmonella*-mediated immunity. The discovery of many of the host effectors has been based on the use of mouse models of infection. The host effectors primarily affecting the innate immune response surrounding the macrophage are summarized in Figure 3.

4.3.1 Innate Immunity: Recognition

The innate immune system needs to be able to detect invading pathogens that breech their protective barriers. To this end, evolutionarily conserved receptors designed to sense microbial molecules have evolved. These include primarily, but not exclusively,

the extracellular sensing TLRs, and the intracellular sensing nucleotide binding oligomerization domain – leucine rich repeat proteins (NOD-LRR).

4.3.1.1 Toll-like Receptors

The first line of evidence hinting at TLRs involvement in host resistance arose from the identification of Toll in *Drosophila melanogaster* immunity to fungal infection, followed by the discovery of the human and mouse orthologs, *TLR4* and *Tlr4* respectively (Lemaitre et al. 1996; Medzhitov et al. 1997; Poltorak et al. 1998; Qureshi et al. 1999). The TLRs are a large family of PRRs involved in the recognition of specific molecular signatures found on microbes and are highly conserved across plants, insects and animals. These proteins have an extracellular leucine rich repeat (LRR) domain involved in ligand binding and a cytosolic Toll/IL-1 receptor-like (TIR) signaling domain that induces inflammatory, antimicrobial and antiviral responses. To date, 10 human and 13 mouse TLRs have been identified. TLR1-9 are conserved in mice and humans, whereas mouse TLR8 and TLR10, and human TLR11 are nonfunctional (Figure 4) (Zhang et al. 2004; Takeda and Akira 2005). Each TLR along with its adaptor proteins recognizes a distinct ligand found exclusively on microbes, leading to the activation of the transcription factors such as NF-κB, activating protein -1 (AP-1), and interferon response factors (IRF) (Takeda and Akira 2003; Takeda and Akira 2004; Kawai and Akira 2006). TLR2 in association with either TLR1 or TLR6, recognizes lipoproteins and peptidoglycans of Gram positive bacteria. Specifically triacyl lipopeptides bind TLR1/2 and diacyl lipopeptides and yeast zymosan bind TLR2/6 (Ozinsky et al. 2000). TLR3 senses viral dsRNA and the synthetic molecule polyinosine-polycytidylic acid (poly-IC) (Alexopoulou et al. 2001). LPS from Gram negative bacteria is the cognate ligand for

TLR4, whereas flagellin signals through TLR5 (Poltorak et al. 1998; Qureshi et al. 1999; Hayashi et al. 2001). The synthetic molecule imidazoquinolone and ssRNA are recognized by TLR7/8, bacterial and viral CpG via TLR9, and uropathogenic bacteria and profilin-like molecules derived from *Toxoplasma gondii* by TLR11 (Jurk et al. 2002; Diebold et al. 2004; Heil et al. 2004; Zhang et al. 2004; Yarovinsky et al. 2005). Receptor activation via ligand binding leads to the induction of inflammatory cytokines such as TNF, IL-1β, IL-6 and IL-12 or to the activation of antiviral type I IFNs including IFNα and β (Kawai and Akira 2006). As part of the innate immune system, TLRs play an important role in the first line of defense against pathogens. Nevertheless, they are also implicated in the priming of the adaptive immune response since TLR signaling also leads to DC maturation and the upregulation of co-stimulatory molecules (CD80, CD86) in antigen presentation (Iwasaki and Medzhitov 2004). The majority of the TLRs are expressed at the cell membrane with the exceptions of TLR3, 7, 8, and 9 which are found in intracellular compartments such as the endosome (Akira and Takeda 2004; Wagner and Bauer 2006). TLR's that have been shown to be implicated in *Salmonella* host response include TLR4, and TLR5 as they respectively recognize molecular patterns present in *Salmonella*, LPS, and flagellin.

4.3.1.2 Tlr4 (Lps locus)

The *Lps* locus was identified in C3H/HeJ mice which were found to be hyporesponsive to stimulation with LPS (Sultzer 1968). LPS, also known as endotoxin, is a glycolipid found in abundance on the cell walls of Gram negative bacteria, including *Salmonella*, and is the ligand for TLR4. The endotoxin tolerance of *Lps* defective mice, in turn rendered them extremely susceptible to *Salmonella* Typhimurium infection, with a

lethal dose 50 (LD50) of <2 bacteria versus >2000 bacteria in wild type mice (O'Brien et al. 1980). Normally, LPS is highly immunogenic to mice leading to septic shock like syndrome, however C3H/HeJ mice could withstand doses of LPS that were 20-40 times higher than that of wild-type mice (Sultzer 1968). The underlying gene, *Tlr4*, was cloned by two independent groups, and a point mutation resulting in a non-synonymous change in a conserved cytoplasmic portion of the protein (P712H) was identified in C3H/HeJ mice (Poltorak et al. 1998; Qureshi et al. 1999). C3H/HeJ mice had an impaired macrophage response to LPS as measured by IL-1, IL-6, TNF secretion, as well as defective splenic B cell mitogenic response (Qureshi et al. 1999). In addition, C57BL10/ScCr and C57BL/6.KB2-mnd mice were also found to be hyporesponsive to LPS. C57BL10/ScCr mice carried a 75kb chromosomal deletion including *Tlr4* and C57BL/6.KB2-mnd mice harbored a deletion of exon 2 at the transcriptional level, leading to a premature stop codon (Poltorak et al. 1998; Qureshi et al. 1999; Poltorak et al. 2000; Bihl et al. 2001). The role of *Tlr4* was further confirmed in $Tlr4^{-/-}$ mice demonstrating its essential role in innate immunity (Hoshino et al. 1999).

In humans, several associations have been made between *TLR4* polymorphisms and infectious disease outcome. Two co-segregating polymorphisms in the extracellular domain of TLR4, Asp299Gly and Thr399Ile, were found to contribute to hyporesponsiveness to inhaled LPS. Furthermore, consistent with this notion, epithelial cells from homozygous or heterozygous individuals showed decreased response to LPS *in vitro* (Arbour et al. 2000). These alleles have been associated with the incidence of septic shock in patients with Gram negative infections, as well as with the increased presence of Gram negative bacteria (Agnese et al. 2002; Lorenz et al. 2002). In addition, these polymorphisms have been linked to susceptibility to respiratory syncytial virus infection,

atherosclerosis, Crohn's disease, and rheumatoid arthritis among many others, but none specifically to *Salmonella* infections (Schroder and Schumann 2005).

4.3.1.3 Tlr5

TLR5 was found to recognize flagellin through the observation that cell culture supernatants from *Listeria monocytogenes* containing flagellin stimulated Chinese hamster ovary (CHO) cells expressing human TLR5 (Hayashi et al. 2001). Flagellin is the monomeric subunit of flagella, found on Gram positive and Gram negative bacteria, involved in bacterial cell motility (Gewirtz 2006). TLR5 is expressed on epithelial cells, monocytes and immature DCs (Cario and Podolsky 2000; Muzio et al. 2000). Flagellin can induce the expression of numerous pro-inflammatory mediators including TNF, IL-1, IL-6, chemokine ligand 20 (CCL20), and NOS2 (Salazar-Gonzalez and McSorley 2005). TLR5 seems to have a more prominent role in intestinal and mucosal immunity. The fact that TLR5 is expressed on human intestinal epithelial cells and responds robustly to flagellin via IL-8 production after *Salmonella* infection provides further evidence for this (Gewirtz et al. 2001). Moreover, in epithelial cells flagellin alone was able to elicit the expression of almost all of the same pro-inflammatory genes as those observed when exposed to *Salmonella* Typhimurium (Zeng et al. 2003). Finally, during murine salmonellosis, aflagellate *Salmonella* strains caused reduced intestinal inflammation (Gewirtz 2006). TLR5 is expressed exclusively at the basolateral surface of intestinal epithelia and not on the apical surface, thus preventing TLR5 signaling due to commensal bacteria in the gut that possess flagellin as well (Gewirtz et al. 2001). TLR5 signaling in human intestinal epithelial cells also induces the expression of the chemoattractant CCL20, leading to the subsequent migration of immature DCs, providing a link between

innate and adaptive immunity as a consequence of TLR signaling (Sierro et al. 2001). TLR5 clearly has an impact on salmonellosis through mucosal immunity, however evidence for its role in systemic infection is weak. In support of this, a stop codon mutation in human *TLR5* was significantly associated with a predisposition to Legionnaires disease, a respiratory illness, but there was no correlation with typhoid fever susceptibility (Hawn et al. 2003; Dunstan et al. 2005).

4.3.1.4 Ipaf (Card12)

Recent work by multiple groups has uncovered a new pathway for *Salmonella* recognition in macrophages through one of the NOD-LRR proteins (also known as BLRs, NODs, CATERPILLAR), IPAF (ICE protease-activating factor also known as CARD12 and CLAN) (Franchi et al. 2006; Miao et al. 2006). The TLRs are known to be involved in the recognition of mainly extracellular bacterial products, whereas NOD-LRR proteins recognize primarily intracellular bacterial products. NOD1 and NOD2 are examples of NOD-LRRs that specifically bind to peptidoglycan and muramyl dipeptides found on bacterial cell walls, leading to the production of pro-inflammatory cytokines or apoptosis. Other members include NALP (NACHT, leucine rich repeat and PYD containing 1), NAIP (neuronal apoptosis inhibitory protein), APAF (apoptotic peptidase activating factor 1), and IPAF proteins. (Inohara et al. 2005). The NOD-LRR proteins consist of a leucine rich repeat domain involved in ligand binding, a nucleotide binding domain which oligomerizes upon activation leading to the activation of caspase via its caspase activation and recruitment domain (CARD). IPAF is thought to interact directly with CASPASE-1 (CASP1), leading to the cleavage of pro-CASPASE-1 into its active form CASP1 (Poyet et al. 2001; Martinon et al. 2002). *Salmonella* Typhimurium signals through IPAF,

leading to the activation of CASP1, and the secretion of IL-1β as well as mediating signals responsible for CASP1 cell death (Mariathasan et al. 2004). Miao et al, and Franchi et al have recently shown that the agonist activating IPAF is cytosolic bacterial flagellin, which was independent of TLR5 activation (Franchi et al. 2006; Miao et al. 2006). Interestingly, the hypothesized mechanism by which *Salmonella* are thought to "contaminate" the cytosol with flagellin is thought to be an inadvertent leakage of flagellin subunits along with the translocation of bacterial effectors into the host cytoplasm via the TTSS.

4.3.2 Innate Immunity: Microbicidal Activities

Not only does the innate immune response have to sense the presence of pathogens but they must be able to unleash various microbicidal effectors to eliminate them. Here, those molecules involved in microbial killing will be further discussed.

4.3.2.1 Slc11a1 (Nramp1)

Solute carrier family 11 member 1, SLC11A1 (also known as natural resistance associated macrophage protein 1 or NRAMP1), is a transmembrane protein expressed predominantly in macrophages and neutrophils. It is recruited to the membranes of phagolysosomal compartments in macrophages triggered by phagocytosis of bacteria (Gruenheid et al. 1997; Searle et al. 1998). SLC11A1 functions as a divalent cation efflux pump at the phagosomal membrane or SCV, leading to the depletion of essential metals such as Mn^{2+} and Fe^{2+} necessary for bacterial replication (Jabado et al. 2000; Forbes and Gros 2003). These observations lead to the hypothesis that the extrusion of cations from

the phagosome by *Slc11a1* contributes to an unfavorable environment for bacterial replication. In fact, the expression of *Slc11a1* has pleiotropic effects, leading to the enhanced recruitment of late endosomal markers such as mannose-6-phosphate receptor on the SCV, which is an indication of phagosome maturation and acidification leading to the demise of the bacterium (Jabado et al. 2000; Cuellar-Mata et al. 2002; Jabado et al. 2003).

Slc11a1 has been identified via positional cloning in inbred strains of mice to govern susceptibility to three unrelated intracellular pathogens, *Salmonella* Typhimurium, *Mycobacterium bovis* BCG (Bacille Calmette-Guerin), and *Leishmania donovani*, (Vidal et al. 1993; Vidal et al. 1996). Initially, this locus on mouse chromosome 1, named *Ity/Lsh/Bcg,* was discovered by three independent groups studying the response of resistant (wild type *Slc11a1*) and susceptible (mutant *Slc11a1*) mice to the respective pathogens, *Salmonella* Typhimurium, *Leishmania donovani, Mycobacterium bovis* BCG, and was subsequently shown to be controlled by the same locus (Bradley et al. 1979; Plant and Glynn 1979; Plant et al. 1982; Skamene et al. 1982). The susceptible strains were found to harbor a point mutation, G169D, in the fourth transmembrane domain of this integral membrane phosphoglycoprotein, leading to the absence of a functional protein (Vidal et al. 1996). The identity of *Slc11a1* underlying *Ity/Bcg/Lsh* was confirmed by targeted disruption, and transgenic rescue of mice with the *Slc11a1* gene (Vidal et al. 1995; Govoni et al. 1996). Mice carrying a mutant allele at *Slc11a1* such as C57BL/6J mice, succumb to infection after only 5 days with intravenous inoculation of *Salmonella* Typhimurium, as compared to those mice that carry the wild type allele, such as 129S6/SvEvTac mice, which are completely resistant to infection. It is clear that *Slc11a1* greatly influences survival time as well as bacterial load in the RES organs during

infection with *Salmonella* Typhimurium in mice. In addition to influencing disease outcome in an acute model of *Salmonella* Typhimurium infection, *Slc11a1* was also found to affect bacterial load in a chronic model of infection with *Salmonella* Enteritidis infection, in which unexpectedly the mutant form of *Slc11a1* (D169) was found to be the protective allele (Caron et al. 2002; Caron et al. 2006).

In humans, the role of *Slc11a1* in infection has been observed mainly in *Mycobacterial* infections (Abel et al. 1998; Greenwood et al. 2000; Malik et al. 2005). Another study has also found that *Slc11a1* can modify HIV susceptibility (Marquet et al. 1999). No association however, was found between typhoid fever susceptibility and *Slc11a1* polymorphisms in a Vietnamese population (Dunstan et al. 2001).

4.3.2.2 Reactive Oxygen Species -NADPH oxidase

The phagocytic NADPH oxidase (phox) is an enzyme expressed in neutrophils and macrophages. It is involved in the production of superoxide anions resulting in the generation of various reactive oxygen species (ROS), which in turn play a crucial role in microbicidal activities of phagocytes during *Salmonella* infection (Shiloh et al. 1999). Superoxide anions on their own do not exert any toxic effects however the enzymatic reactions they undergo result in the generation of reactive intermediates. Hydrogen peroxide is highly toxic and is generated via superoxide dismutase, hypochlorous acid via myeloperoxidase, hydroxyl radicals through the Fenton reaction, and peroxynitrite through the reaction between reactive nitrogen species (RNS) and superoxide (Beutler 2004). The enzyme is a complex of multiple subunits, including the membrane bound gp91phox and gp22phox (comprising the cytochrome b558), as well as the cytosolic members p47phox, p67phox, p40phox, and RacI proteins. Activation of the enzyme

complex through microbial stimuli requires the recruitment of the cytosolic members to the membrane where they will assemble and form a functional enzyme. Targeted knock-out of gp91phox encoded by *Cybb* in mice resulted in greatly increased susceptibility to *Salmonella* Typhimurium infection with high bacterial load in the RES (Mastroeni et al. 2000). In humans, mutations in genes that encode four of the phox subunits, *CYBB, CYBA, NCF1* and *NCF2* result in the primary immunodeficiency chronic granulomatous disease, rendering them susceptible to recurrent bacterial and fungal infections including *Salmonella* (Cross et al. 2000; Winkelstein et al. 2000; Heyworth et al. 2001). Therefore, phagocytic NADPH oxidase is instrumental to the suppression of bacterial replication in the first phases of infection.

4.3.2.3 Reactive Nitrogen Species

In addition to the generation of reactive oxygen species, phagocytes release RNS, such as nitric oxide, which have microbicidal activity. Nitric oxide is produced as a result of the oxidation of L-arginine via nitric oxide synthase, and in phagocytes this is primarily accomplished by the gene inducible nitric oxide synthase or *Nos2* (Nathan and Hibbs 1991). This gene is induced after stimulation with bacterial products, or other cytokines such as IL-1, TNF, and IFNγ (Vazquez-Torres and Fang 2001). Not surprisingly, *Nos2* knockout mice have higher mortality rates, as well as a greater bacterial burden in the spleen and liver after infection with *Salmonella* Typhimurium. The average survival was 15-20 days as opposed to *Cybb* targeted knockout mice that succumb to infection within 4-5 days at a lower infectious dose, suggesting that *Nos2* plays an important role later in infection (Shiloh et al. 1999; Mastroeni et al. 2000).

4.3.2.4 Caspase-1

Caspases are cysteine proteases that are involved in the induction of apoptosis, however there is a subset of caspases known as the inflammatory caspases, such as CASPASE1(CASP1) and 5, that have a role in the immune response mounted against pathogens (Martinon and Tschopp 2004). These caspases are activated by the assembly of the inflammasome, an intracellular complex of adaptor and scaffold proteins, which results in the cleavage of the pro-inflammatory cytokines pro-interleukin-1β and pro-interleukin-18 into their mature, active forms to be secreted from the cell (Thornberry et al. 1992; Miller et al. 1993; Martinon et al. 2002; Gracie et al. 2003). IL-1β has been reported to be mainly responsible for the induction of fever *in vivo*, as well as in the secondary upregulation of cytokines such as IL-6, colony stimulating factor (CSF) and several chemokines (Dinarello 1997; Fantuzzi 2001). Similarly, IL-18 is involved in pro-inflammatory cytokine induction, as well as in the stimulation of IFNγ in splenocytes, activating natural killer cells and in recruiting adhesion molecules (Gracie et al. 2003). The importance of CASP1, also known as ICE (IL-1β converting enzyme), has been demonstrated by the absence of the mature form of IL-1β in C*asp1* knockout mice, who were more susceptible to *Salmonella* Typhimurium, *Shigella flexneri*, *Listeria monocytogenes* and *Francisella tularensis* infections (Kuida et al. 1995; Li et al. 1995; Monack et al. 2000; Sansonetti et al. 2000; Tsuji et al. 2004; Mariathasan et al. 2005; Lara-Tejero et al. 2006).

Salmonella Typhimurium is known to induce a *Salmonella*-specific macrophage cell death that is distinct from that of classical apoptosis, named pyroptosis (Chen et al.

1996; Monack et al. 1996; Brennan and Cookson 2000). Associated with this rapid cell death or pyroptosis, is the release of pro-inflammatory cytokines. In *Casp1* knockout macrophages, this type of macrophage cell death was not observed suggesting the involvement of *Casp1* in this process (Los et al. 1999). This *Casp1* dependent cell death was also found to occur only in the presence of SipB, a TTSS encoded by *Salmonella* (Hersh et al. 1999). Recent work has revealed that this is due to flagellin-induced activation of IPAF, which leads to CASP1 activation described earlier in this chapter (Franchi et al. 2006; Miao et al. 2006). Another adaptor protein of the inflammosome ASC (apoptosis-associated speck-like protein containing a CARD or PYCARD) is also essential in CASP1 dependent IL-1β production during *Salmonella* infection since *Asc* knockout mice derived macrophages showed an impaired response to *Salmonella* Typhimurium infection (Mariathasan et al. 2004).

4.3.3 Cytokine Response

Communication is crucial for the appropriate induction of an immune response. This is accomplished by the secretion of cytokines which are signaling molecules that can orchestrate immune cell activation, microbicidal activities, chemotaxis, and proliferation.

4.3.3.1 IFNγ

IFNγ is a pleiotropic cytokine secreted by T helper cells, NK cells, and $CD8^{+}$T cells leading to the activation of innate and adaptive immune responses. IFNγ primes macrophages leading to the induction of bactericidal activity via the generation of ROI and RNIs. (Boehm et al. 1997; Shiloh et al. 1999). In addition to these functions, IFNγ is

also involved in antibody isotype switching and in the production of immunoglobulins by B cells (Huang et al. 1993) . The importance of IFNγ in activation of macrophages during *in vitro Salmonella* Typhimurium infection has also been clearly demonstrated by the enhanced bactericidal activity, hydrogen peroxide production and phagolysosomal fusion (Kagaya et al. 1989). The absence of IFNγ results in increased susceptibility to *Salmonella* Typhimurium due to a failure to suppress bacterial growth at the onset of the plateau phase of infection leading to compromised survival of the host. This was demonstrated by *Ifng* knockout mice infected with avirulent *Salmonella* strains, as well as through the administration of anti-IFNγ antibodies (Muotiala and Makela 1990; Nauciel and Espinasse-Maes 1992; Hess et al. 1996). Notably, humans with mutations in the IFNγ receptor 1 and 2 (IFNGR1, IFNGR2) develop atypical mycobacterial infections, with reported chronic *Salmonella* infections (Jouanguy et al. 1996; Casanova and Abel 2004). This suggests that IFNγ, is crucial in the suppression of bacterial replication and containment of the infection.

4.3.3.2 IL-12

IL-12 is a pro-inflammatory cytokine primarily involved in the induction of IFNγ. IL-12 is comprised of two subunits, the constitutively transcribed p35 and the inducible p40 subunits, which make up the biologically active p75 form of IL-12. *Il12b* (*Il12p40*) transcription is triggered by bacterial and viral antigens in antigen presenting cells (D'Andrea et al. 1992; Ma et al. 1997; Murphy et al. 2000). The importance of *Il12b* during *Salmonella* infection is demonstrated by the increased susceptibility and spread of infection in anti-IL-12 treated mice (Bost and Clements 1995; Chong et al. 1996; Kincy-Cain et al. 1996). Mice treated with neutralizing IL-12 antibodies not only had higher

bacterial loads in the spleens and livers when infected with an attenuated strain of *Salmonella* Typhimurium but also had reduced IFNγ, MHC class II, and nitric oxide production (Mastroeni et al. 1998). The induced Il12p40 subunit, seems to be linked to the anti-infectious activity of IL-12 since *Il12b* knockout mice were found to be more susceptible to *Salmonella* Enteritidis infection than *Il12a* (*Il12p35*) knockout mice (Lehmann et al. 2001). The production of IL-12 during intracellular infection has been shown to influence T_H1 polarization, leading to the production of cytokines such as IFNγ and TNF (Germann et al. 1993; Romagnani 1996). In addition, a role for IL-12 has also been established in human non typhoidal *Salmonella* infections. Humans with *IL12RB1* and *IL12B* deficiency have multiple recurring *Salmonella* infections as well as the predisposition to infections with poorly virulent *Mycobacteria* (Altare et al. 1998; de Jong et al. 1998; Casanova and Abel 2004).

4.3.3.3 TNF

TNF is another multi-faceted pro-inflammatory cytokine, essential in the activation of bactericidal activities as well as in the development of septic shock (Tracey and Cerami 1993). TNF is recognized by two receptors, p55 and p75, which initiate signaling and activation of various immune effectors (Bazzoni and Beutler 1996). The abrogation of TNF signaling in *Tnfrsf1a* (tumor necrosis factor receptor superfamily member 1a, encoding for the p55 receptor) knockout mice, leads to reduced survival during infection with *Salmonella* (Everest et al. 1998). In addition, the administration of anti-TNF antibodies prior to and during infection resulted in increased susceptibility to *Salmonella* Typhimurium infection due to unrestricted bacterial growth and the lack of organized granuloma formation (Nauciel and Espinasse-Maes 1992; Mastroeni et al.

1995). Moreover, TNF has been shown to regulate recruitment of the NADPH oxidase complex to SCVs, leading to reduced bactericidal activity in *Tnfrsf1a* knockout derived macrophages (Vazquez-Torres et al. 2001). Collectively, these studies suggest that TNF is involved in regulating the spread of infection through granuloma formation in the RES, as well as controlling NADPH oxidase mediated killing of *Salmonella*. In humans, a correlation between increased susceptibility to typhoid fever and a variant in TNF has been established in a Southern Vietnamese population (Dunstan et al. 2001).

4.3.3.4 Other cytokines involved in Salmonella Infection

The IL-1 family of cytokines is comprised of IL-1α, IL-1β and IL-18. They are pro-inflammatory cytokines known to induce genes involved in the inflammatory response. IL-1α and IL-1β increase the production of nitric oxides, prostaglandins, platelet activating factor, inducing fever and the promotion of leukocyte infiltration into tissues. IL-1β but not IL-1α requires proteolytic cleavage into its bioactive form through CASP1 (Dinarello 2002). IL-1β mediated inflammation is involved in sepsis caused by Gram negative pathogens (Cannon et al. 1990). It is also produced by macrophages after *Salmonella* Typhimurium infection (Kita et al. 1992). Exogenous IL-1α administered either prior to or post-infection has been shown to improve response to infection (Morrissey and Charrier 1991; Morrissey et al. 1995). IL-18, like IL-1β, requires CASP1 for cleavage into a bioactive form (Biet et al. 2002). Similar to IL-12, IL-18 is capable of inducing T_H1 mediate response via production of IFNγ as well as inducing enhanced cell mediated cytotoxicity. IL-18 is important in murine typhoid models, as neutralizing IL-18 antibodies compromises host response to infection with an attenuated *Salmonella* Typhimurium strain (Dybing et al. 1999; Mastroeni et al. 1999).

Finally, IL-6 is known to contribute to the inflammatory response with a wide range of effects in the body including hematopoeisis, acute phase reactions, mucosal B-cell differentiation as well as in the proliferation and differentiation of T cells (Van Snick 1990; Kishimoto 2005). Typhoid patients were found to have increased serum levels of IL-6, in addition to findings that IL-6 is secreted by epithelial cells infected with *Salmonella* (Keuter et al. 1994; Weinstein et al. 1997).

Two anti-inflammatory cytokines that are produced during *Salmonella* infection are IL-4 and IL-10. IL-10 downregulates other cytokines and the level of macrophage activation, although the neutralization of IL-10 during *Salmonella* infection did not affect the host response. However, it is thought to be an indicator of disease severity as it is positively correlated with higher bacterial load (Pie et al. 1996). IL-4 similarly counters inflammatory cytokines such as IL-12 and its absence during infection leads to increased resistance to *Salmonella* infection (Everest et al. 1997; Naiki et al. 1999).

4.3.4 Acquired Immunity

4.3.4.1 T cells

The importance of the adaptive arm of the immune system is demonstrated by the compromised immune response to attenuated *Salmonella* strains in TCRαβ deficient (αβTcell deficient), MHC class II deficient ($CD4^+$ deficient) and athymic nude mice (Hess et al. 1996; Sinha et al. 1997; Weintraub et al. 1997). Studies suggest that $CD4^+$ T cells play a more important role in *Salmonella* immunity since $CD4^+$ T cell depletion has a greater impact on bacterial clearance and infection outcome than does $CD8^+$ T cell depletion (Nauciel 1990).

The athymic nude mice, *nu*, have a congenital absence of hair in addition to being naturally deficient in $CD4^+$ and $CD8^+$ T cells, and show difficulty in mounting an appropriate immune response to *Salmonella* resulting in the absence of IgG1, IgG2a and IgG2b antibodies (O'Brien and Metcalf 1982; Segre et al. 1995; Sinha et al. 1997; Mittrucker et al. 1999). The *Foxn1* (also known as *Hfh11*, *whn*) gene, part of the forkhead/winged helix family of transcription factors exclusively expressed in the thymus and skin, was subsequently identified as the causal gene of the *nu* locus by positional cloning, due to single nucleotide deletion resulting in a premature stop codon (Nehls et al. 1994). The only known human mutations in *WHN* gene have been reported in a small South Italian community, causing a severe combined immunodeficiency disease (SCID) characterized by alopecia, nail dystrophy and T cell defects (Pignata et al. 1996; Frank et al. 1999; Pignata et al. 2001). The manifestation of the human mutation, a nonsense mutation (R255X), is very reminiscent of the *nude* mice phenotype, and recurrent bronchopneumonia infections have been reported (Pignata et al. 2001).

4.3.4.2 B cells

The role of B cells during infection has been mainly attributed to antibody production (Casadevall 1998). Mice with B cell impairment carrying the *xid* mutation, as well as B cell deficient mice present with increased susceptibility to infection with virulent *Salmonella* (O'Brien et al. 1981; Mittrucker et al. 2000). Furthermore, the importance of antibodies during *Salmonella* infection impacts the systemic spread of infection, since the administration of *Salmonella* specific IgA in mice prevented oral infection. Circulating antibodies leads to enhanced phagocytosis of bacteria, and studies indicate that Fc receptor mediated uptake increases antibacterial activity (Mosser 1994).

The antibodies can also activate the complement cascade through the classical pathway, leading to complement opsonization and phagocytosis (Brown 1991). In fact, studies have shown that mice pretreated with *Salmonella* specific antibodies cleared bacteria from the general circulation more rapidly (Cheers and Ho 1983; Saxen 1984).

CBA/N mice are known to carry a spontaneous mutation in the gene *Btk* (Bruton's tyrosine kinase also known as x-linked immunodeficiency, *xid*) that renders them unable to clear infection with *Salmonella* Typhimurium due to lack of antibody production (O'Brien et al. 1981; Thomas et al. 1993). CBA/N or F1 male mice derived from CBA/N females have very low immunoglobulin production, impaired B cell maturation, and reduced T cell responses. They were subsequently found to carry a missense mutation at a conserved arginine (R28C) in *Btk* located on the X chromosome which abolishes their ability to translocate to the membrane and transduce signals essential in B cell survival (Rawlings et al. 1993; Kang et al. 2001). *Btk* is part of the Tec family of non-receptor protein tyrosine kinases, involved in signal transduction of the B cell antigen receptor and the Toll-like receptor RP105 signaling (Chan et al. 1998; Rawlings 1999). Humans with X-linked agammaglobulinemia (XLA) also have a B cell immunodeficiency, similar to these mice, and carry mutations in *BTK* (Tsukada et al. 1993; Vetrie et al. 1993). There are reported cases of salmonellosis in XLA patients however *Streptococcus*, *Haemophilus*, *Staphylococcus* and *Pseudomonas* infections are most commonly observed (Ochs and Smith 1996).

4.3.4.3 H2 complex

Finally, the genes of the mouse major histocompatibility complex, *H2* complex, have also been implicated in host defense of *Salmonella* infections. This was analyzed

using C57BL/10 congenic mouse strains carrying different combinations of alleles at the H2 locus, representing distinct H2 haplotypes. B10.H2^b, H2^d congenic mice were more susceptible to infection than H2^a, H2^k and H2^f mice (Hormaeche et al. 1985). Mice possessing the *H2^j*, *H2^q*, *H2^u* haplotypes have highest clearance rates of bacteria as opposed to those with *H2^b* with the lowest clearance rates (Nauciel et al. 1988).

Consistent with findings in mouse models, association studies in humans have identified certain MHC class II and III haplotypes, HLA-DRB1*0301/6/8, HLA-DQB1*0201-3, that were correlated with higher susceptibility to typhoid fever, as opposed to the protective haplotypes HLSDRB1*04, HLADQB1*0401/2 (Dunstan et al. 2001).

SECTION 5: MOUSE GENETICS OF *SALMONELLA* MEDIATED IMMUNITY

The host response to infectious disease is a complex trait, influenced by multiple factors: exposure to the pathogen, the virulence and strain of the pathogen, the route of infection, exposure status of the host, and the host genetic make-up. Teasing out the multiple factors that contribute to infection in humans is tedious as many of the variables remain unknown. The mouse model ensures that all mice receive the same dose and strain of *Salmonella*, through the same route of infection, and all mice are considered naïve. The major difference in disease susceptibility can then be attributed to the genetic make up of the homogeneous inbred strains of mice, facilitating the dissection of the genetic factors that may influence the outcome of infection. Mice have the advantage over invertebrate models of infection such as *Caenorhabditis elegans* and *Drosophila melanogaster*, since they have both arms of the immune system, innate and acquired, and

because they have a broader host range to other human pathogens. As early as the 1930's, scientists have observed that differential susceptibilities to infectious diseases in mice are polygenic in nature (Gruneberg 1952).

The mouse model is ideal for genetic analysis. This is due to the availability of numerous homozygous inbred strains, congenic strains in which one genetic locus from a donor strain is transferred to a recipient strain, recombinant inbred strains, recombinant congenic strains, spontaneous and induced mutants, as well as the availability of mouse genome sequences, and dense polymorphic genetic markers including microsatellite and single nucleotide polymorphisms (SNPs) (Flint et al. 2005). In general, mice can be used for genetic studies through three distinct approaches – creation of gene targeted mutants, positional cloning of simple mutant phenotypes (spontaneous or induced), and quantitative locus (QTL) mapping using an informative mouse cross (Lengeling et al. 2001).

The description of *Salmonella* pathogenesis given previously in this Chapter has largely been based on the insight gained from the generation of targeted mutant mice. However, several loci have also been identified due to naturally-occurring mutations in classical inbred laboratory mice, accounting for strain specific differences in resistance, such as C57BL/6J (*Slc11a1*), C3H/HeJ (*Tlr4*), CBA/N (*Btk*), and nude (*Foxn1*) mice described above. Moreover, the continuous distribution of survival to systemic *Salmonella* Typhimurium infection in mice seems to indicate the involvement of multiple genes (Figure 5). In the acute model of infection, 1,000 CFUs of *Salmonella* Typhimurium are injected intravenously, resulting in the rapid replication of bacteria in the RES organs leading to death in susceptible C57BL/6J and C3H/HeJ mice within a week of infection due to mutations in *Slc11a1* and *Tlr4* respectively. In resistant mice

such as 129/SvEvTac, there is partial clearance of bacteria and low bacterial counts in the RES organs however the mice remain chronic carriers of the pathogen (Sebastiani et al. 2002). In addition to these extreme phenotypes, there are strains of mice that present with intermediate survival, such as the A/J mice (Roy and Malo 2002).

5.1 QTL Mapping

At least four strains of mice (C57BL/6J, C3H/HeJ, CBA/N, and nude mice), each harboring distinct naturally-occurring spontaneous mutations in a gene, have been successfully used to map and clone the causative *Salmonella* susceptibility gene in a genetic screen of a simple Mendelian trait. Gene identification in such simple monogenic traits have been very successful, however not all genes contributing to *Salmonella* infection manifest themselves as such. In fact, susceptibilities to infection and to common diseases are considered to be complex, such that the relative contribution of genes and environment leads to the the final phenotypic outcome. The genetic study of complex traits in model organisms has been mainly accomplished by the use of QTL mapping in which the phenotype under study is a quantitative trait defined as a numerically measurable phenotype such as CFUs in the spleen or days of survival. A quantitative trait tends to be multifactorial in nature leading to the discovery of one or more QTLs or genes that determine the manifestation of the particular trait. Monogenic or Mendelian loci governing dichotomous traits, and QTLs governing complex traits, should be viewed as extremes on a continuous spectrum of genetic effects as opposed to two distinct entities as QTL mapping can be used to identify loci in both scenarios. Within this continuous spectrum, scenarios of a quantitative trait with merely one QTL, or others with multiple

QTLs may exist. Where multiple QTLs are involved, each QTL may not necessarily contribute equally to the effect of the phenotype. To complicate matters further, the QTLs involved in the quantitative trait may be influenced by the genetic background of the individual due to interactions of the QTLs with the rest of the genome (Abiola et al. 2003; Flint et al. 2005; Mott 2006).

Despite the complexity underlying these traits, QTL mapping has provided a means by which to dissect the genetic contributions to complex traits. This approach involves the identification of a quantitative trait phenotype that segregates in a cross of two strains of mice, followed by the generation of an informative cross (F2 or backcross). The mice progeny are phenotyped and genotyped with markers across the whole genome using a genome scan approach, revealing chromosomal segments linked to the phenotype in question. A genome scan employing F2 mice usually provides linkage to 10-40cM chromosome intervals including approximately 200-800 genes although this number can vary considerably depending on the region of the genome. Statistical significance for linkage is assessed via interval mapping through the use of specific analysis software such as MapManager and R/Qtl (Lander and Botstein 1989; Jansen and Stam 1994; Lander and Kruglyak 1995; Darvasi 1998; Manly et al. 2001; Broman et al. 2003). The results are expressed as a logarithm of odds (LOD) statistic which represents the logarithm of the ratio of likelihoods under the null and alternative hypotheses, that there is no linkage versus that there is linkage between the trait and the genotype respectively (Lander and Kruglyak 1995; Flint and Mott 2001; Abiola et al. 2003).

Validation of linkage can be accomplished through the generation of additional crosses distinct from the original cross in which the same trait of interest segregates, as well as through the generation of congenic mice. Confirmation in an additional cross

typically only requires linkage to be established on the chromosomes on which the QTL(s) was found, as opposed to a whole genome scan (Lander and Kruglyak 1995; Abiola et al. 2003). Congenic strains specific for the QTLs detected can be generated by introgressing the candidate interval from the donor strain into a homogenous genetic background of the recipient strain. This is achieved through repeated backcrossing, ideally for 10 generations to remove any other QTL effects, to the recipient strain while using marker assisted selection for the QTL in the donor strain. Finally, the mice carrying the QTL interval are intercrossed to create homozygous individuals. Testing of the phenotype can be carried out in the congenic mouse to validate the predicted effect of the locus (Abiola et al. 2003).

Gene identification directly from an initial genome scan is formidable at best, and fine mapping must be carried out to further gene identification. Fine mapping aims to narrow the interval identified through genome wide association to a smaller region in which candidates can be identified and further tested for functional relevance. The creation of subcongenic strains that carry different recombinant intervals of the QTL has been used successfully to minimize candidate intervals (Darvasi 1998; Fehr et al. 2002). Another method that can be used to minimize the interval is the use of haplotype mapping among the parental strains. As inbred strains share ancestry, the genomes reflect ancestral haplotypes accounting for most of the genetic variation (Wade et al. 2002). Thus, haplotype blocks that differed in the QTL interval can be the focus of further investigation (Flaherty et al. 2005). Using this method, the QTL can be reduced to a 1cM (~2Mb) region containing approximately 20 genes, also reducing the number of candidate genes to consider (Abiola et al. 2003; Flint et al. 2005).

The next step in QTL mapping involves the identification of candidates. The complex trait community has suggested a number of approaches that would be reasonable proof of a gene underlying the QTL. Obviously, positional candidates that have a known role in the phenotype in question, or are expressed in the relevant tissues are attractive candidates. Sequencing of candidates can be done to identify any coding or regulatory variants. In cases where a sequence variant is found, *in vitro* studies may be carried out to assess its biological relevance. Even though there are cases in which sequence variants have been identified in QTLs, the impact of such sequence variants especially those in non-coding regions may be difficult to assess (Glazier et al. 2002; Abiola et al. 2003; Yalcin et al. 2004; Yalcin et al. 2004; Flint et al. 2005). Expression profiling has also been used successfully to identify candidates for specific QTLs, especially when QTL specific congenics are used (Rozzo et al. 2001; Gu et al. 2002; McBride et al. 2003; Flint et al. 2005). The creation of transgenics and knock-ins is also suggested however the gene must be introduced on the appropriate genetic background which may not always be feasible, complicating the interpretation of results (Abiola et al. 2003). Ideally, a QTL-knockout interaction (also known as quantitative complementation) would be the final proof. This involves testing the QTL against a null allele of the candidate gene within the QTL interval in a co-isogenic animal (Flint et al. 2005).

The use of this QTL mapping approach has lead to the discovery of over 2000 QTLs and although only under 1% of these have lead to gene identification, it provides a method by which we can get a glimpse of the complex genetic architecture underlying complex traits (Flint et al. 2005).

5.2 Wild-derived Inbred Mouse – MOLF/Ei

The Japanese *Mus musculus molossinus* (MOLF/Ei) subspecies is a hybrid between the *Mus musculus musculus* subspecies originating in East Europe, Russia, and North China, and *Mus musculus castaneous* from West Asia, Southeast Asia and South China (Yonekawa et al. 1988; Wade et al. 2002) (Figure 6). Investigations into the genomic structure of inbred mouse strains revealed that most of the standard inbred strains of mice carry a mosaic structure consisting of contributions from *M.m. domesticus* and *M.m. musculus* (Wade et al. 2002; Wiltshire et al. 2003; Frazer et al. 2004; Zhang et al. 2005). The *M.m. molossinus* subspecies is comprised of at least 10 other strains all of which originated in Japan. The first *M.m. molossinus* or Japanese tree mouse was given to Michael Potter at the NIH in 1969 who subsequently provided them to Eva M. Eicher at the Jackson Laboratory in the same year. Eicher's colony then became known as MOLF/Ei (Ei for Eicher).

Wild derived-inbred strains such as MOLF/Ei differ from the classical inbred strains in that they have been separated by a million years of divergent evolution and are subsequently of a different subspecies (*Mus musculus molossinus* versus *Mus musculus domesticus*). The use of wild-derived mice shares all the same advantages as other classical inbred strains, having a short generation time (10-12 weeks), homogenous genetic background, being small and relatively simple to maintain, and being established as a model of multiple human diseases. The evolutionary difference between wild-derived and classical inbred mice has numerous ramifications in the use of wild-derived inbred strains in genetic studies, namely as a reservoir of novel genetic polymorphisms. The frequency of genetic polymorphisms such as single nucleotide polymorphisms (SNPs)

and microsatellite markers, is much higher than in intraspecific crosses or than that between humans, on the order of 1 polymorphism every 100 base pairs, in wild-derived inbred strains as compared to classical inbred strains of mice (Guenet and Bonhomme 2003; Abe et al. 2004; Ideraabdullah et al. 2004). At the intitiation of the study when microsatellites were more commonly used, mapping a particular locus in an intersubspecific cross was much easier and more precise as a much denser panel of microsatellite markers could be used as compared to using a cross of *M.m. domesticus* classical inbred strains. Clearly, the high frequency of polymorphisms also has its pitfalls, making hybridization of primers, based on the sequence of classical laboratory strains, problematic at times. In the study of epistatic interactions, it is also clear that the maintenance of some combinations of alleles are essential for the proper manifestation of a phenotype in an intersubspecific cross (Montagutelli et al. 1996).

The strain specific susceptibility to *Salmonella* Typhimurium infection has been assessed in a selection of wild-derived mice (Figure 5). CAST/Ei mice were relatively resistant to infection with half of infected mice surviving up to 25 days, whereas SPRET/Ei had a mean survival time of 7.2 days and all MOLF/Ei mice succumbed to infection within 6 days (Sebastiani et al. 1998). This prompted the investigation into the host factors governing the outcome of *Salmonella* infection in MOLF/Ei mice as they presented with the most severe phenotype.

5.3 *Ity, Ity2, Ity3*

A QTL mapping approach has been used by our laboratory to identify novel loci affecting *Salmonella*-induced immunity, using an acute model of systemic *Salmonella*

Typhimurium infection of the wild-derived inbred strain MOLF/Ei mice. These mice, despite carrying functional *Slc11a1* and *Tlr4* alleles, were found to be highly susceptible to intravenous *Salmonella* Typhimurium infection, succumbing to infection 6 days post-infection (Figure 7). C57BL/6J mice are a classical inbred strain, sharing this extreme susceptibility to *Salmonella* infection, and are known to harbor a non-functional allele at *Slc11a1* leading to their demise. A C57BL/6J x MOLF/Ei cross was initiated with the aim of identifying modifiers of *Slc11a1* in the MOLF/Ei mice (Sebastiani et al. 1998). An F1 cross between the C57BL/6J x MOLF/Ei, resulted in progeny that were more resistant than either one of the parents due to the segregation of the functional MOLF/Ei derived *Slc11a1* allele in these mice, suggesting that *Slc11a1* is a major gene in this model. An F2 intercross of these mice confirmed that the phenotype of survival was a complex trait with the involvement of multiple loci, with the largest effect contributed by *Ity* or *Slc11a1* (Figure 8). No evidence was found that suggested the existence of modifiers of the major gene *Slc11a1*. A subsequent genome scan revealed two novel QTLs, in addition to *Ity*, controlling systemic *Salmonella* Typhimurium infection in MOLF/Ei mice, *Ity2* and *Ity3* (Sebastiani et al. 1998).

A *Salmonella*-resistant phenotype inherited in an additive fashion was linked to a region on mouse chromosome 11 located between the markers *D11Mit79* and *D11Mit13* (*Ity2*) with a maximum LOD score of 7.0 at *D11Mit5*. A second QTL conferring recessive susceptibility was located on mouse chromosome 1 *(Ity3)*, approximately 25 cM distal to *Slc11a1*, with a maximum LOD score of 4.8 at *D1Mit100.* These two novel QTLs, in addition to the effect of *Slc11a1*, which segregates in the cross, were found to collectively account for approximately 55% of the variance in survival time following *Salmonella* Typhimurium infection (Sebastiani et al. 1998).

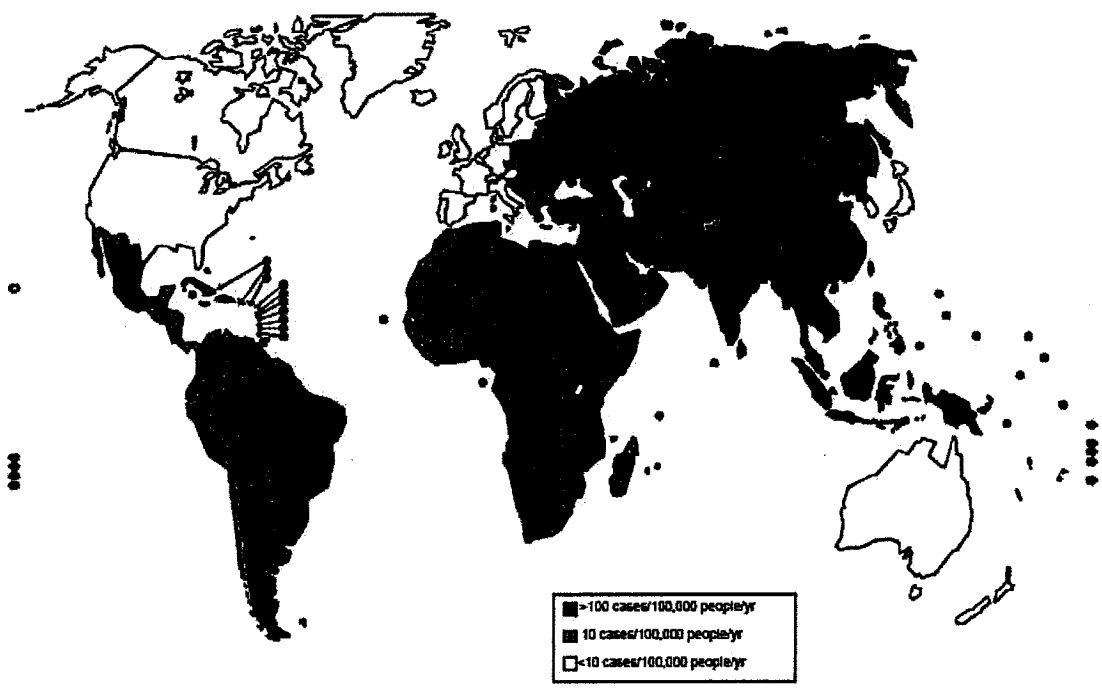
THESIS OBJECTIVES

The use of the wild-derived inbred strain MOLF/Ei has led to the identification of novel QTL's affecting the host response to *Salmonella* infection in mice enabling us to tap into the genetic diversity represented by this strain. QTL mapping has revealed the complexity of the genetic architecture that underlies this phenotype, establishing the existence of 3 loci, two resistant (*Ity* and *Ity2*) and one susceptible (*Ity3*) that contribute to *Salmonella*-induced immunity in MOLF/Ei mice. *Ity* represents a known *Salmonella* resistance gene *Slc11a1*, however *Ity2* and *Ity3* have never been described before. We have hypothesized that genes located within the chromosomal regions defined by *Ity2* and *Ity3* affect the outcome of *Salmonella* Typhimurium infection in the wild-derived mouse, MOLF/Ei.

This dissertation represents efforts aimed at further defining the regions on mouse chromosome 11 (*Ity2*) and 1 (*Ity3*). Fine mapping of the two loci as well as the prioritization of candidates are presented in Chapter 2, and candidate gene analyses for *Ity3* are the subject of Chapter 3 (*Tlr5*) and Chapter 4 (*Ncf2*). The dissection of protective immunological factors to bacterial infections can ultimately contribute towards the further characterization and our understanding of the complex immune response to infectious disease.

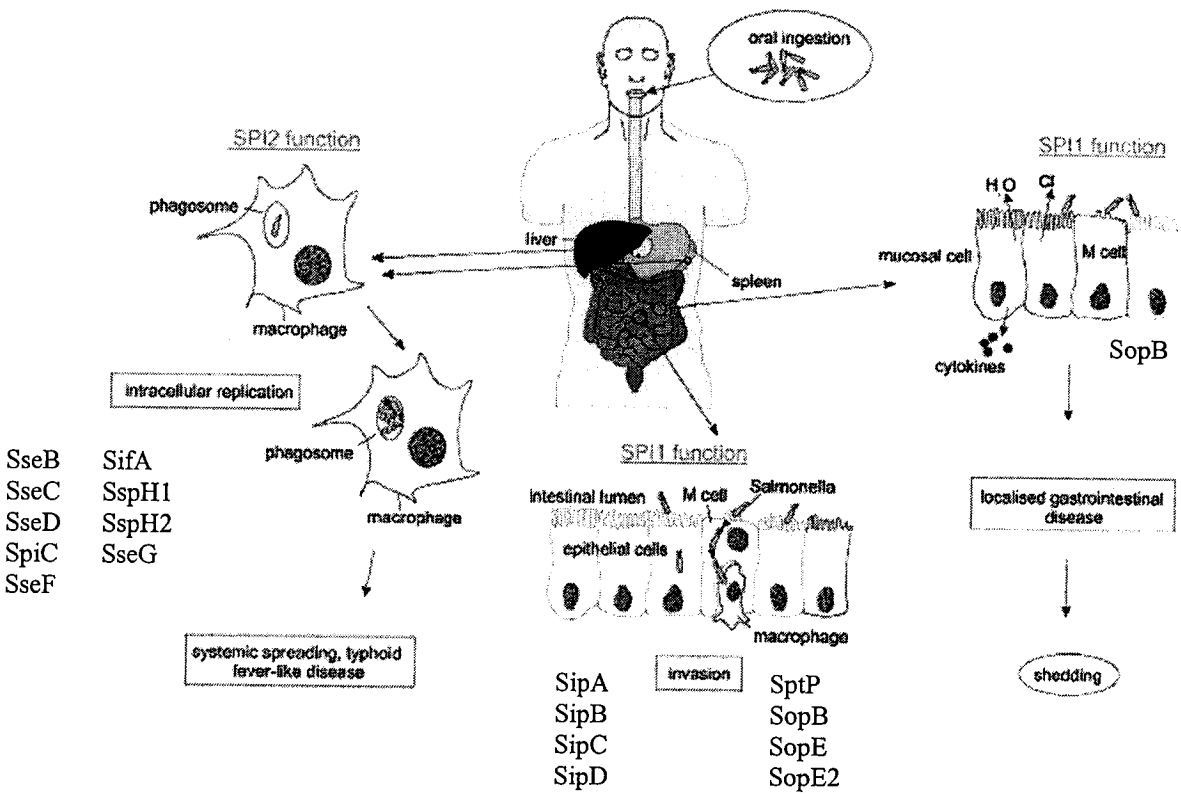
FIGURES

Figure 1: The global distribution of typhoid fever based on the number of cases per 100,000 people per year. Regions of high incidence are indicated in gray, intermediate incidence in green and low incidence in white (Crump et al. 2004).



Adapted from (Crump et al. 2004)

Figure 2: *Salmonella* pathogenesis and virulence factors during infection. SPI-1 TTSS is required for the entry and invasion of intestinal cells, as well as in the onset of diarrhea in salmonellosis. SPI-2 mediated TTSS is necessary for the intracellular survival and replication of *Salmonella*. A selection of SPI-1 and SPI-2 encoded genes and translocated effectors are listed here.



SseB SseC SseD SpiC SseF

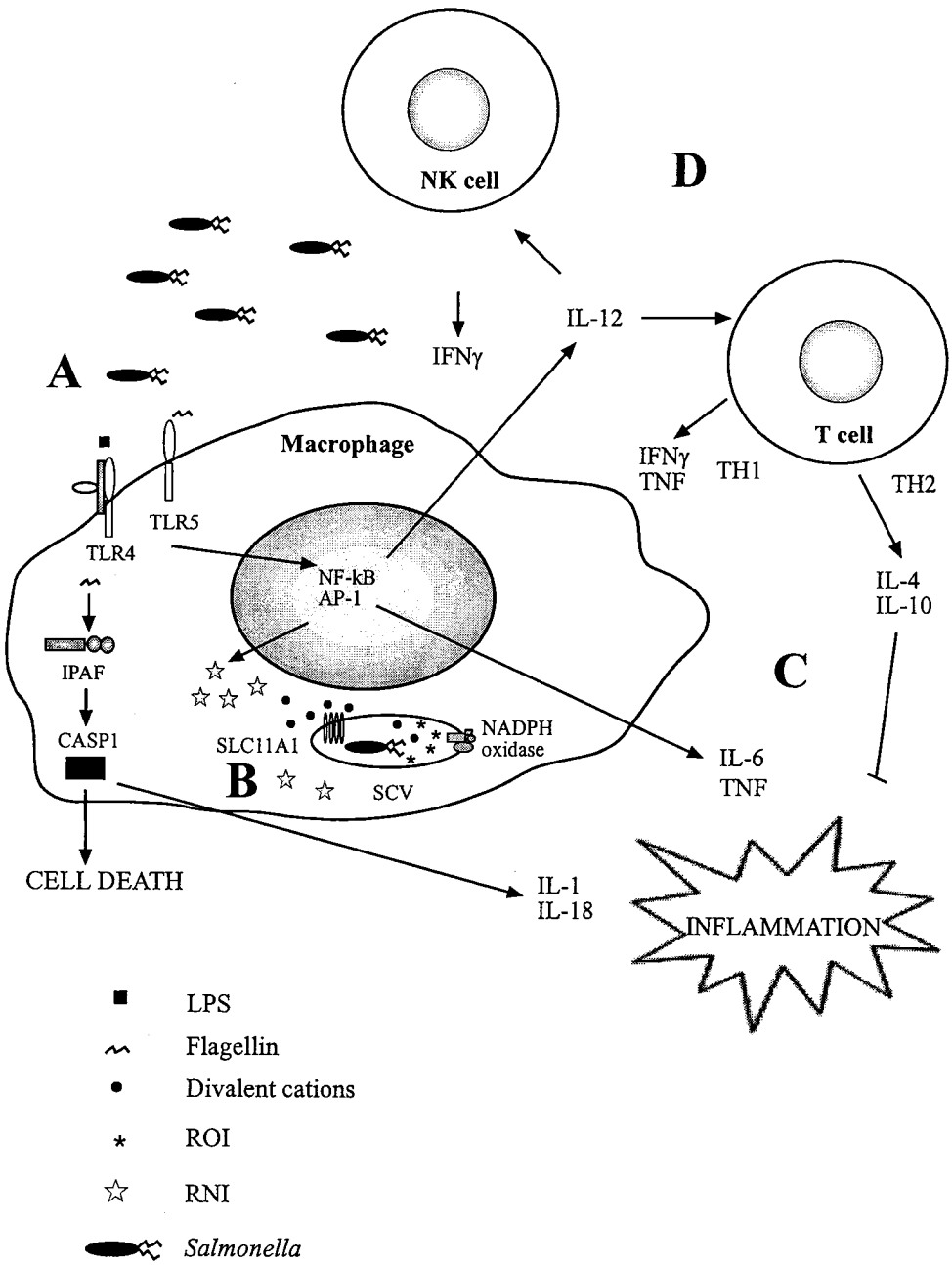
SifA SspH1 SspH2 SseG

SipA SipB SipC SipD

SptP SopB SopE SopE2

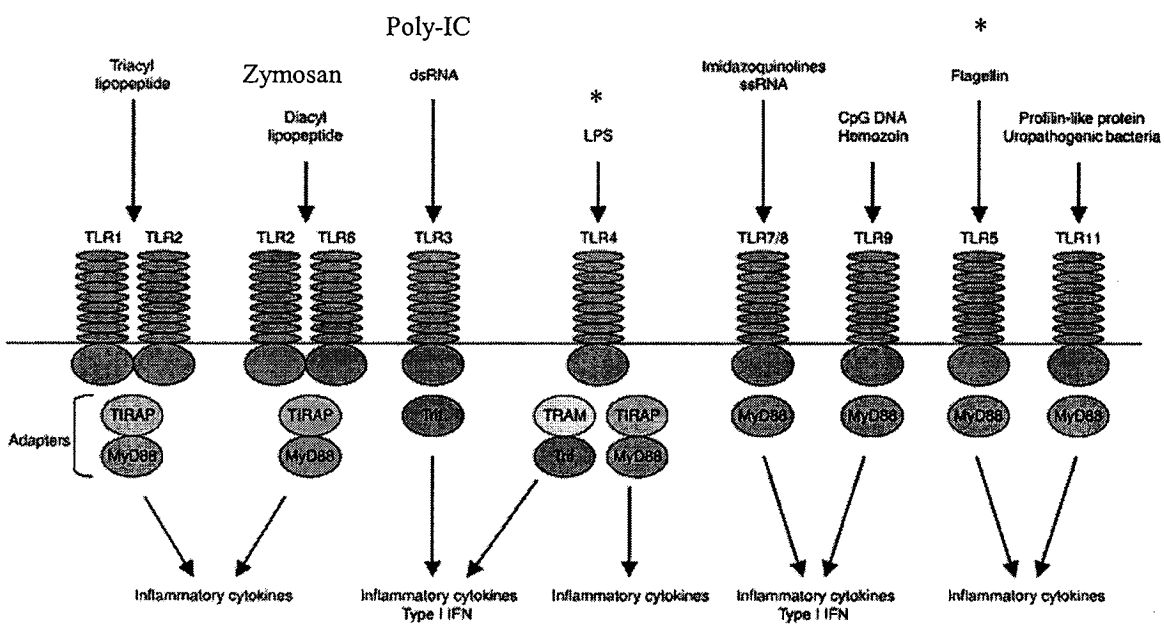
Adapted from (Hansen-Wester and Hensel 2001)

Figure 3: An overview of the host response to *Salmonella* infection in the macrophage (Lalmanach and Lantier 1999). *Salmonella* is recognized by macrophages via the TLRs as well as through the interaction of cytosolic flagellin with IPAF (A); microbicidal functions including the activities associated with the expression of *Slc11a1*, the induction of ROIs, RNIs and CASP1 (B); various cytokines mediating inflammation are produced (C); priming adaptive immunity, including T-cell activation and T_H1 polarization, is illustrated here (D).



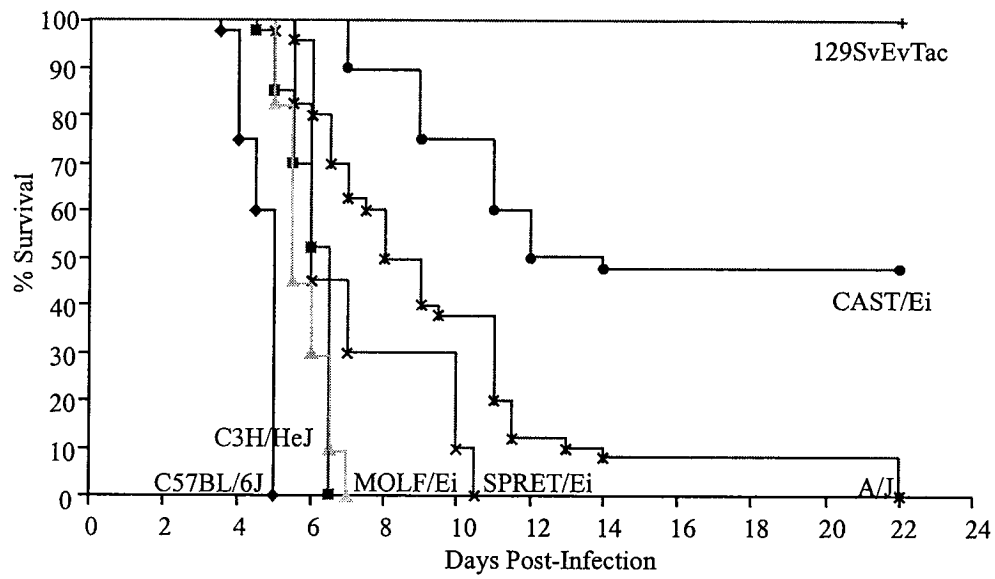
Adapted from (Lalmanach and Lantier 1999)

Figure 4: Characterized TLRs and their ligands. Asterisks indicate TLRs through which *Salmonella* have been demonstrated to signal (Kawai and Akira 2006).



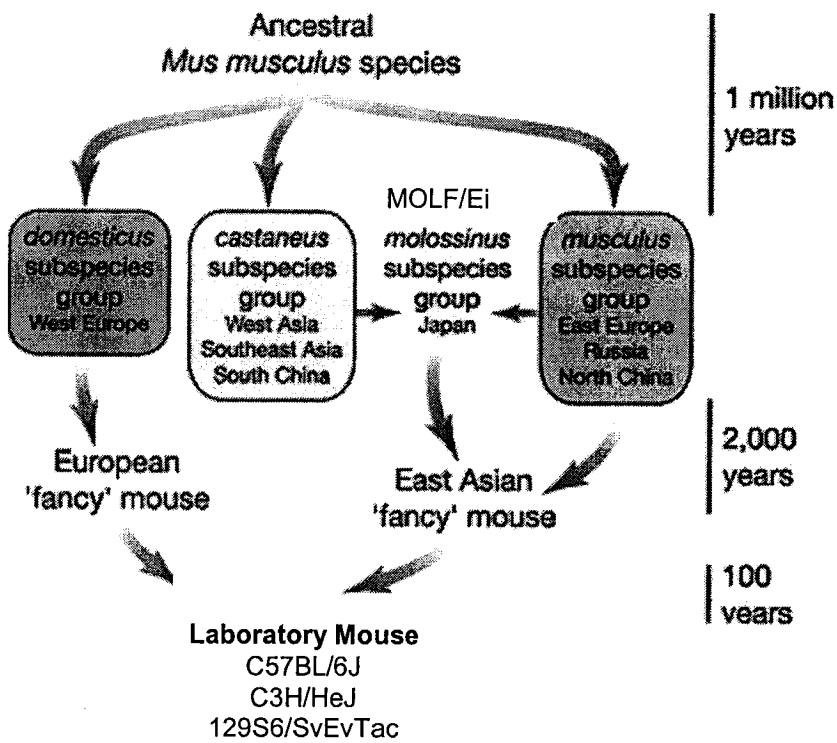
Adapted from (Kawai and Akira 2006)

Figure 5: Survival of various inbred mouse strains to intravenous infection with *Salmonella* Typhimurium (Roy and Malo 2002).



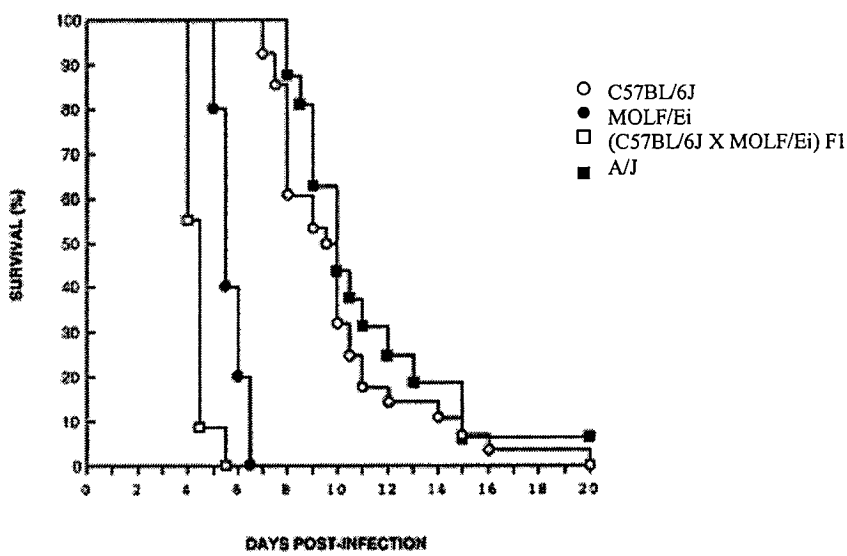
Adapted from (Roy and Malo 2002)

Figure 6: The historical derivation and geographical location of *Mus musculus* subspecies that contributed to the present day classical inbred laboratory mice (Wade et al. 2002).



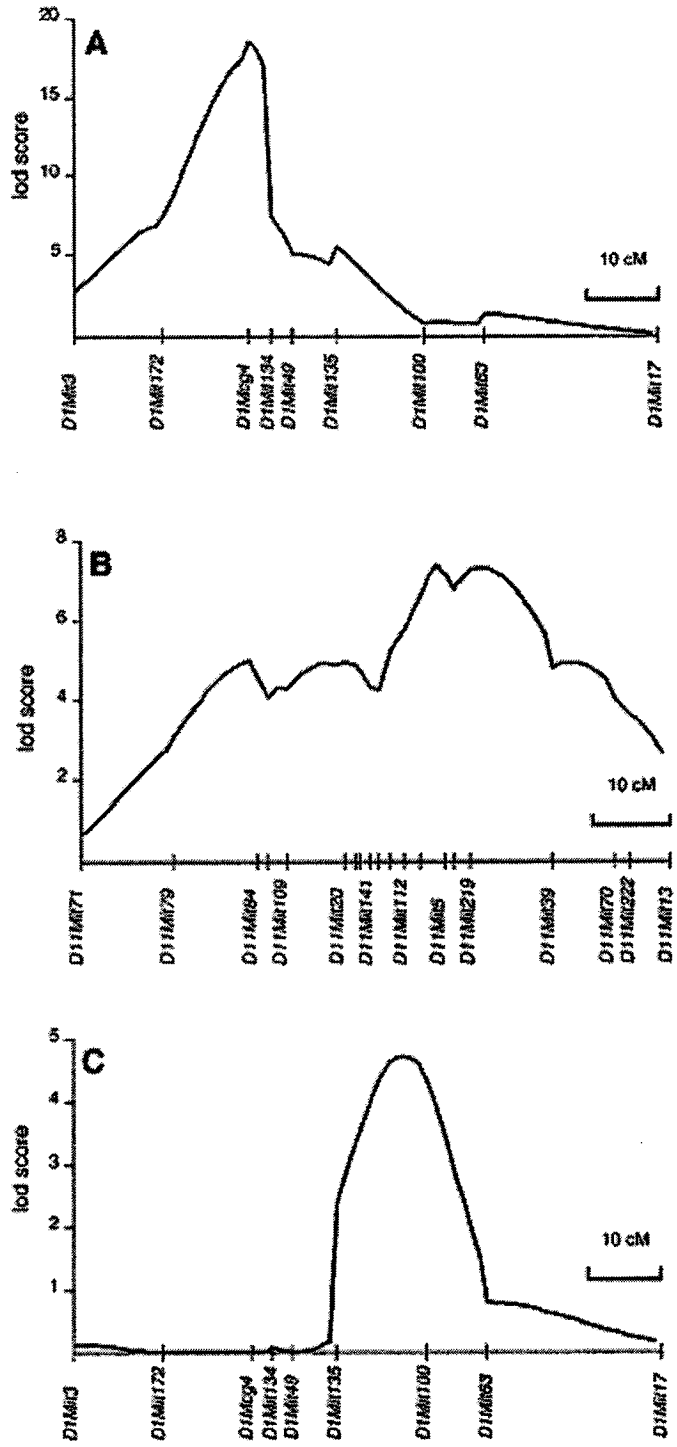
Adapted from (Wade et al. 2002)

Figure 7: Survival analysis of C57BL/6J, MOLF/Ei, (C57BL/6J x MOLF/Ei)F1, and A/J mice following infection with *Salmonella* Typhimurium. The F1 mice are more resistant to infection than their parental counterparts due to the additive effect of the functional MOLF/Ei *Slc11a1* allele (Sebastiani et al. 1998).



Adapted from (Sebastiani et al. 1998)

Figure 8: LOD score plots for chromosomes 1 and 11 in 191 (C57BL/6J x MOLF/Ei)F2 intercross mice. A resistance locus, *Ity,* on chromosome 1 with a peak LOD score at *D1Mcg4*, a marker located 400bp upstream from *Slc11a1* (A). *Ity2* is a resistance locus contributed by the MOLF/Ei allele on chromosome 11 inherited additively (B), and *Ity3* is a recessively inherited susceptibility locus contributed by the MOLF/Ei allele on distal chromosome 1 (C) (Sebastiani et al. 1998).



Adapted from (Sebastiani et al. 1998)

CHAPTER 2:

MOLECULAR GENETIC ANALYSIS OF TWO LOCI (*ITY2* AND *ITY3*) INVOLVED IN THE HOST RESPONSE TO INFECTION WITH *SALMONELLA* TYPHIMURIUM USING CONGENIC MICE AND EXPRESSION PROFILING

RATIONALE

Systemic infection of mice with *Salmonella* Typhimurium results in a disease reminiscent of typhoid fever in humans, making it an excellent model to study the etiology of disease (Santos et al. 2001). Susceptibility to *Salmonella* infection is considered to be a complex trait, involving multiple genetic contributions as well as environmental factors. At least two major genes, *Slc11a1* and *Tlr4*, have been identified that influence the innate immune response to *Salmonella* infection in inbred strains of mice (Vidal et al. 1993; Poltorak et al. 1998; Qureshi et al. 1999). Using a wild-derived inbred strain MOLF/Ei, two novel loci *Ity2* and *Ity3* have been identified in a (C57BL/6J X MOLF/Ei)F2 cross that contribute to systemic *Salmonella* Typhimurium infection in the context of a protective allele at *Slc11a1* or *Ity* (Sebastiani et al. 1998). These two loci cover relatively large intervals including numerous genes with immunological implications. In order to further define the critical intervals, fine mapping was carried out followed by expression profiling with the aim of creating a prioritized list of candidate genes to be further analyzed, bringing us closer to the identification of the causative gene(s).

Molecular genetic analysis of two loci (*Ity2* and *Ity3*) involved in the host response to infection with *Salmonella* Typhimurium using congenic mice and expression profiling

Vanessa Sancho-Shimizu[1,2], Serge Mostowy[2], Line Lariviere[2], Noemie Riendeau[2], Marcel Behr[2], Danielle Malo[1,2,3]

1 Department of Human Genetics, McGill University, Montreal, QC H3G 1A4

2 Center for the Study of Host Resistance, McGill University Health Center, Montreal, QC H3G 1A4

3 Department of Medicine, McGill University, Montreal, QC H3G 1A4

ABSTRACT

To date, numerous genes have been identified that contribute to the host response to systemic *Salmonella* Typhimurium infection in mice. We have previously identified two loci *Ity2* and *Ity3* that control survival to *Salmonella* infection in the wild-derived inbred MOLF/Ei mice, using a (C57BL/6J X MOLF/Ei)F2 cross. We validated the existence of these two loci by creating congenic mice carrying each QTL in isolation. Moreover, linkage analysis of a new segregating cross (129S6 X MOLF/Ei)F2 confirmed the contribution of *Ity2* as a resistant locus and *Ity3* as a susceptibility locus in MOLF/Ei mice. Subcongenic mice generated for each locus allowed us to define the critical intervals underlying *Ity2* and *Ity3*. Furthermore, expression profiling was carried out with the aim of identifying differentially expressed genes within the critical intervals as potential candidate genes. Genome wide expression arrays were used to interrogate expression differences in the *Ity2* congenics, leading to the identification of seven new candidate genes (*Sqstm1, Itk, Cyfip2, Hmmr, Butr1*, *Tgtp*, *Havcr2*). Interval specific oligonucleotide arrays were created for *Ity3*, identifying three differentially regulated genes (*Chi3l1, Lax1*, *Sft2d2*) to be pursued further as candidate genes. The combination of the use of congenics in QTL confirmation and fine mapping, and the identification of candidate genes by expression profiling has been successful and represents a step towards quantitative gene(s) identification.

INTRODUCTION

Salmonella enterica serovar Typhimurium (*Salmonella* Typhimurium) is a Gram-negative intracellular bacterium that is responsible for a gastrointestinal illness known as salmonellosis in humans and a typhoid-like systemic disease in mice. Typhoid fever, caused by the host specific *Salmonella* Typhi in humans, is a generalized systemic enteric fever, characterized by headache, nausea, abdominal pain, and diarrhea or constipation with case fatality of 16% without appropriate antibiotic treatment (Ohl and Miller 2001). Susceptibility to such infectious diseases is considered a complex trait involving numerous genetic and environmental factors the interactions of which determine the ultimate outcome of infection. Only a few genes that control the host response to *Salmonella* infection have been identified in humans and include *IFNGR1, IFNGR2, IL12B*, *IL12RB1*, and *STAT1* (Casanova and Abel 2004). However, the understanding of immunity to *Salmonella* infection in humans has progressed considerably through the use of mouse models of infection.

A wide range of susceptibilities to intravenous infection with *Salmonella* Typhimurium have been reported among various laboratory mouse strains. The commonly used inbred mice strain C57BL/6J are known to succumb to intravenous *Salmonella* Typhimurium infection 5 days post-infection due to a single point mutation within *Slc11a1* (previously known as *Nramp1* and *Ity* for Immunity to Typhimurium), a gene having a major impact in controlling the replication of *Salmonella* Typhimurium within the macrophage (Vidal et al. 1995). Another particular strain of interest to our laboratory was the wild-derived inbred mice MOLF/Ei, which were extremely susceptible to infection despite harboring functional alleles at *Slc11a1* and at another known

Salmonella susceptibility locus, *Toll-like receptor 4* (*Tlr4*). Using an F2 panel of (C57BL/6J x MOLF/Ei) mice, three quantitative trait loci (QTL) linked to the host response to *Salmonella* Typhimurium infection were identified: *Slc11a1* (*Ity*), *Ity2* and *Ity3* with respective LOD scores of 18.8, 7.0 and 5.0. The *Ity2* and *Ity3* QTLs were only detected once the analysis controlled for the large effect of *Slc11a1*. MOLF/Ei contributed resistant additive allele at *Ity2* on chromosome 11 and susceptible recessive allele at *Ity3* on distal chromosome 1 (Sebastiani et al. 1998).

Gene identification using quantitative trait loci (QTL) analysis is a challenging task and is based on establishing, as precisely as possible, the chromosomal position of the QTL and identifying the genes that are located in the target region. Genes are then selected according to function and sequence variants that correspond to the known effects and strain distribution of the QTL alleles. The creation of congenic and subcongenic strains is commonly used in the validation and in the fine mapping of QTLs underlying complex traits in mice. Through repeated marker-assisted backcrossing, a congenic mouse is generated such that it carries the QTL interval from the donor strain on a homogenous background representing the recipient strain. The generation of congenics allows the assessment of the effect of a unique QTL on the disease phenotype (Rogner and Avner 2003). This congenic approach has proven fruitful in the genetic dissection of diseases such as seizure susceptibility (Ferraro et al. 2004), systemic lupus erythematosus (Haywood et al. 2004; Subramanian et al. 2005) and type 1 diabetes (Lyons et al. 2000). A combination of this classical approach to fine mapping with genome-wide expression profiling techniques has been used successfully to prioritize candidate genes (Rozzo et al. 2001; Gu et al. 2002; McBride et al. 2003; Klein et al. 2004; Johannesson et al. 2005; de Buhr et al. 2006). In this paper, we describe our efforts to fine map the large *Ity2* and *Ity3*

intervals combining two genetic dissection tools, the use of congenic strain mapping and expression profiling (genome-wide and QTL interval-specific oligonucleotide arrays).

MATERIALS AND METHODS

Mice: The parental mouse strains, MOLF/Ei and C57BL/6J, were purchased from The Jackson Laboratories at 4-6 weeks of age. 129S6/SvEvTac (129S6) were obtained from Taconic (Germantown, NY, USA). The congenic mice were successively backcrossed for 10 generations selecting mice carrying the MOLF/Ei allele at *D1Mcg4* (*Ity*) and between the markers *D11Mit84* and *Mpo* (*Ity2*), resulting in the generation of B6.MOLF-*Ity/Ity2* congenic mice. Two distinct congenic strains were created additionally as a result of recombinants selected within the *Ity2* interval during the intercrossing of N10 mice to homozygosity (B6.MOLF-*Ity/Ity2.RecD*, B6.MOLF-*Ity/Ity2.RecI*). These mice carry homozygous MOLF/Ei alleles at their respective recombinant *Ity2* intervals. The construction of *Ity3* congenic mice has been described previously (Sancho-Shimizu and Malo 2006). The *Ity3* congenic and recombinant subcongenic strains (B6.MOLF-*Ity/Ity3*, B6.MOLF-*Ity/Ity.RecA*, B6.MOLF-*Ity/Ity3.RecB*, B6.MOLF-*Ity/Ity3.RecC*, B6.MOLF-*Ity/Ity3.RecE*) used in the present study differ from the previously published strains in that they have been backcrossed for over 10 generations. The recombinant subcongenic strains were obtained by breeding recombinant chromosomes generated during the intercrossing of the N10 B6.MOLF-*Ity/Ity3* strains to homozygosity. A novel segregating cross was generated between MOLF/Ei and 129S6/SvEvTac mice to produce (129S6 X MOLF/Ei)F1, and 105 (129S6 X MOLF/Ei)F2 mice. All mice were maintained in a ventilated cage with free access to food and water on a 12hr/12hr light/dark cycle. All animal procedures were performed in accordance with regulations of the Canadian Council of Animal Care.

Genotyping of Congenic Mice: At every generation, *Ity2* mice were genotyped using microsatellite markers, *D1Mcg4*, *D11Mit84*, *D11Mit109*, *D11Mit20*, *D11Mit141*, *D11Mit142*, *D11Mit112*, *D11Mit193*, *D11Mit219*, *D11Mit39*, as well as an SSLP marker for *Nos2* (due to an additional proline in MOLF/Ei) and an SSCP marker for *Mpo*. *Nos2* primers, 5'-gtatggtgtgaggttatagagatt-3' and 5'-gtcatgcaaaatctctccactgcc-3' and *Mpo* primers, 5'-gctcactcttgcagatgtgttgac-3' and 5'-tcgccacaggcaagacctagaccc-3' were used for PCR amplification of genomic DNA. Animals heterozygous for the target region of chromosome 11 were further bred to obtain the next generation of mice. Additional markers, *D1Mit3, D1Mit5, D1Mit318, D1Mit213, D1Mit46, D1Mit134, D1Mit216, D1Mit135, D1Mit218, D1Mit193, D1Mit17, D11Mit110, D11Mit174, D11Mit347, D11Mit208, D11Mit26, D11Mit156, D11Mit113, D11Mit5, D11Mit30, D11Mit7, D11Mit91, D11Mit8, D11Mit354, D11Mit326, D11Mit178* and *D11Mit70* were used to fine map the congenic boundaries. *Ity3* mice were genotyped using *D1Mit3, D1Mit5, D1Mcg4, D1Mit216, D1Mit218, D1Mit193, D1Mit63*, and *D1Mit17* for PCR amplification at every generation. Additional restriction digests specific for *Slc11a1* and *Ncf2* were also carried out. *Slc11a1* primers 5'-agctatttgggttcctgact-3' and 5'-ggtcaaagccattatggtaa-3' specific for the G169D point mutation were used to amplify genomic DNA, followed by a restriction digest using HpyCH4III. The C57BL/6J allele (D169) produces two fragments of 453bp and 81bp whereas the MOLF/Ei allele (G169) is uncut with a fragment of 534bp. *Ncf2* primers 5'-atgccttacatgctcaaggtg-3' and 5'-catgctttcttcggacaggagcagaagc-3' specific for a point mutation (R394Q), were used to amplify genomic DNA. Digestion with BsaHI restriction enzyme was carried out resulting in two products of 278bp and 41bp for C57BL/6J allele (R394), and one for the

MOLF/Ei allele (Q394) of 319bp. The digested products were resolved on a 2% agarose gel.

Sequencing: Slfn1, *Slfn3* and *Slfn4* genes were sequenced using spleen cDNA isolated from B6.MOLF-*Ity/Ity2.RecG*, B6.MOLF-*Ity/Ity2.RecD* and MOLF/Ei strains to further refine the boundaries of the congenic intervals. The entire coding sequence was sequenced for *Slfn1*, and primers used include 5'-actgtagctcatccctcaaa-3', 5'-caattctttgcttcaaaacc-3', 5'-acgggggatatttgtttatt-3', 5'-tgttacgaaaagcaagaggt-3'. The coding region of *Slfn3* and *Slfn4* were partially sequenced using the following primers for *Slfn3* 5'-gcctatgaggagacattctg-3' and 5'-tttcaacgagagctttttct-3', and *Slfn4* 5'-tgtctgcgtttttaaatgtg-3' and 5'-aaatggcagaacctttgtta-3'.

Infection and Survival Analysis: Mice between the ages of 6-16 weeks were infected intravenously with ~1,000 CFUs of *Salmonella* Typhimurium strain Keller through the caudal vein as described by us previously (Bihl et al. 2001; Sancho-Shimizu and Malo 2006). Mice were monitored daily, survival recorded, and moribund animals sacrificed by carbon dioxide asphyxiation. Survival data was analyzed using the Kaplan-Meier survival analysis.

Linkage Analysis: Genetic linkage was carried out using MapManagerQTX on 232 (C57BL/6J X MOLF/Ei) F2 mice using 8 additional markers on chromosome 11 – *Il12b* (5'-ttcatgtgctcgtggcctgatcca-3' and 5'gtacccttctaaagaaggccctgg-3'), *D11Mit22*, *D11Mit164*, *D11Mit156*, *D11Mit30*, *Nos2*, *Mpo*, and *D11Mit41*. Linkage analysis was carried out by one locus interval mapping using the two part model with the R/Qtl

program on 105 (129S6 X MOLF/Ei) F2 mice using markers on chromosome 1 (*D1Mit5, D1Mit216, D1Mit218, D1Mit193, Ncf2, D1Mit63, D1Mit17*) and chromosome 11 (*D11Mit20, D11Mit141, D11Mit156, D11Mit30, D11Mit70*) (Boyartchuk et al. 2001; Broman 2003). The genome wide significance threshold was assessed based on 1,000 permutations.

Bacterial Load Enumeration: Infected mice were sacrificed at days 3 and 7 in addition to non-infected control mice. Their spleens and livers were aseptically removed and placed in tubes with 0.9% saline solution. The tissues were homogenized and plated on trypticase soy agar plates in duplicate using at least 3 serial dilutions. Plates were incubated at 37C overnight and the colonies enumerated the following day.

Whole-Genome Expression Profiling: Total RNA was isolated from the spleens of uninfected controls and infected B6.MOLF-*Ity/Ity2* and B6.MOLF-*Ity/Ity2.RecD* mice on days 3 and 7 post-infection using Trizol reagent (Invitrogen). RNA from 3 males and 3 females was pooled per strain per time point for the first experiment and 1 male and 1 female pooled per strain per time point for the second experiment. The quality of the RNA was verified using BioAnalyzer. 10ug of each sample was used to hybridize to the Affymetrix Mouse Chip 430v.2.0 (Affymetrix, Santa Clara, CA, USA). Labeling of the probe, hybridization and scanning of the microarrays were done at the McGill and Genome Quebec Innovation Center (Montreal, Canada) as previously described by us (Caron et al. 2006).

Construction of Ity3 Interval-Specific 70-mer Oligonucleotide Microarray: Seventy-mer oligonucleotide probes were designed by Scienion AG (Berlin, Germany) for all transcripts possessing an accession ID and mapping to the *Ity3* interval (Ensembl v19.30.1 and Celera) delineated by *D1Mit135* and *D1Mit63*, resulting in a total of 375 transcripts. In addition to these probes, also added were probes for six other genes of interest during infection (*Slc11a1, Il1b, Il6, Nos2, Mpo, Tlr2*), four probes representing housekeeping genes (*18srRNA, Hprt, Tbp, Gapdh*), and two genes as negative controls for infection based on other array experiments carried out in our laboratory (*Actg2, Vcam1*). The 70-mer oligos were synthesized for all transcript probes by MetaBion GmbH (Martinsried, Germany). Finally, hybridization controls, two positive (Herring sperm DNA 10mg/ml, Cot1DNA 1mg/ml), and three negative controls (water, 3XSSC, 14XSSC) were added to the panel. Lyophilized oligos were resuspended in 3XSSC, and spotted at a concentration of 70uM on Sigmascreen™ microarray slides (Sigma) using the Virtek Chipwriter™ model SDDC2 to print oligonucleotides. The assembled *Ity3* array contained a total of 2304 spots. Each transcript probe was spotted in triplicate (1152spots), the housekeeping genes and positive hybridization controls were spotted 24 times (144 spots), and the remaining 1008 spots were represented by the negative controls. The quality of the *Ity3* array was verified after each batch of prints, using the SpotQC Kit (Integrated DNA Technologies (Coralville, IA, USA).

Interval-Specific Expression Profiling: Total RNA from spleens of uninfected and infected B6.MOLF-*Ity/Ity3*$^{MOLF/B6}$ and B6.MOLF-*Ity/Ity3* mice (at the 5th generation of backcross) was isolated as described above. Following RNA extraction, residual DNA was removed using the DNAse-I kit from Ambion and subsequent RNAeasy on-column

digestion following the manufacturer's instructions (Qiagen, Mississauga, Canada). The quality of RNA was confirmed by denaturing gel electrophoresis (formaldehyde). Microarray hybridization was performed as previously described (Charlet et al. 2005). In brief, 5-20ug of total RNA extracted from control and infected mice was labeled with Cy3 or Cy5 dUTP by reverse-transcriptase (Amersham Biosciences). Labeled cDNA was applied to a post-processed array, covered with a glass slip, and placed into a hybridization chamber overnight at 42°C. Arrays were placed into 37°C 1X SSC, 0.2% SDS to remove the cover glass, then washed in 1X SSC, 0.2% SDS for 15 min, 0.1X SSC, 0.2% SDS for 15 min, and 0.1X SSC for 15 min. Hybridized arrays were scanned with ScanArray 5000XL and hybridization results were quantified with ScanArray software (PerkinElmer, Freemont, CA). For each hybridization, one RNA sample from an individual B6.MOLF-*Ity/Ity3* $^{MOLF/B6}$ was hybridized against one RNA sample from an individual B6.MOLF-*Ity/Iy3* mouse at each time point per tissue. A single reciprocal Cy-dye swap (Cy3/Cy5 and Cy5/Cy3) experiment was minimally carried out for all hybridizations, resulting in at least a single replicate for each experiment, resulting in a total of 11 arrays studied.

Microarray Data Analysis: For whole-genome microarrays, the expression values were generated by probe-level analyses using the robust multi-array analysis (RMA) procedure (Irizarry et al. 2003). For *Ity3* arrays, analysis was performed as previously described (Charlet et al. 2005). All spots flagged as misrepresentative by ScanArray (array artifacts, etc) were analytically ignored. Subtracting total spot intensity minus the surrounding background produced a corrected spot intensity. Negative corrected spot intensities were set to +1. Intensity ratios (Cy3/Cy5 or Cy5/Cy3) were determined using

corrected spot intensities and log10 transformed. Fold change is calculated from a normalized log-ratio of that gene. Values for each gene were obtained in triplicate for each array (inherent to array design) and averaged. Only genes with fold changes of 2.0 or greater in replicate hybridization experiments are reported.

Real-time PCR: Expression of *Slfn1,4,5* in B6.MOLF-*Ity/Ity2* and B6.MOLF-*Ity/Ity2.RecD* was determined by real-time PCR using Chromo4 (MJResearch). cDNAs were obtained from reverse transcription of infected and control spleen RNAs. The cDNAs were amplified using the primers *Slfn1* 5'- GGGAACGTGCTCAGTAGA-3' and 5'-CCTGCATTTAGAATCAGCA-3', *Slfn4* 5'- AGGTTTACCACAGAGGAATG-3' and 5'-TCTGGAGAGCATATCACCTT-3', *Slfn5* 5'-GGCCTCTCGGATGATAGAAA -3' and 5'-GGTCTTGCTGCAGGGTGT -3'and cycled at 95°C for 30sec, 55°C for 30sec, 72°C for 30 sec a total of 40 cycles. *Tbp* was used as a housekeeping gene control and amplified using the primers 5'- CCCTTGTACCCTTCACCAAT-3' and 5'-ACAGCCAAGATTCACGGTAG -3' using the same cycling conditions. Stratagenes's Brilliant® SYBR® Green QPCR Master Mix was used for the PCR reactions. All samples were run in duplicate along with a standard curve of four 10-fold serial dilutions of template cDNA. The expression data are expressed in relative fold change units using uninfected B6.MOLF-*Ity/Ity2* as the referent according to the following $2^{-\Delta\Delta Ct}$ equation $2^{-((Slfn \text{ treatment Ct} - Tbp \text{ treatment Ct})-(Slfn \text{ reference Ct} - Tbp \text{ reference Ct}))}$ (Livak and Schmittgen 2001). The level of significance was assessed using the Student's t-test ($p<0.05$).

RESULTS

Ity2 and Ity3 Linkage Analysis: We have previously reported the existence of two QTLs, *Ity2* on chromosome 11 and *Ity3* on distal chromosome 1, affecting the host response of MOLF/Ei mice to infection with *Salmonella* Typhimurium. The MOLF/Ei allele at *Ity2* confers resistance to infection in an additive fashion, accounting for 10% of the phenotypic variance. The MOLF/Ei allele at *Ity3* was found to contribute to the susceptibility of MOLF/Ei mice recessively, and explained 7% of the phenotypic variance (Sebastiani et al. 1998). To confirm the location of these QTLs, we have added additional progeny as well as extra chromosome 11 specific markers to the existing (C57BL/6J X MOLF/Ei)F2 panel used in the initial linkage analysis. We have also created a new F2 population using the highly resistant 129S6/SvEvTac strain and MOLF/Ei. This was of particular importance for the creation of congenic mice especially for the QTL on distal chromosome 1 because linkage between the region harboring *Slc11a1* or *Ity* (proximal chromosome 1) may have interfered with the true position of *Ity3* located on distal chromosome 1.

For the chromosome 11 QTL harboring *Ity2*, linkage was re-analyzed using eight novel markers (*Il12b, D11Mit22, D11Mit164, D11Mit156, Inos, Mpo,* and *D11Mit41*) on a total of 232 (C57BL/6J X MOLF/Ei) F2 mice, including an additional 41 mice and the original set of 191 mice. The *Ity2* locus yielded a significant peak LOD score of 7.8 at *Nos2* under a model of free regression, with a 2 LOD support interval (99% confidence interval) spanning *D11Mit112* to *Mpo* (Figure 1A). The addition of 8 markers and additional F2 mice, lead to an increase in the peak LOD score from 7.0 to 7.8 and to a

minor repositioning of the relatively large *Ity2* interval as compared to the initial analysis, placing the peak slightly more distally.

Similarly, the *Ity3* QTL on distal chromosome 1 was re-evaluated using eight additional markers, *D1Mit5, D1Mit135, D1Mit218, D1Mit99, D1Mit193, D1Mit 201, Ncf2* and *Tlr5*, and 41 additional mice, as previously described (Sancho-Shimizu and Malo 2006). The peak LOD score of 4.1 was obtained at *D1Mit218* and *D1Mit100*, encompassing approximately the same 2 LOD support interval from *D1Mit135* to *D1Mit201* as detected in the initial analysis (*D1Mit135* to *D1Mit63*) (Figure 1B) (Sebastiani et al. 1998).

Linkage analysis in 105 (129S6 X MOLF/Ei)F2 progeny was carried out using one locus interval mapping using the two part model in R/Qtl involving two methods of analysis (Boyartchuk et al. 2001; Broman 2003). One part of the analysis represented by LODμ considers days of survival (or days to death) conditional on a non censored phenotype (excluding those mice that survive) whereas the other focuses on a binary trait, survival or death due to infection represented by LODp. In addition the assessment of linkage based on the combined score of the two analyses is shown by LODp,μ. The MOLF/Ei allele at *Ity2* on chromosome 11 conferred resistance to infection with a LODp,μ score of 3.6 ($p<0.05$) in the combined analysis, affecting the binary trait of survival represented by LODp (Figure 1C). *Ity3* was found to influence the survival phenotype such that the MOLF/Ei derived allele conferred susceptibility with a peak LOD score of 2.9 ($p<0.05$) at *D1Mit63* in the combined analysis, controlling the time to death (LODμ) as opposed to the binary trait. The impact of the *Ity2* (based on the genotype at *D11Mit156*) and *Ity3* (based on the genotype at *D1Mit63*) loci on the survival of (129S6 X MOLF/Ei)F2 mice is shown in Figures 1D and 1E. The survival analysis

found a significant difference between all genotypes at *Ity2* ($p<0.05$), consistent with an additive mode of inheritance (Figure 1D). Analysis of survival (excluding mice that survive) for *Ity3* found a significant difference ($p<0.001$) only between mice homozygous MOLF/Ei at *D1Mit63* (MST=9.2±0.7 days) versus homozygous 129S6 or heterozygous mice (MST=13.1±0.8 days; MST=15.0±1.3 days respectively) at this marker, suggesting a recessive mode of inheritance with a role in affecting only the days to death and not on surviving the infection (Figure 1E).

Generation of Congenic Mice: Congenic mice were created for *Ity2* and *Ity3* by producing F1 hybrids between C57BL/6J and MOLF/Ei, followed by at least 10 successive backcross generations to the C57BL/6J parental strain. The target *Ity/Ity2* and *Ity/Ity3* segments were maintained using marker-assisted genotyping. Homozygous founders were established by brother-sister matings of N10 mice. Due to the impact of *Slc11a1* (*Ity*) on the detection of *Ity2* and *Ity3,* congenic B6.MOLF-*Ity* were created by transferring the wild-type allele at *Slc11a1* originating from the MOLF/Ei mice onto a C57BL/6J genetic background, who naturally carry the mutant form of this well characterized *Salmonella* susceptibility gene (Vidal et al. 1993). The largest *Ity2* interval transferred spans from *D11Mit110* to *D11Mit91*, a 39.0Mb interval and the largest *Ity3* interval is 62.0Mb in size located from *D1Mit218* to *D1Mit17*. Both regions extend well beyond the 2 LOD support interval to ensure that all genetic elements contributing to the QTL- associated phenotype will be transferred to the resulting congenic strains. Moreover the presence of numerous immunological relevant genes that map within the relatively large *Ity2* and *Ity3* intervals suggest the possibility that more than one gene may be involved in the disease phenotype underlying the QTL interval.

The recombinant congenics were generated at the same time as the B6.MOLF-*Ity/Ity2* and B6.MOLF-*Ity/Ity3* congenics, by selecting mice sharing recombinant chromosomes at the N10 generation, that were further bred to homozygosity through brother-sister matings. This led to the establishment of two recombinant B6.MOLF-*Ity/Ity2* strains (*RecD* and *RecI*), and four recombinant B6.MOLF-*Ity/Ity3* strains (*RecA*, *RecB*, *RecC*, and *RecE*) shown in Figure2A and 2B.

Phenotypic Characterization of Ity2 Congenic Strains: All congenic strains were infected with *Salmonella* Typhimurium intravenously and their survival noted. As expected, the C57BL/6J mice were the most susceptible to infection and the transferal of a wild-type MOLF/Ei allele at *Slc11a1* (B6.MOLF-*Ity*) improved resistance to infection significantly (Figure 3A) (Sancho-Shimizu and Malo 2006). Survival analysis revealed that only the B6.MOLF-*Ity/Ity2* congenic (MST =11±1.1 days) improved survival time over the control B6.MOLF-*Ity* mice (MST=8.7±0.4 days) ($p<0.05$) (Figure 3A). The remaining *Ity2* congenic strains, B6.MOLF-*Ity/Ity2.RecD* and B6.MOLF-*Ity/Ity2.RecI* (MST=7.9±0.4 and MST=8.3±0.6 days respectively) did not differ significantly from the B6.MOLF-*Ity* controls suggesting that the portion of *Ity2* that these mice carry does not contribute to improved resistance.

In an attempt to further sub-phenotype the *Ity2* congenic mice, the bacterial load in the spleen and liver was determined at various time points upon infection. C57BL/6J mice had approximately ten-fold higher bacterial loads in the spleen and liver at day 3 compared to the congenic mice and no C57BL/6J mice survived after day 5. This observation is also attributable to the fact that there mice carry a non functional mutation in *Slc11a1*, as previously reported (Vidal et al. 1995; Sancho-Shimizu and Malo 2006).

No significant difference was observed between any of the *Ity2* congenics and the B6.MOLF-*Ity* controls at all time points (Figures 3B and 3C) strongly suggesting that the impact of *Ity2* on survival to infection is not related to the level of bacterial load in the target organs.

Consistent with previously published results, survival of B6.MOLF-*Ity/Ity3* (MST=7±0.3 days) mice was significantly reduced in comparison to the B6.MOLF-*Ity* control (MST=10.2±0.3 days) ($p<0.05$) (Figure 3D). Data presented here represents B6.MOLF-*Ity/Ity3* mice that have been backcrossed for over 10 generations as opposed to previously published data which tested B6.MOLF-*Ity/Ity3* mice intercrossed after only 5 generations (Sancho-Shimizu and Malo 2006). The survival analysis represents three independent experiments that have been pooled due to the consistent mean survival times obtained among the B6.MOLF-*Ity* mice in all experiments (MST=10.7±1.3; MST=10.1±0.4 and MST=9.8±0.3 days). B6.MOLF-*Ity/Ity3.RecC* (MST=9.8 SEM 0.3 days) did not differ significantly from the B6.MOLF-*Ity* controls suggesting that this portion of chromosome 1 did not contribute to the susceptibility phenotype, whereas all other *Ity3* recombinant congenics were more susceptible to infection. Based on this analysis the B6.MOLF-*Ity/Ity3* congenics (MST=7.0±0.3 days) were more susceptible than *RecA*, *RecB*, and *RecE*, (MST=8.5±0.4; MST=8.8±0.3; MST=8.1±0.2 days respectively $p<0.05$ for all comparisons) which can be explained by the effect of more than one gene within the *Ity3* interval on the disease phenotype.

Refinement of Ity2 and Ity3 Critical Intervals: The centromeric boundary of the congenic *Ity2* interval in B6.MOLF-*Ity/Ity2* was resolved to a 2.3Mb region between *D11Mit110* and *D11Mit109*. The distal end was delimited by a 700kb region located

between *D11Mit91* and *D11Mit8*. The *Ity2 RecD* interval was resolved proximally by a 1.9 Mb region between *D11Mit112* and *D11Mit26* and distally, by a 600kb interval delimited by *Slfn3* and *D11Mit326*. Based on the survival analysis of the *Ity2* congenics, we were able to further restrict the large interval to two more defined regions (Figure 2A, *Ity2.A* and *Ity2.B*). The thick black lines represent specific regions that are necessary but not sufficient in the resistance phenotype. The two intervals (*Ity2.A* and *Ity2.B*) are present in the resistant strain B6.MOLF-*Ity/Ity2*, and one of the two regions absent in the susceptible strains B6.MOLF-*Ity/Ity2.RecD* (missing *Ity2.A*) and B6.MOLF-*Ity/Ity3.RecI* (missing *Ity2.B*). The proximal region, *Ity2.A*, is flanked by *D11Mit109* and *D11Mit26*, an interval spanning approximately 22Mb, encoding for 333 genes according to the latest update of the Ensembl genome browser (Ensembl build 39). The distal region, *Ity2.B*, is flanked by *D11Mit5* and *D11Mit8*, an interval of approximately 13Mb comprised of 364 genes (Ensembl build 39). Even though the survival data seem to point out these two particular regions as critical areas of interest, the region between these two intervals cannot be disregarded in any future analysis and may harbor additional genes impacting on the phenotype.

The B6.MOLF-*Ity/Ity3* congenics harbor the *Ity3* interval spanning 62.0Mb between *D1Mit218* and *D1Mit17*, with a proximal boundary between *D1Mit216* and *D1Mit218* (Figure 2B). The *Ity* interval in B6.MOLF-*Ity/Ity3* mice spans *D1Mcg4* to *D1Mit5* with region boundaries extending proximally to *D1Mit3* and distally to *D1Mit216*. The *Ity* interval in the B6.MOLF-*Ity* and B6.MOLF-*Ity/Ity3* recombinant strains (*RecA, RecB, RecC* and *RecE*) carry MOLF/Ei alleles at *D1Mcg4* and have region boundaries spanning from *D1Mcg4* to *D1Mit3*, and between *D1Mcg4* and *D1Mit216*. The survival analysis of the recessively inherited susceptibility locus *Ity3*, suggests that the region of

3.8Mb between *D1Mit218* and *D1Mit193* in B6.MOLF-*Ity/Ity3.RecC* strain does not contribute to the susceptibility of the mice, and could be eliminated as harboring genes for *Ity3* susceptibility. The remaining *Ity3* strains were all susceptible to infection suggesting they all carry intervals necessary for the manifestation of the phenotype. The smallest interval can be deduced from the overlap between the *Ity3 RecB* and *RecE* strains, surrounding *Ncf2* and delineated by *D1Mit193* and *D1Mit63* representing the critical interval (Figure2B). Within this 34Mb critical interval, there are a total of 320 genes according to Ensembl build 39.

Ity2 Interval-Specific Expression Profiling: We have used transcriptional profiling to help the identification of candidate genes underlying *Ity2* and *Ity3*. Disease QTLs have been shown previously to be linked to the heritability of variation in gene expression of positional candidate genes (Aitman et al. 1999; Hubner et al. 2005). Expression profiles of spleens from B6.MOLF-*Ity/Ity2* (resistant) and B6.MOLF-*Ity/Ity2.RecD* (susceptible) mice were interrogated using the Affymetrix Mouse 430v2.0 chip, at days 0, 3 and 7 of infection (Table 1,Figure 4A). Genes that differed by 2 fold and mapped to chromosome 11 were the primary focus of analysis. Differentially regulated genes were clustered within two chromosomal regions (*D11Mit110-D11Mit26* and *Nos2-D11Mit178*) of different parental origin in the two congenic strains (Figure 4A). The proximal region delineated by *D11Mit110* and *D11Mit26* corresponds to the critical minimal *Ity2.A* interval although the distal region (*Nos2-D11Mit178*) does not. In fact, no genes located within the *Ity2.B* interval were differentially regulated.

Among those differentially regulated, there were a total of 5 genes that were differentially expressed at all time points (Table1, Figure 4A). Three genes, *Slfn8*, *Crlf3*,

and *Ccdc16* were consistently upregulated in the resistant B6.MOLF-*Ity/Ity2* strain and 2 genes, *Zfp207* and *Sqstm1* downregulated at all time points. Of these consistently differentially regulated genes, only *Sqstm1* (sequestosome 1) was mapped to the critical minimal *Ity2.A* interval. *Sqstm1* is of interest as it is involved in the polyubiquitination of TRAF6 (TNF receptor-associated factor 6) and in the regulation of NF-κB signaling (Babu et al. 2005). Many of the pathways that lead to the upregulation of pro-inflammatory cytokine expression are dependent on proper NF-κB signaling, suggesting a role for *Sqstm1* in the immune response.

In control mice (day 0), there were a total of 12 chromosome 11 specific genes that were differentially regulated (Table 1). Of the 12 genes, 7 were located within the minimal critical *Ity2.A* interval and included in addition to *Sqstm1*, *Zfp62* (zinc finger protein 62), *Itk* (IL-2 inducible T-cell kinase), *Cyfip2* (cytoplasmic FMR1 interacting protein 2) and three ESTs. The gene *Itk* is clearly involved in T-cell activation (Au-Yeung et al. 2006) and *Cyfip2*, may also play a role in T-cell regulation based on recent work showing that high expression of *CYFIP2* in multiple sclerosis patients is associated with increased T-cell adhesion (Mayne et al. 2004).

At day 3, we detected an increase in the number of differentially regulated genes on chromosome 11, for a total of 22 genes, of which 14 were downregulated and 8 upregulated. Ten genes were located within the minimal *Ity2.A* interval including several genes involved in the regulation of transcription (*Ublcp1, Ankrd43, Hist3h2ba*) and genes with potential relevance to the immune response, *Hmmr* (hyaluronan mediated motility receptor), *Tgtp* (T-cell specific GTPase), *Butr1* (butyrophilin related 1). *Hmmr* is a receptor involved in cell motility and in various kinase signaling cascades, including the ERK1 kinase (Turley et al. 2002), *Tgtp* is implicated in anti-viral responses (Carlow et al.

1998), and *Butr1* according to UniProtKB/Swiss-Prot (http://ca.expasy.org/sprot/) is predicted to be a member of the immunoglobulin gene family.

At day 7, there were 5 down regulated and 12 upregulated transcripts between the two congenic strains and 7 were located within the minimal *Ity2.A* interval. We observe an upregulation of two additional transcripts involved in T-cell activation, *Tgtp* and *Havcr2* (hepatitis A virus cellular receptor 2) a gene involved in dampening T_H1 immune responses and immunological tolerance (Carlow et al. 1998; Sanchez-Fueyo et al. 2003).

For all time points, the downregulated genes were almost exclusively found in the proximal portion of the minimal *Ity2.A* interval and high transcriptional activity was observed at the distal limit of the *Ity2* QTL interval, outside of the critical interval. This distal region contains members of the Schlafen family of genes (*Slfn1, Slfn4, Slfn5, Slfn8,* and *Slfn10*) that were upregulated at different time points during infection in the resistant B6.MOLF-*Ity/Ity2* mice. *Slfn1,* and more recently *Slfn8,* has been demonstrated to be involved in the negative regulation of peripheral T-cell growth (Schwarz et al. 1998; Geserick et al. 2004). The differential regulation observed here may represent either epigenetic effects on gene expression due to rearrangement in the chromatin structure at the limit of the congenic interval or downstream cis effects due to the *Ity2* QTL gene(s).

Validation of Ity2 microarray data: In general, alleles of MOLF/Ei origin present lower levels of expression for several genes located within the *Ity2* QTL interval. We have measured mRNA levels of specific transcripts by quantitative PCR (QPCR) in the congenic strains to validate the observed differential expression in congenic mice and to determine if the interstrain differences in gene expression may be caused by the high genetic diversity known to be present between MOLF/Ei and C57BL/6J mice. We

initially focused our validation on the *Slfn* family of genes that were consistently upregulated in the resistant B6.MOLF-*Ity/Ity2* mice. We first sequenced the target region for *Slfn1* and found numerous important sequence variants between MOLF/Ei and C57BL/6J alleles that could have interfered with probe binding, accounting for the apparent differential expression observed using the microarray. Primers were then carefully designed to ensure that MOLF/Ei and C57BL/6J alleles were appropriately amplified by QPCR for all genes tested. We confirmed an expression difference for *Slfn1, Slfn4* and *Slfn5* (Figure 5). The expression of all genes was significantly increased in B6.MOLF-*Ity/Ity2* during infection at day3. Consistent with microarray results, *Slf5* was upregulated in B6.MOLF-*Ity/Ity2* mice on day3, and *Slfn4* was upregulated at day 7 but also on day 3. *Slfn1* results did not correlate with microarray data due to the numerous sequence variants within the Affymetrix probe.

Seven positional candidate genes (*Sqstm1, Itk, Cyfip2, Hmmr, Butr1, Tgtp* and *Havcr2*) have been selected based on their differential levels of expression and on their potential role in the host immune response, for future QPCR analyses.

Ity3 Interval-Specific Expression Profiling: In order to investigate expression differences in the *Ity3* interval, we employed a slightly different approach by creating a custom oligonucleotide array including probes representing genes that map within the *Ity3* interval. The assembled array included 375 genes mapping between the markers *D1Mit135* and *D1Mit63* (based on Ensembl build v19.30.1) which corresponds to the region approximating the 2 LOD support interval. The quality of the array hybridization experiments was confirmed by positive hybridization signals emitted by the positive controls (*18srRNA, Hprt, Tbp, Gapdh,* Herring sperm DNA, cot1 DNA). Spleen RNA

from B6.MOLF-*Ity/Ity3* and B6.MOLF-*Ity/Ity3*$^{MOLF/B6}$ uninfected (day 0), day 1, day3 and day5 *Salmonella* Typhimurium infected mice was hybridized to the arrays for analysis. The recessively inherited susceptibility phenotype that the *Ity3* locus confers, has previously been established using B6.MOLF-*Ity/Ity3* and B6.MOLF-*Ity/Ity3*$^{MOLF/B6}$ mice, such that B6.MOLF-*Ity/Ity3*$^{MOLF/B6}$ were more resistant to infection and performed as B6.MOLF-*Ity* mice do as shown in Figure 3D (Sancho-Shimizu 2006). Probes that were differentially regulated by 2 fold in dye swap experiments were considered for further analysis.

No genes were found to be differentially regulated at day 0, however two genes were found to be upregulated in the susceptible B6.MOLF-*Ity/Ity3* mice one day after infection with *Salmonella* Typhimurium, *Bcl2* (B-cell leukemia/lymphoma 2) and *Lax1* (lymphocyte transmembrane adaptor 1) (Table 2 and Figure 4B). *Bcl2* is involved in suppressing apoptosis and it has been implicated in apoptosis associated with infections, however this gene was found outside the critical candidate region for *Ity3* (Kroemer 1997; Rios-Barrera et al. 2006). *Lax1* has yet to be mapped definitively on the Ensembl mouse genome browser build 39, however it is syntenic to human 1p32.1, placing it on mouse chromosome 1 around 135.5Mb within the *Ity3* minimal interval, making it an excellent candidate gene based on its role in lymphocyte signaling (Zhu et al. 2002).

Only one gene, chitinase 3-like 1 (*Chi3l1*) was upregulated in the B6.MOLF-*Ity/Ity3* mice on day 3. *Chi3l1* is located within the critical *Ity3* interval, and has been shown recently to mediate bacterial adhesion and invasion in intestinal epithelial cells and to influence the outcome of oral *Salmonella* Typhimurium infections in mice, making it an interesting candidate gene to pursue (Mizoguchi 2006).

Seven genes were upregulated and one downregulated on day 5 of infection in B6.MOLF-*Ity/Ity3* mice as detected by array experiments. The only gene found to be downregulated in B6.MOLF-*Ity/Ity3* mice, was *Fcamr* (FcαμR or Fc receptor, IgA, IgM, high affinity) the Fc receptor responsible for binding to IgM and IgA however it was found to be outside the critical interval (Shibuya et al. 2000). Of the seven upregulated genes, six mapped within the critical *Ity3* interval identified through fine mapping and include, *Rbbp5* (Retinoblastoma binding protein 5), *Cdc73* (also known as *Hprt2*), *Sft2d2* (SFT2 domain containing 2), *Niban*, *1200016B10Rik*, and *1190005F20Rik* (Table 2 and Figure 4B). Among these genes, only *Sft2d2* presented with a relevant putative function in *Salmonella* infection, as it is predicted to be involved in the retrograde vesicle transport by similarity to the yeast Sft2p protein (Conchon et al. 1999).

Based on position and function, three differentially regulated genes, *Lax1, Chi3l1,* and *Sft2d2* will be further validated by QPCR for future experiments as candidate targets for *Ity3*.

DISCUSSION

Ity2 and *Ity3* are two QTLs implicated in survival to systemic *Salmonella* Typhimurium infection, that have been identified in the wild-derived inbred mouse MOLF/Ei (Sebastiani et al. 1998).We have used congenic mouse strains in combination with expression profiling to prioritize candidate genes for each QTL, an approach that has been used successfully in finding disease-causing genes in QTL mapping studies by other groups (Rozzo et al. 2001; Gu et al. 2002; McBride et al. 2003; Klein et al. 2004; Johannesson et al. 2005; de Buhr et al. 2006). A weakness of the expression profiling approach to keep in mind however, is that the causative effect underlying quantitative traits such as survival to *Salmonella* Typhimurium infection, may not be due to differential gene expression and this approach may be associated with the potential risk of not identifying the disease gene(s). In fact several important *Salmonella*-susceptibility loci including *Slc11a1* and *Tlr4* are not regulated at the transcript levels (Vidal et al. 1993; Qureshi et al. 1999). On the other hand, differential expression of a candidate gene does not necessarily imply causation. An excellent example of this situation is provided by the gene *Tlr5* in *Salmonella* susceptibility of MOLF/Ei mice. *Tlr5* was clearly shown to be downregulated in the liver of MOLF/Ei mice, however *in vitro* and *in vivo* functional analyses clearly showed that *Tlr5* was not the gene underlying the *Ity3* locus (Sebastiani et al. 2000; Sancho-Shimizu et al. 2006). However the use of congenic mice in microarray analyses remains extremely valuable in the prioritization of candidate genes for future analysis.

We have confirmed the genetic effect of *Ity2* on resistance to infection and *Ity3* on susceptibility to infection using congenic and subcongenic mouse strains and a novel

segregating cross between 129S6 and MOLF/Ei mice. The impact of *Ity2* and *Ity3* on the disease phenotype was similar to that observed previously in the (C57BL/6J X MOLF/Ei)F2 cross. The existence of the *Ity2* resistance QTL in the new (129S6 X MOLF/Ei)F2 population was somewhat surprising considering the fact that 129S6 mice are extremely resistant to infection and that the chromosome 11 alleles contributed by the highly *Salmonella*-susceptible MOLF/Ei mice are more protective than the 129S6 allele. The two part analysis in R/Qtl used in the novel cross has been able to attribute the effects of *Ity2* in controlling primarily the binary trait of death or survival. The *Ity3* QTL was also confirmed, influencing the time to death in this novel cross. *Ity3* was also found previously in a third cross between C3H/HeJ and MOLF/Ei (Sebastiani G., Sancho-Shimizu V and Malo D. unpublished data). In this particular cross *Ity3* accounts for a larger portion of the phenotypic variance (25%). The importance of the validation in the novel cross is highlighted by the fact that in previous crosses, with C57BL/6J and C3H/HeJ, major genes involved in *Salmonella* host response, *Slc11a1* and *Tlr4*, were segregating, possibly impacting on the effects of other *Salmonella* response loci.

Congenic and subcongenic mouse strains were also very useful to map more precisely *Ity2* and *Ity3*, and in defining the minimal critical region to search for candidate genes. Congenics carrying the chromosome 11 *Ity2* interval as well as *Ity3* on distal chromosome 1 were successfully created, in the context of a protective allele at *Slc11a1* within *Ity* (Sancho-Shimizu and Malo 2006). B6.MOLF-*Ity/Ity2* congenics showed improved survival as compared to the B6.MOLF-*Ity* controls, consistent with the prediction from linkage analyses, in which the MOLF/Ei allele at *Ity2* conferred protection and was inherited additively (Sebastiani et al. 1998). The two subcongenics, B6.MOLF-*Ity/Ity2.RecD* and B6.MOLF-*Ity/Ity2RecI*, enabled us to identify the minimal

intervals necessary for resistance, *Ity2.A* and *Ity2.B*. *Ity2.A* covers a 22Mb proximal portion spanning *D11Mit109* to *D11Mit26* containing 333 genes, and *Ity2.B* consists of a13Mb distal interval from *D11Mit5* to *D11Mit8* including 364 genes.

B6.MOLF-*Ity/Ity3* and subcongenics were used to further delineate the critical *Ity3* interval to 34Mb comprised of 320 genes. The region boundaries marked by *D1Mit218* and *D1Mit216*, as well as region boundaries surrounding the *Ity* interval will be resolved further in future experiments as their contribution to the susceptibility of B6.MOLF-*Ity/Ity3* mice could not be determined. In spite of this, a clear and consistent phenotype was observed in the *Ity3* congenic strains tested (B6.MOLF-*Ity/Ity3, Rec.A, Rec.B, Rec.E*) implicating that the homozygous MOLF/Ei interval that they carry is responsible for the observed susceptibility.

Faced with a large number of candidate genes within the *Ity2* and *Ity3* intervals and the laborious and time-consuming creation of high resolution congenic mapping, we have used genome wide expression (*Ity2*) and QTL-specific arrays (*Ity3*) to obtain a list of candidate genes for each QTL. The advantage of microarrays lies partly in its unbiased approach to identify differentially expressed genes, at times leading to the discovery of genes that may not have been previously considered as candidates. For *Ity2*, the primary focus was to identify genes dysregulated in the QTL interval with the aim of identifying pathways that may be implicated in the phenotype. We noticed first of all, a bias of differentially regulated transcripts that appear as clusters on chromosomes 1 and 11 (Supplemental Tables 1-3). These happen to correspond to the chromosomes that carry the congenic intervals. This is probably due to the introduction of a MOLF/Ei genomic interval, and may represent epigenetic effects on transcription. The mere introduction of a portion of the chromosome may affect the chromatin structure for example, affecting the

transcription of a number of genes. Resolving expression differences due to the different congenic fragments in the context of *Salmonella* infection can be complicated by these extraneous influences.

Within the chromosome 11 interval, we have identified two clusters of differentially regulated genes, a cluster of genes around *Ity2.A* and another just outside the distal interval *Ity2.B*. We observed an overall trend that the differentially regulated genes in the proximal interval were almost always upregulated in the spleens of the resistant B6.MOLF-*Ity/Ity2* mice, whereas those that were at the distal region were always downregulated. An example of differentially regulated genes in the distal region, are the *Slfn* family of genes. Upon sequencing of *Slfn1*, numerous sequence variants were identified (21SNPs/1kb coding sequence) suggesting that difference in intensity signal is probably due to poor probe hybridization in the B6.MOLF-*Ity/Ity2.RecD* mice rather than low transcript levels. Although some expression differences were confirmed upon infection (Figure 6), it is unlikely that these genes are the primary genes underlying *Ity2* as they map outside the target congenic interval. The differential regulation may be due to the allelic differences in the genes since the susceptible B6.MOLF-*Ity/Ity2.RecD* mice carry the MOLF/Ei allele in this interval, or due to epigenetic effects as discussed above.

The candidates of the *Ity2* locus would be those that are differentially regulated in the proximal interval coincident with *Ity2.A* since the B6.MOLF-*Ity/Ity2* mice carry the resistant MOLF/Ei allele in this interval as compared to the susceptible B6.MOLF-*Ity/Ity2.RecD* mice that harbor the C57BL/6J allele. We have identified 7 such genes in the spleen. Of the consistently dysregulated genes, *Sqstm1* is of interest as it maps in the proximal interval and is involved in the polyubiquitination of TRAF6 and is thought to regulate NF-κB signaling (Babu et al. 2005). Many of the pathways that lead to the

upregulation of pro-inflammatory cytokine signaling are dependent on proper NF-κB signaling suggesting a role in the immune response. The fact that this gene is consistently downregulated in the resistant B6.MOLF-*Ity/Ity2* may suggest that they have somewhat reduced signaling capacity resulting in less inflammatory molecules being secreted, which in excess may be detrimental to the host. However the genome-wide data does not corroborate this hypothesis since we do in fact find immune response genes to be upregulated in the resistant strain at days 3 and 7 (Supplemental Tables 2-3). *Itk*, is another candidate gene that is downregulated in B6.MOLF-*Ity/Ity2* at day 0 and has a role in T cell development and immunity however it has an essential role in T_H2 type responses which are primarily involved in extracellular infections, making it a less attractive candidate in this infection model (Au-Yeung et al. 2006). *Cyfip2*, the cytoplasmic interacting partner to fragile X protein, has recently been implicated in patients with multiple sclerosis (Mayne et al. 2004). It was found to be upregulated in $CD4^+CD8^+$ cells, and Mayne and colleagues have hypothesized that *Cyfip2* acts to facilitate T cell adhesion. Another T cell associated gene, *Tgtp*, known to be an immediate-early gene of IFNγ in T cells as well as in macrophages, was upregulated upon infection. This gene has been demonstrated to be essential in RNA-virus mediated immunity (Carlow et al. 1998). *Hmmr*, downregulated in B6.MOLF-*Ity/Ity2* mice on day 3, is a receptor involved in cell motility and in various kinase signaling cascades, in particular the ERK1 kinase (Turley et al. 2002). The function of *Butr1* remains unknown however it has been shown to harbor an immunoglobulin-like domain suggesting its involvement in the immune system (http://ca.expasy.org/sprot/). Finally, on day 7, *Havcr2* (also known as *Tim3*) was found to be upregulated in the resistant congenic B6.MOLF-*Ity/Ity2*. *Havcr2* is specifically expressed on T_H1 cells and thought to

negatively regulate T_H1 responses, and has been linked to asthma susceptibility (Meyers et al. 2005). This may indicate that the resistant B6.MOLF-*Ity/Ity2* mice are more efficient at controlling the inflammatory response than their susceptible counterparts, suggesting a crucial role in the response to infection and leading to their prolonged survival.

Many of the genes that were differentially expressed and discussed above were almost exclusively involved in T cell activities. We have assayed for the expression in the spleens of these mice, in which lymphocytes are the predominant cell type. Consequently, the bias towards T cell activities may be merely due to the fact that we have used spleens in the microarray experiments, however it may also strongly suggest that this is the cellular compartment that warrants further analysis. The apparent lack of differentially expressed genes in the *Ity2.B* interval may suggest that it may contain gene(s) with a protein defect that may in turn affect the expression of the genes within the *Ity2.A* interval. This may be a proposed mechanism by which these two critical intervals interact and are hence both necessary for the resistance phenotype observed in the B6.MOLF-*Ity/Ity2* mice.

Custom oligonucleotide arrays were constructed for the *Ity3* interval, and led to the identification of three new candidates, *Lax1, Chi3l1* and *Sft2d2* based on position, expression and potential function in the host response to *Salmonella* infection. These three genes are all upregulated in the susceptible B6.MOLF-*Ity/Ity3* mice. *Sft2d2*, is believed to participate in retrograde vesicular trafficking by similarity to the yeast protein (Conchon et al. 1999). The potential relevance of this gene in *Salmonella* infection can be easily foreseen, as it may affect components of the endosomal pathway, crucial to the establishment of the *Salmonella* containing vacuole, in which intracellular *Salmonella*

reside and replicate (Holden 2002; Knodler and Steele-Mortimer 2003). *Lax1* has been shown to function as a negative regulator of lymphocyte activation, and overexpression of *Lax1* in T cells inhibited TCR mediated p38MAPK and NFAT/AP-1 activation (Zhu et al. 2005). Upregulation of these pathways are an integral part of the host immune response, and may affect disease outcome. Finally, *Chi3l1* appears to be a very promising candidate based on its function to enhance bacterial invasion and adherence in intestinal epithelial cells. In fact, the administration of *Chi3l1* neutralizing antibodies prior to oral *Salmonella* Typhimurium infection was protective in mice (Mizoguchi 2006). Moreover, overexpression of this gene *in vitro* resulted in the presence of greater numbers of intracellular *Salmonella* Typhimurium (Mizoguchi 2006). Therefore the upregulation of *Chi3l1* may be consistent with increased susceptibility to infection as observed in B6.MOLF-*Ity/Ity3* mice.

In the current study, we have restricted the *Ity2* and *Ity3* intervals and proposed a list of candidate genes to be investigated further. The wild-derived inbred mouse MOLF/Ei has been separated by over 1 million years of evolution from the classical strains such as C57BL/6J and 129S6 mice, within which time they have accumulated numerous sequence variants, on the order of 1 SNP/100bp. The evolutionary divergence between the two strains is well illustrated in this study as reflected by the high sequence variation between C57BL/6J and MOLF/Ei alleles at specific genes, such as the *Slfn's,* which has the potential of affecting interpretation of array studies. Other than sequence variation, the possibility to impact on transcriptional regulation based on chromatin structure or due to genetic background effects is also demonstrated through the cluster of differentially regulated genes found distal to the *Ity2.B* interval. The existence of the resistance locus in *Ity2* in the (129S6 X MOLF/Ei)F2 population, further reveals the

complexity of the host response to infection in MOLF/Ei where one would expect to find strong susceptibility determinants. A complete genome scan of the novel fully informative (129S6 X MOLF/Ei)F2 cross may further resolve the intricacies of *Salmonella* pathogenesis and lead to the identification of other loci in the MOLF/Ei strain contributing to our understanding of their extreme susceptibility to infection.

ACKNOWLEDGEMENTS

We thank Rosalie Wilkinson for her assistance with the animal work, and Beatrice Kenol for her technical assistance. Makeda Semret for helpful discussions in the creation of the *Ity3* custom oligonucleotide arrays, Fiona MacIntosh for her help in the printing of the arrays as well as in several hybridization experiments. Marie France Roy for her assistance in R/Qtl analysis. This project was supported by research grants to Danielle Malo from the Howard Hughes Medical Institute and the Canadian Institute for Health Research. Danielle Malo is a McGill William Dawson Scholar. Vanessa Sancho-Shimizu was supported by studentships from the McGill University Health Center Research Institute and by the McGill University Faculty of Medicine.

TABLES

Table 1: *Ity2* Whole Genome Array List of Differentially Regulated Genes on Chromosome 11

Up/Down*	Affymetrix ID	Gene Name	Position (Mb)**	Fold Change	Function
		- DAY 0 -			
UP	1451655_at	*Slfn8*	82.8	10.8	T cell Development
	1438235_at	*Crlf3*	79.9	8.0	Cytokine receptor
	1418612_at	*Slfn1*	83.1	5.6	T cell Development
	1450942_at	*Ccdc16*	82.6	3.9	Cell cycle
	1451730_at	*Zfp62*	49.1	2.2	Skeletal muscle
DOWN	1423546_at	*Zfp207*	80.2	-3.3	Metal binding Nuclear Protein
	1440076_at	*Sqstm1*	50.0	-3.3	Regulates NF-κB signaling
	1443435_at	*3732413I11Rik*	44.4	-3.3	Ubiquitin Conjugation
	1456836_at	*Itk*	46.2	-2.5	T cell Development
	1430177_at	*2610301N02Rik*	51.8	-2.5	Unknown
	1427108_at	*9530068E07Rik*	52.2	-2.0	Unknown
	1449273_at	*Cyfip2*	46.0	-2.0	Fragile X / T cell Adhesion
		- DAY 3 -			
UP	1451655_at	*Slfn8*	82.8	15.6	T cell Development
	1438235_at	*Crlf3*	79.9	7.1	Cytokine receptor
	1450942_at	*Ccdc16*	82.6	5.5	Cell cycle
	1418612_at	*Slfn1*	82.9	5.1	T cell Development
	1425728_at	*Tgtp*	48.7	3.4	T cell GTPase
	1419684_at	*Ccl8*	81.9	2.3	Chemotaxis
	1458458_at ,1456288_at	*Slfn5*	82.8	2.2, 2.0	T cell Development
	1444350_at	*Slfn10*	82.8	2.0	T cell Development
DOWN	1451257_at	*Acsl6*	54.1	-3.3	Fatty acid metabolism
	1445845_at	*Ublcp1*	44.3	-3.3	RNA polymerase II CTD
	1423546_at	*Zfp207*	80.2	-3.3	Metal binding Nuclear Protein
	1429871_at ,1427541_x_at	*Hmmr*	40.5	-3.3,-2.5	Cell Motility/ERK Kinase
	1429606_at	*4930527B16Rik*	44.3	-2.5	Unknown
	1436998_at	*Ankrd43*	53.3	-2.5	DNA binding
	1418311_at	*Fn3k*	121.3	-2.5	Fructosamine kinase
	1419074_at	*2510006C20Rik*	30.9	-2.5	Unknown
	1421264_at	*Butr1*	58.7	-2.5	Immunoglobulin-like
	1449482_at	*Hist3h2ba*	58.8	-2.5	Histone/Nucleosome
	1440076_at	*Sqstm1*	50.0	-2.5	Regulates NF-κB signaling
	1458440_at	*LOC432572*	62.0	-2.5	Unknown
	1443435_at	*3732413I11Rik*	44.4	-2.0	Ubiquitin Conjugation
	1425704_at	*BC022224*	84.6	-2.0	Short chain dehydrogenase/reductase
		- DAY 7 -			
UP	1451655_at	*Slfn8*	82.8	10.2	T cell development
	1418612_at	*Slfn1*	82.9	4.5	T cell development
	1425728_at	*Tgtp*	48.7	3.8	T cell GTPase
	1438235_at	*Crlf3*	79.9	3.8	Cytokine receptor
	1418126_at	*Ccl5*	83.3	3.0	T cell/Macrophage Chemokine
	1450942_at	*Ccdc16*	82.6	3.0	Cell cycle
	1417789_at	*Ccl11*	81.9	2.4	Eosinophil Chemokine
	1451584_at	*Havcr2*	46.3	2.3	Macrophage / TH1 response
	1444875_at	*Ppp2ca*	51.9	2.1	Kinase
	1424501_at	*4732497O03Rik*	79.8	2.0	RNA processing
	1444350_at	*Slfn10*	82.8	2.0	T cell development
	1427102_at	*Slfn4*	83.0	2.0	T cell development
DOWN	1440076_at	*Sqstm1*	50.0	-3.3	Regulates NF-κB signaling
	1436789_at	*Ccnjl*	43.4	-2.0	Cyclin/Cell cycle
	1423546_at	*Zfp207*	80.2	-2.0	Metal binding Nuclear Protein
	1427108_at	*9530068E07Rik*	52.2	-2.0	Unknown
	1451257_at	*Acsl6*	54.1	-2.0	Fatty acid metabolism

* Up- or down- regulated in B6.MOLF-*Ity/Ity2* compared to B6.MOLF-*Ity/Ity2.RecD*

** Physical map positions based on Ensembl build 39

Table 2: *Ity3* Interval Specific Oligonucleotide Array List of Differentially Regulated Genes on Chromosome 1 in Spleen

Up/Down*	Accession ID	Gene Name	Position (Mb)**	Fold Change Avg±SEM)	Function
				- DAY 1 -	
UP	NM_172842	*Lax1*	***	2.7±0.7	Regulates TCR/BCR signaling
	NM_009741	*Bcl2*	108.4	2.3±0.2	Suppresses apoptosis
				- DAY 3-	
UP	NM_007695	*Chi3l1*	136.0	2.4 ±0.3	Bacterial adhesion/invasion
				- DAY5 -	
UP	NM_145512	*Sft2d2*	167.0	2.4± 0.2	Retrograde Vesicle Transport
	NM_022018	*Niban*	153.3	2.3± 0.2	Unknown
	NM_02876	*1190005F20Rik*	153.2	2.3± 0.3	N(2),N(2)-dimethylguanosine tRNA methyltransferase
	NM_025819	*1200016B10Rik*	153.1	2.2± 0.1	Unknown
	NM_145991	*Cdc73*	145.4	2.2± 0.4	Tumor suppressor; Cell cycle regulation
	NM_010709	*Lgtn*	133.0	2.1± 0.2	Trafficking receptor for phosphoglycoproteins
	NM_172517	*Rbbp5*	134.3	2.1± 0.4	Binds Retinoblastoma protein
DOWN	NM_144960	*Fcamr*	132.6	-3.7±1.1	Fc Receptor (IgA, IgM)

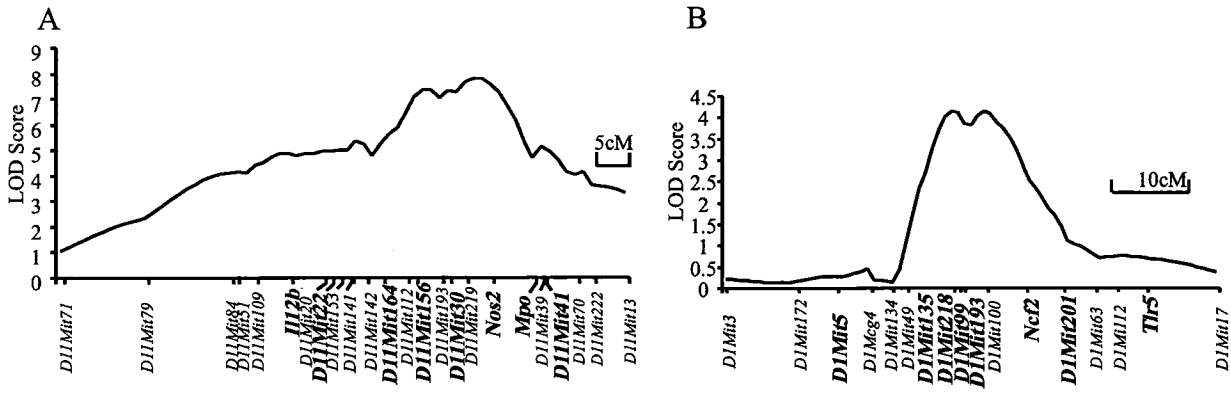
*Up- or down-regulated in B6.MOLF-*Ity/Ity3* compared to B6.MOLF-*Ity/Ity3*$^{MOLF/B6}$

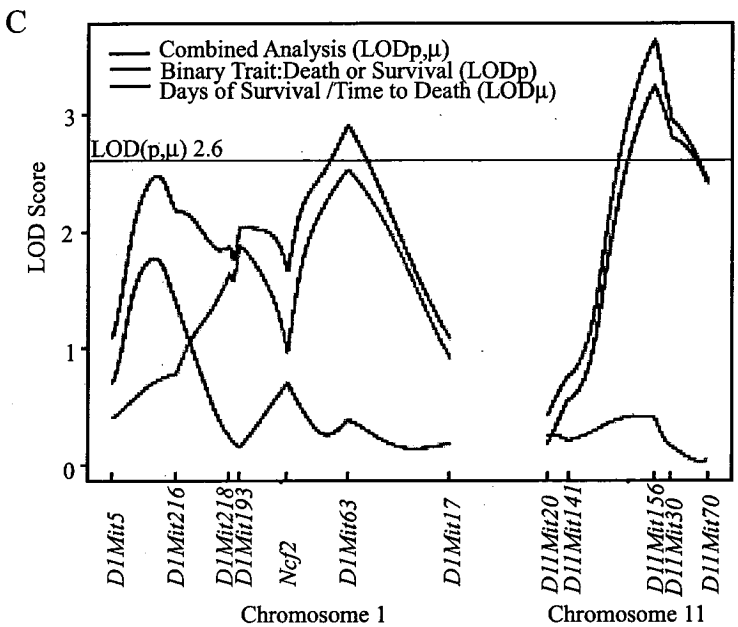
** Physical map positions based on Ensembl build 39

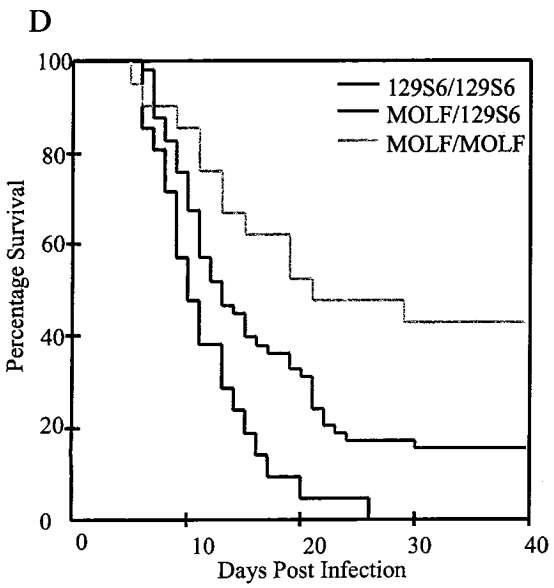
***Physical map position is unknown based on Ensembl build 39 however it is syntenic to human 1q32.1 corresponding to a region around 135Mb on mouse chromosome 1.

FIGURES

Figure 1: LOD score plots of the *Ity2* and *Ity3* regions in (C57BL/6J X MOLF/Ei)F2 cross (A and B) and (129S6 X MOLF/Ei)F2 cross (C). The markers in bold are the additional markers used in the linkage analysis of *Ity2* and *Ity3* (C57BL/6J X MOLF/Ei)F2 cross as assessed by MapManagerQTX (A). The LOD score plot for *Ity3* in (C57BL/6J X MOLF/Ei) F2 cross is taken from Sancho-Shimizu et al, 2006 (also in Chapter 4). LOD score plots for the *Ity2* (chromosome 11) and *Ity3* (chromosome 1) intervals in a novel (129S6 X MOLF/Ei)F2 cross using R/Qtl (C). The black horizontal line represents the genome wide threshold for significance for the combined analysis. The impact of the genotypes at *D11Mit156* (*Ity2*) (D) on (129S6 X MOLF/Ei)F2 mice. The impact of genotypes at *D1Mit63* (*Ity3*) (E) using only (129S6 X MOLF/Ei)F2 mice that die during infection is shown.


A
LOD Score
5cM
D11Mit71
D11Mit79
Il12b
Nos2
Mpo
D11Mit41
D11Mit13
B
LOD Score
10cM
D1Mit3
D1Mit5
D1Mit49
D1Mit135
Ncf2
D1Mit201
Tlr5
D1Mit17


C
Combined Analysis (LODp,μ)
Binary Trait:Death or Survival (LODp)
Days of Survival /Time to Death (LODμ)
LOD(p,μ) 2.6
LOD Score
D1Mit5
D1Mit216
D1Mit218
D1Mit193
Ncf2
D1Mit63
D1Mit17
D11Mit20
D11Mit141
D11Mit156
D11Mit30
D11Mit70
Chromosome 1
Chromosome 11


D
129S6/129S6
MOLF/129S6
MOLF/MOLF
Percentage Survival
Days Post Infection

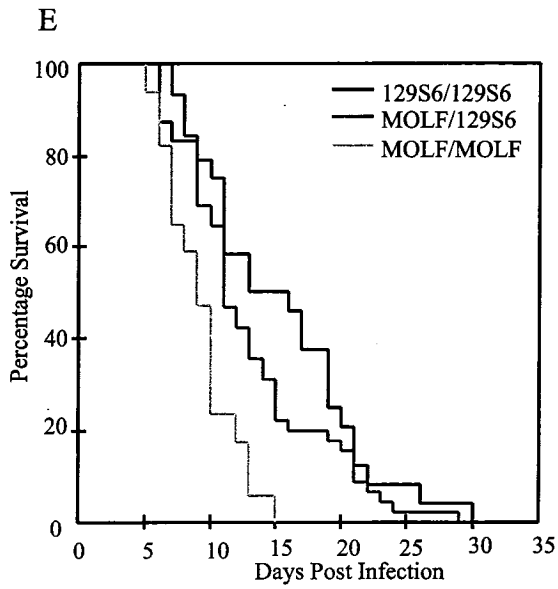
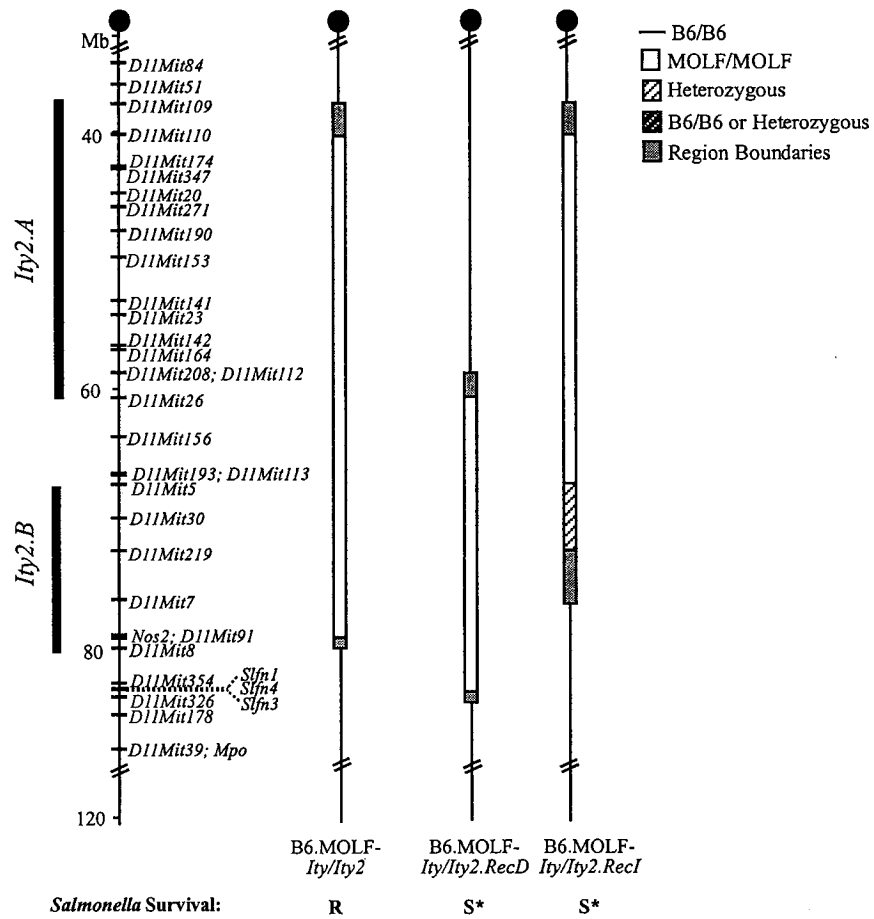

E
129S6/129S6
MOLF/129S6
MOLF/MOLF
Percentage Survival
Days Post Infection

Figure 2: Fine mapping of *Ity2* and *Ity3* loci. A schematic representation of the *Ity2* congenics on chromosome 11 (A), and the *Ity3* congenics on chromosome 1. The white box represents regions of MOLF/Ei homozygosity (MOLF/MOLF), the black line regions of C57BL/6J homozygosity (B6/B6), and the hatched segment on white background, heterozygous intervals. Congenics in which a mixed population of mice including individuals with intervals of C57BL/6J homozygosity and heterozygosity (B6/B6 or Heterozygous), are indicated by hatched segments on black background. Unresolved intervals at the boundaries of congenic fragments, where the genotypes have not been fixed or remain undetermined, are indicated by grey boxes. All *Ity2* congenic mice also carry the MOLF/Ei congenic fragment, *Ity*, on chromosome 1. The bold line by the chromosomes, indicate the restricted interval defined by phenotyping the congenic mice. The relative susceptibility of each congenic strain is indicated at the bottom of each chromosome as compared to the B6.MOLF-*Ity* referent (R=more resistant, S=more susceptible). An asterisk by the R or S indicates that the survival does not differ from the B6.MOLF-*Ity* referent strain.

A


Mb
D11Mit84
D11Mit51
D11Mit109
40
D11Mit110
D11Mit174
D11Mit347
D11Mit20
D11Mit271
D11Mit190
D11Mit153
D11Mit141
D11Mit23
D11Mit142
D11Mit164
D11Mit208; D11Mit112
60
D11Mit26
D11Mit156
D11Mit193; D11Mit113
D11Mit5
D11Mit30
D11Mit219
D11Mit7
Nos2; D11Mit91
80
D11Mit8
Slfn1
Slfn4
D11Mit326
Slfn3
D11Mit178
D11Mit39; Mpo
120
Ity2.A
Ity2.B
B6/B6
MOLF/MOLF
Heterozygous
B6/B6 or Heterozygous
Region Boundaries
B6.MOLF-Ity/Ity2
B6.MOLF-Ity/Ity2.RecD
B6.MOLF-Ity/Ity2.RecI
Salmonella Survival:
R
S*
S*

B

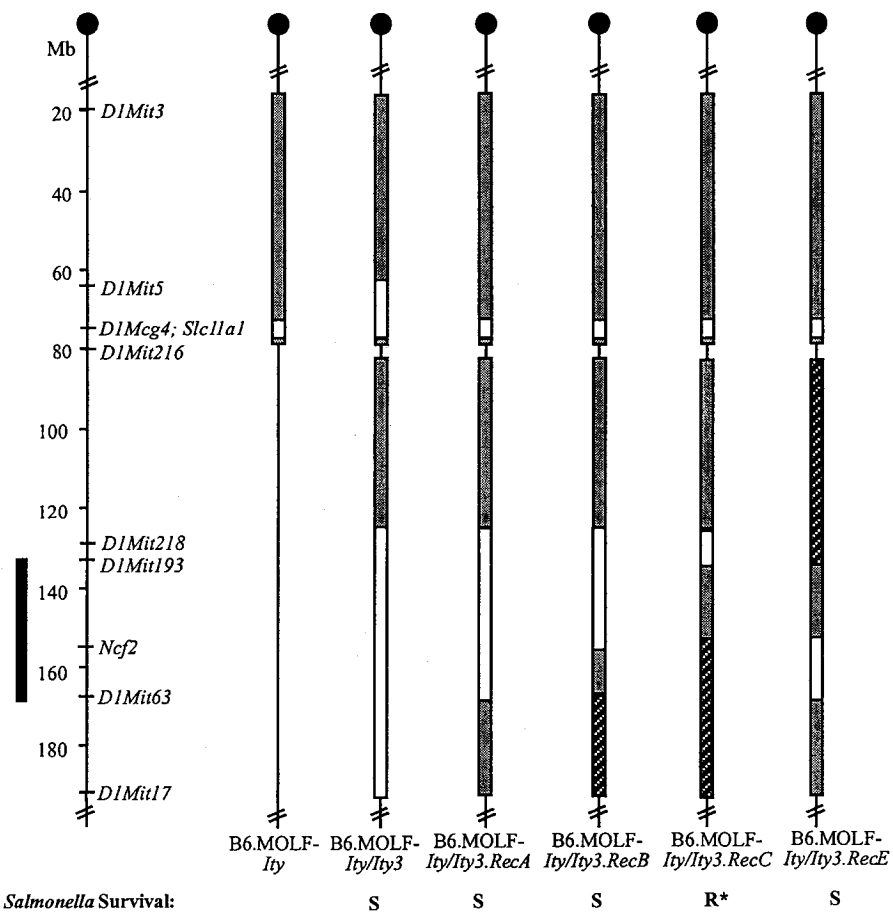

Mb
20
D1Mit3
40
60
D1Mit5
D1Mcg4; Slc11a1
80
D1Mit216
100
120
D1Mit218
D1Mit193
140
Ncf2
160
D1Mit63
180
D1Mit17
B6.MOLF-Ity
B6.MOLF-Ity/Ity3
B6.MOLF-Ity/Ity3.RecA
B6.MOLF-Ity/Ity3.RecB
B6.MOLF-Ity/Ity3.RecC
B6.MOLF-Ity/Ity3.RecE
Salmonella Survival:
S
S
S
R*
S

Figure 3: Phenotypic characterization of the *Ity2* and *Ity3* congenic mice. Survival curves for *Ity2* congenic mice are shown in (A). One representative survival experiment of seven is shown here. There were at least 6 mice in each group of mice tested. Bacterial load in the spleen (B) and the liver (C) of the *Ity2* congenics are represented as log (CFU/g of organ). Groups of 3-4 mice were used for CFU enumeration for each time point, medians for each group is shown as a bar. Survival of *Ity3* congenic mice are shown in (D). Samples from three independent experiments using at least 8 mice per group were pooled and represented here.

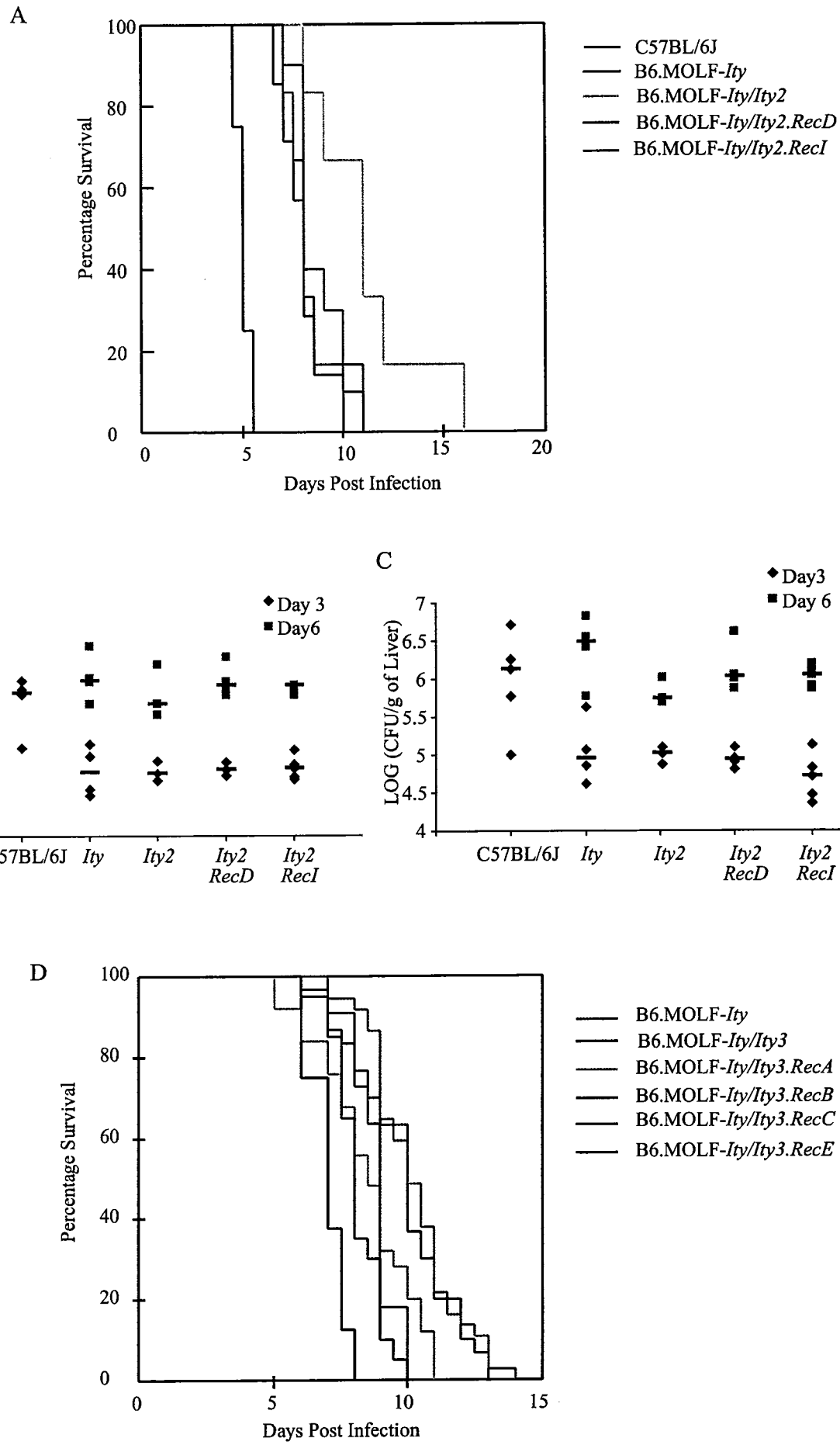
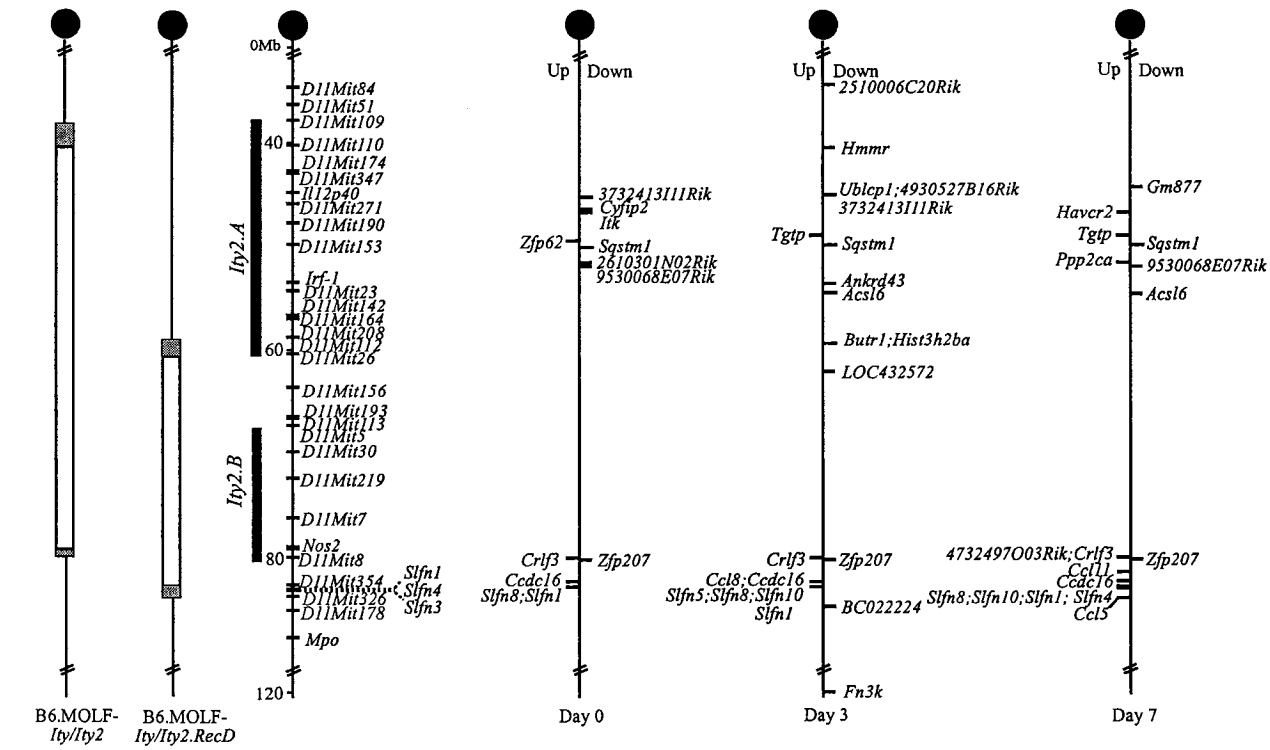

A
100
80
60
40
20
0
Percentage Survival
0
5
10
15
20
Days Post Infection
C57BL/6J
B6.MOLF-Ity
B6.MOLF-Ity/Ity2
B6.MOLF-Ity/Ity2.RecD
B6.MOLF-Ity/Ity2.RecI
B
Day 3
Day6
LOG (CFU/g of Spleen)
8
7.5
7
6.5
6
5.5
5
C57BL/6J
Ity
Ity2
Ity2 RecD
Ity2 RecI
C
Day3
Day 6
LOG (CFU/g of Liver)
7
6.5
6
5.5
5
4.5
4
C57BL/6J
Ity
Ity2
Ity2 RecD
Ity2 RecI
D
100
80
60
40
20
0
Percentage Survival
0
5
10
15
Days Post Infection
B6.MOLF-Ity
B6.MOLF-Ity/Ity3
B6.MOLF-Ity/Ity3.RecA
B6.MOLF-Ity/Ity3.RecB
B6.MOLF-Ity/Ity3.RecC
B6.MOLF-Ity/Ity3.RecE

Figure 4: Location of differentially expressed genes on chromosome 11, *Ity2* (A) and on chromosome 1, *Ity3* (B) during infection. Upregulated genes in the resistant B6.MOLF-*Ity/Ity2* in (A) or B6.MOLF-*Ity/Ity3* strain in (B) are on the left of the chromosome and downregulated genes on the right. * The position of *Lax1* is approximated based on the fact that it is syntenic to human 1q32.

A


0Mb
40
60
80
120
Ity2.A
Ity2.B
D11Mit84
D11Mit51
D11Mit109
D11Mit110
D11Mit174
D11Mit347
Il12p40
D11Mit271
D11Mit190
D11Mit153
Irf-1
D11Mit23
D11Mit142
D11Mit164
D11Mit208
D11Mit112
D11Mit26
D11Mit156
D11Mit193
D11Mit113
D11Mit5
D11Mit30
D11Mit219
D11Mit7
Nos2
D11Mit8
D11Mit354
D11Mit326
D11Mit178
Mpo
Slfn1
Slfn4
Slfn3
B6.MOLF-Ity/Ity2
B6.MOLF-Ity/Ity2.RecD
Up
Down
3732413I11Rik
Cyfip2
Itk
Zfp62
Sqstm1
2610301N02Rik
9530068E07Rik
Crlf3
Zfp207
Ccdc16
Slfn8;Slfn1
Day 0
2510006C20Rik
Hmmr
Ublcp1;4930527B16Rik
3732413I11Rik
Tgtp
Sqstm1
Ankrd43
Acsl6
Butr1;Hist3h2ba
LOC432572
Zfp207
Ccl8;Ccdc16
Slfn5;Slfn8;Slfn10
Slfn1
BC022224
Fn3k
Day 3
Gm877
Havcr2
Tgtp
Sqstm1
Ppp2ca
9530068E07Rik
Acsl6
4732497O03Rik;Crlf3
Zfp207
Ccl11
Ccdc16
Slfn8;Slfn10;Slfn1;
Slfn4
Ccl5
Day 7

B

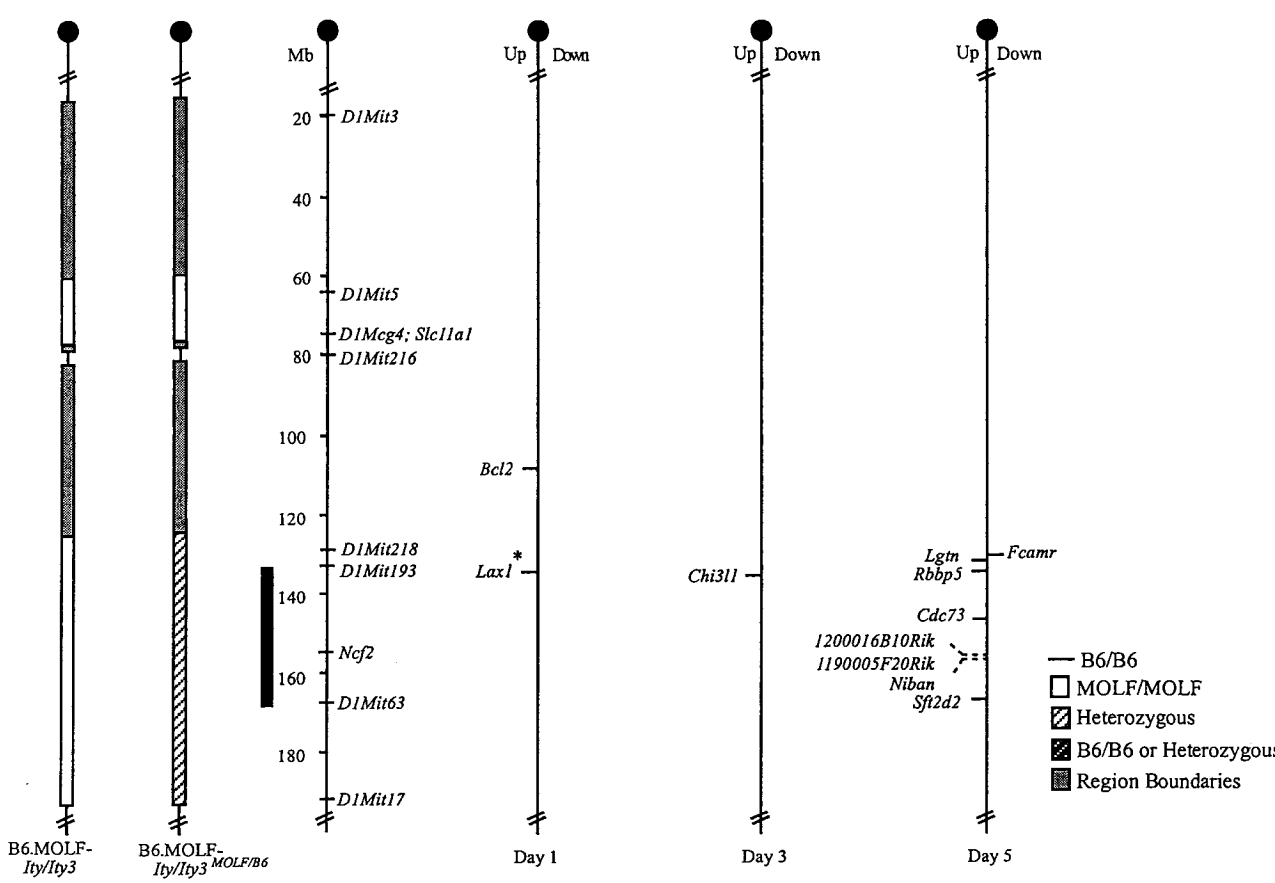
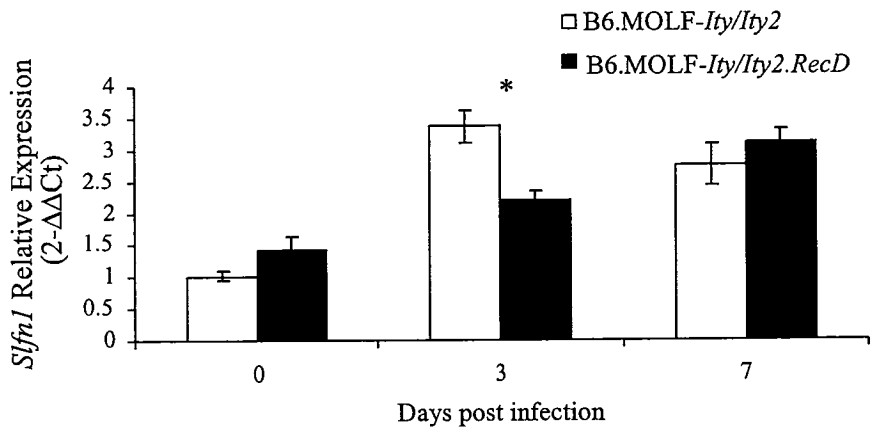
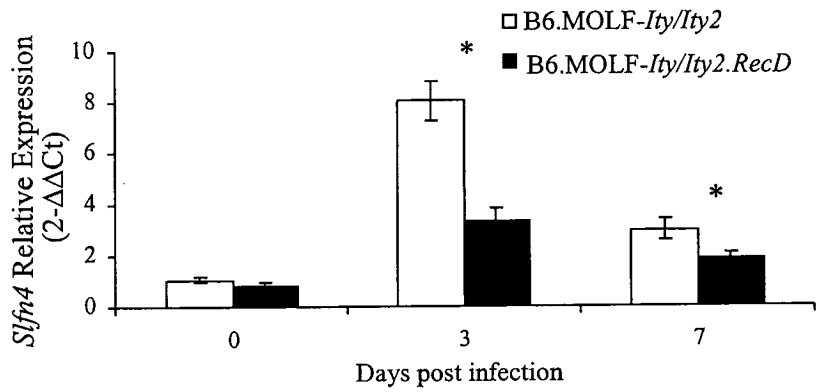

Mb
20
40
60
80
100
120
140
160
180
D1Mit3
D1Mit5
D1Mcg4; Slc11a1
D1Mit216
D1Mit218
D1Mit193
Ncf2
D1Mit63
D1Mit17
B6.MOLF-Ity/Ity3
B6.MOLF-Ity/Ity3MOLF/B6
Up
Down
Bcl2
Lax1
*
Day 1
Chi3l1
Day 3
Lgtn
Fcamr
Rbbp5
Cdc73
1200016B10Rik
1190005F20Rik
Niban
Sft2d2
Day 5
B6/B6
MOLF/MOLF
Heterozygous
B6/B6 or Heterozygous
Region Boundaries

Figure 5: Real time PCR validation of the *Slfn* cluster of genes during infection. Expression was determined using uninfected B6.MOLF-*Ity/Ity2* mice as the referent. All values are expressed as $2^{-\Delta\Delta Ct}$ units, where the referent is set to 1, and the house keeping gene used was *Tbp*. Statistical significance was assessed using the student's t-test and is indicated by an asterisk ($p<0.05$). *Slfn1* (A), *Slfn4* (B), *Slfn5* (C) are shown here.

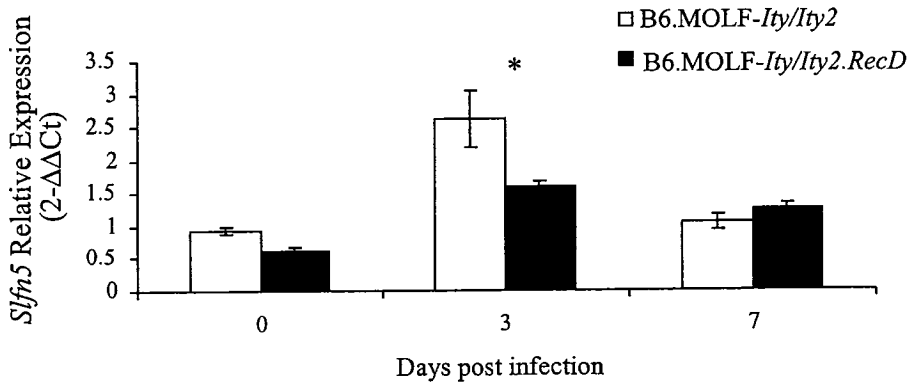
A


B6.MOLF-Ity/Ity2
B6.MOLF-Ity/Ity2.RecD
Sifn1 Relative Expression (2-ΔΔCt)
4
3.5
3
2.5
2
1.5
1
0.5
0
*
0
3
7
Days post infection

B


B6.MOLF-Ity/Ity2
B6.MOLF-Ity/Ity2.RecD
Sifn4 Relative Expression (2-ΔΔCt)
10
8
6
4
2
0
*
*
0
3
7
Days post infection

C


B6.MOLF-Ity/Ity2
B6.MOLF-Ity/Ity2.RecD
Sifn5 Relative Expression (2-ΔΔCt)
3.5
3
2.5
2
1.5
1
0.5
0
*
0
3
7
Days post infection

SUPPLEMENTAL TABLES

Supplemental Table 1: *Ity2* Differentially Regulated Genes at Day 0

AffymetrixID	Fold change	UniGene ID	Gene Symbol	Chromosome
1423546_at	0.3	Mm.102253	Zfp207	11
1440076_at	0.3	Mm.40828	Sqstm1	11
1419616_at	0.3	---	Bmpr2	1
1443435_at	0.3	Mm.156771	3732413I11Rik	11
1437192_x_at	0.3	Mm.3555	Vdac1	19
1456836_at	0.4	---	Itk	11
1430177_at	0.4	---	2610301N02Rik	11
1449838_at	0.5	Mm.347391	Crisp3	17
1427820_at	0.5	---	---	
1427108_at	0.5	Mm.291979	9530068E07Rik	11
1449273_at	0.5	Mm.154358	Cyfip2	11
1438084_at	2.0	Mm.302274	Adam23	1
1416139_at	2.0	Mm.46360	Reg2	6
1452536_s_at	2.0	---	---	12
1457307_at	2.0	Mm.17957	A330102K04Rik	15
1416055_at	2.0	Mm.331311	Amy2	3
1417682_a_at	2.0	Mm.276926	Prss2	6
1433573_x_at	2.0	Mm.276926	Prss2	6
1456320_at	2.0	Mm.26272	BC049806	1
1426936_at	2.0	---	BC005512	
1448220_at	2.1	Mm.34374	Ctrb1	8
1437438_x_at	2.1	Mm.212333	Pnliprp2	19
1447329_at	2.1	---	---	
1445148_at	2.2	Mm.100884	Akr1b3	12
1428359_s_at	2.2	Mm.21835	1810010M01Rik	7
1422436_at	2.2	Mm.153729	2210010C04Rik	6
1454623_at	2.2	Mm.268855	LOC232680	6
1428062_at	2.2	Mm.25377	Cpa1	6
1422434_a_at	2.2	Mm.153729	2210010C04Rik	6
1417257_at	2.2	Mm.236017	Cel	2
1451730_at	2.2	Mm.16650	Zfp62	11
1436317_at	2.3	Mm.103539	D230012E17Rik	1
1438612_a_at	2.4	Mm.21160	Clps	17
1433431_at	2.4	Mm.20407	Pnlip	19
1448281_a_at	2.4	Mm.45316	Ela2	4
1428102_at	2.5	---	---	3
1422651_at	2.5	Mm.3969	Acdc	16
1417270_at	2.6	Mm.281079	Wdr12	1
1427883_a_at	2.6	Mm.249555	Col3a1	1
1458543_at	2.6	---	---	
1456257_at	2.7	Mm.373670	C130065N10Rik	1
1450997_at	2.7	Mm.25559	Stk17b	1
1429607_at	2.8	Mm.222887	Als2cr3	1
1416523_at	2.8	Mm.235538	Rnase1	14

Supplemental Table 1: *Ity2* Differentially Regulated Genes at Day 0 (cont'd)

AffymetrixID	Fold change	UniGene ID	Gene Symbol	Chromosome
1459956_at	2.8	Mm.133473	Dock10	1
1449434_at	2.8	Mm.300	Car3	3
1440214_at	2.9	Mm.116691	A630001G21Rik	1
1417867_at	2.9	Mm.4407	Adn	10
1434137_x_at	3.0	Mm.21835	1810010M01Rik	7
1415905_at	3.0	Mm.142731	Reg1	6
1456635_at	3.1	---	---	1
1458924_at	3.2	---	D430013B06Rik	1
1419356_at	3.5	Mm.29466	Klf7	1
1446017_at	3.8	Mm.368533	---	Y
1415954_at	3.9	Mm.333022	Try4	6
1450942_at	3.9	Mm.29622	Ccdc16	11
1418612_at	5.6	Mm.10948	Slfn1	11
1440481_at	7.5	---	---	1
1438235_at	8.0	---	Crlf3	11
1441444_at	8.1	Mm.336604	---	1
1451655_at	10.8	Mm.347694	Slfn8	11
1456736_x_at	14.1	Mm.370263	5230400G24Rik	1

Supplemental Table 2: *Ity2* Differentially Regulated Genes at Day 3

AffymetrixIDs	Fold change	UniGene ID	Gene Symbol	Chromosome
1449452_a_at	0.3	Mm.46403	Gp2	7
1458609_at	0.3	Mm.353315	---	17
1451257_at	0.3	Mm.267478	Acsl6	11
1418287_a_at	0.3	Mm.4138	Dmbt1	7
1415954_at	0.3	Mm.333022	Try4	6
1415905_at	0.3	Mm.142731	Reg1	6
1445845_at	0.3	Mm.338784	BC002236	11
1441054_at	0.3	Mm.125650	Apol2	15
1428359_s_at	0.3	Mm.21835	1810010M01Rik	7
1428102_at	0.3	---	---	3
1438612_a_at	0.3	Mm.21160	Clps	17
1428062_at	0.3	Mm.25377	Cpa1	6
1424649_a_at	0.3	Mm.22270	Tm4sf3	10
1437192_x_at	0.3	Mm.3555	Vdac1	19
1416523_at	0.3	Mm.235538	Rnase1	14
1423546_at	0.3	Mm.102253	Zfp207	11
1448281_a_at	0.3	Mm.45316	Ela2	4
1427677_a_at	0.3	Mm.323365	Sox6	7
1415777_at	0.3	Mm.10753	Pnliprp1	19
1454193_at	0.3	Mm.362016	5430401H09Rik	12
1454623_at	0.3	Mm.268855	LOC232680	6
1429871_at	0.3	Mm.116997	Hmmr	11
1433431_at	0.3	Mm.20407	Pnlip	19
1429606_at	0.4	---	4930527B16Rik	11
1437438_x_at	0.4	Mm.212333	Pnliprp2	19
1448186_at	0.4	Mm.212333	Pnliprp2	19
1436998_at	0.4	Mm.145208	A830006N08Rik	11
1434137_x_at	0.4	Mm.21835	1810010M01Rik	7
1422434_a_at	0.4	Mm.153729	2210010C04Rik	6
1417337_at	0.4	Mm.240051	Epb4.2	2
1418311_at	0.4	Mm.266448	Fn3k	11
1421868_a_at	0.4	Mm.20407	Pnlip	19
1448220_at	0.4	Mm.34374	Ctrb1	8
1416139_at	0.4	Mm.46360	Reg2	6
1434501_at	0.4	Mm.358908	Ypel4	2
1417257_at	0.4	Mm.236017	Cel	2
1417682_a_at	0.4	Mm.276926	Prss2	6
1435814_at	0.4	Mm.152987	Xpo7	14
1415883_a_at	0.4	Mm.297477	Ela3b	4
1437326_x_at	0.4	Mm.297477	Ela3b	4
1419074_at	0.4	Mm.32656	2510006C20Rik	11
1421278_s_at	0.4	Mm.200611	Spna1	1
1430839_at	0.4	Mm.159919	9430076G02Rik	18
1448290_at	0.4	Mm.2553	Pap	6
1436930_x_at	0.4	Mm.247676	Hmbs	9
1421264_at	0.4	Mm.22540	MGI:2182073	11

Supplemental Table 2: *Ity2* Differentially Regulated Genes at Day 3 (cont'd)

AffymetrixIDs	Fold change	UniGene ID	Gene Symbol	Chromosome
1449057_at	0.4	Mm.19958	Kel	6
1422435_at	0.4	Mm.153729	2210010C04Rik	6
1434553_at	0.4	Mm.26088	4930577M16Rik	3
1425288_at	0.4	Mm.246385	Samd11	6
1433573_x_at	0.4	Mm.276926	Prss2	6
1447655_x_at	0.4	Mm.323365	Sox6	7
AFFX-TransRecMur/X57349_3_at	0.4	---	---	
1421277_at	0.4	Mm.200611	Spna1	1
1448300_at	0.4	Mm.218286	Mgst3	1
1423016_a_at	0.4	Mm.13123	Gypa	8
1417338_at	0.4	Mm.240051	Epb4.2	2
1433966_x_at	0.4	Mm.2942	Asns	6
1422930_at	0.4	Mm.30220	Icam4	9
1415805_at	0.4	Mm.21160	Clps	17
1422966_a_at	0.4	Mm.28683	Tfrc	16
1421802_at	0.4	Mm.86948	Ear1	14
1417049_at	0.4	Mm.195461	Rhced	4
1418199_at	0.4	Mm.25793	Hemgn	4
1434803_a_at	0.4	Mm.25210	Sycn	7
1449482_at	0.4	Mm.28022	Hist3h2ba	11
1417689_a_at	0.4	Mm.30181	MGI:1914432	4
1427541_x_at	0.4	Mm.116997	Hmmr	11
1435287_at	0.4	Mm.104155	Add2	6
1424722_at	0.4	Mm.18802	1300017J02Rik	9
1439018_at	0.4	Mm.331107	6330505N24Rik	3
1440076_at	0.4	Mm.40828	Sqstm1	11
1437015_x_at	0.4	Mm.20190	Pla2g1b	5
1428952_at	0.4	Mm.32631	Pdip	17
1458440_at	0.4	---	LOC432572	11
1443657_at	0.4	Mm.331107	6330505N24Rik	3
1422754_at	0.4	Mm.249594	Tmod1	4
1453136_at	0.4	Mm.276229	Fbxo30	10
1422920_at	0.4	Mm.86652	Cldn13	5
1435611_x_at	0.5	Mm.297477	Ela3b	4
1458667_at	0.5	Mm.293091	4930519N13Rik	2
1416468_at	0.5	Mm.250866	Aldh1a1	19
1425643_at	0.5	Mm.13123	Gypa	8
AFFX-TransRecMur/X57349_M_at	0.5	---	---	18
1436827_at	0.5	Mm.312276	Gm944	18
1453137_at	0.5	Mm.276229	Fbxo30	10
1427221_at	0.5	Mm.27208	MGI:2143217	9
1443435_at	0.5	Mm.156771	3732413I11Rik	11
1416297_s_at	0.5	Mm.2553	Pap	6
AFFX-TransRecMur/X57349_5_at	0.5	---	---	
1419014_at	0.5	Mm.12961	Rhag	17

Supplemental Table 2: *Ity2* Differentially Regulated Genes at Day 3 (cont'd)

AffymetrixIDs	Fold change	UniGene ID	Gene Symbol	Chromosome
1452666_a_at	0.5	Mm.273785	Tmcc2	1
1453139_at	0.5	---	---	17
1428358_at	0.5	Mm.21835	1810010M01Rik	7
1455618_x_at	0.5	Mm.225289	1300010A20Rik	6
1418600_at	0.5	---	---	8
1451228_a_at	0.5	Mm.25210	Sycn	7
1425704_at	0.5	Mm.192213	BC022224	11
1460223_a_at	0.5	Mm.210863	Epb4.9	14
1450009_at	0.5	Mm.282359	Ltf	9
1433459_x_at	0.5	Mm.276926	Prss2	6
1449519_at	0.5	Mm.1236	Gadd45a	6
1419311_at	0.5	Mm.299155	Trim10	17
1450354_a_at	0.5	Mm.293591	Ptdss2	7
1454622_at	0.5	Mm.6055	Slc38a5	X
1424009_at	0.5	Mm.359401	MGI:1353426	6
1426917_s_at	0.5	Mm.247457	Scrn3	2
1424968_at	0.5	Mm.26580	2210023G05Rik	8
1439422_a_at	0.5	Mm.29140	1110035L05Rik	4
1438202_at	0.5	Mm.354748	C920005C14Rik	15
1435012_x_at	0.5	Mm.297477	Ela3b	4
1429146_at	0.5	Mm.41279	6620401M08Rik	7
1418909_at	0.5	---	---	4
1452001_at	0.5	Mm.255151	Nfe2	15
1443849_x_at	0.5	Mm.46484	Urod	4
1443962_at	0.5	Mm.17977	Tfdp2	9
1428108_x_at	0.5	Mm.273785	Tmcc2	1
1426475_at	0.5	Mm.247676	Hmbs	9
1459692_at	0.5	---	---	17
1428304_at	0.5	---	---	14
1444051_at	0.5	Mm.236287	1700019D03Rik	10
1435507_x_at	0.5	Mm.276926	Prss2	6
1416055_at	0.5	Mm.331311	Amy2	3
1420499_at	0.5	Mm.10651	Gch1	14
1443673_x_at	0.5	---	---	3
1431763_a_at	0.5	Mm.2745	Ctrl	8
1451095_at	0.5	Mm.2942	Asns	6
1417206_at	0.5	Mm.46484	Urod	4
1444350_at	2.0	Mm.341645	Slfn10	11
1431214_at	2.0	---	---	
1456288_at	2.0	Mm.42147	Slfn5	11
1438084_at	2.0	Mm.302274	Adam23	1
1426174_s_at	2.1	Mm.291537	Ighg	12
1450188_s_at	2.1	Mm.299647	Lipg	18
1422837_at	2.1	Mm.244003	Scel	14
1422651_at	2.1	Mm.3969	Acdc	16
1452536_s_at	2.1	---	---	12
1458458_at	2.2	Mm.42147	Slfn5	11

Supplemental Table 2: *Ity2* Differentially Regulated Genes at Day 3 (cont'd)

AffymetrixIDs	Fold change	UniGene ID	Gene Symbol	Chromosome
1427883_a_at	2.2	Mm.249555	Col3a1	1
1450826_a_at	2.2	Mm.14277	Saa3	7
1418028_at	2.3	Mm.19987	Dct	14
1419684_at	2.3	Mm.42029	Ccl8	11
1456320_at	2.3	Mm.26272	BC049806	1
1417270_at	2.4	Mm.281079	Wdr12	1
1456635_at	2.4	---	---	1
1425871_a_at	2.4	Mm.304150	LOC384413	6
1449434_at	2.5	Mm.300	Car3	3
1436317_at	2.7	Mm.103539	D230012E17Rik	1
1425738_at	2.7	Mm.335657	LOC243469	6
1427870_x_at	2.7	Mm.373639	Igh-4	12
1445399_at	2.7	Mm.14489	Klrb1d	6
1458924_at	2.8	---	D430013B06Rik	1
1417867_at	2.9	Mm.4407	Adn	10
1456257_at	3.0	Mm.373670	C130065N10Rik	1
1450997_at	3.0	Mm.25559	Stk17b	1
1425247_a_at	3.0	Mm.373639	Igh-4	12
1427756_x_at	3.1	Mm.373639	Igh-4	12
1425728_at	3.4	Mm.213453	---	11
1419356_at	3.8	Mm.29466	Klf7	1
1425324_x_at	3.9	Mm.373639	Igh-4	12
1440481_at	4.1	---	---	1
1418612_at	5.1	Mm.10948	Slfn1	11
1450942_at	5.5	Mm.29622	Ccdc16	11
1427757_at	6.0	Mm.372601	Igk-V21	6
1441444_at	6.2	Mm.336604	---	1
1456736_x_at	6.9	Mm.370263	5230400G24Rik	1
1438235_at	7.1	---	Crlf3	11
1451655_at	15.6	Mm.347694	Slfn8	11

Supplemental Table 3: *Ity2* Differentially Regulated Genes at Day 7

AffymetrixIDs	Fold Change	UniGene ID	Gene Symbol	Chromosome
1440076_at	0.3	Mm.40828	Sqstm1	11
1419616_at	0.3	---	Bmpr2	1
1437192_x_at	0.4	Mm.3555	Vdac1	19
1444051_at	0.4	Mm.236287	1700019D03Rik	10
1436789_at	0.5	Mm.247595	Gm877	11
1423546_at	0.5	Mm.102253	Zfp207	11
1437060_at	0.5	Mm.26456	Olfm4	14
1427108_at	0.5	Mm.291979	9530068E07Rik	11
1442646_at	0.5	---	---	3
1451257_at	0.5	Mm.267478	Acsl6	11
1427102_at	2.0	Mm.38192	Slfn4	11
1441192_at	2.0	Mm.25724	Scly	1
1444350_at	2.0	Mm.341645	Slfn10	11
1424501_at	2.0	Mm.274961	4732497O03Rik	11
1444875_at	2.1	Mm.260288	Ppp2ca	11
1435127_a_at	2.1	Mm.185144	Osgepl1	1
1438239_at	2.1	Mm.34441	Mid1	X
1417270_at	2.1	Mm.281079	Wdr12	1
1447227_at	2.2	Mm.28756	Slc40a1	1
1456918_at	2.2	Mm.87452	9430025M21Rik	1
1456320_at	2.3	Mm.26272	BC049806	1
1451584_at	2.3	Mm.72168	Havcr2	11
1427883_a_at	2.3	Mm.249555	Col3a1	1
1417789_at	2.4	Mm.4686	Ccl11	11
1427884_at	2.4	Mm.249555	Col3a1	1
1419431_at	2.5	Mm.4791	Ereg	5
1450297_at	2.5	Mm.1019	Il6	5
1428557_a_at	2.6	Mm.185144	Osgepl1	1
1456635_at	2.6	---	---	1
1450942_at	3.0	Mm.29622	Ccdc6	11
1418126_at	3.0	---	---	11
1456257_at	3.1	Mm.373670	C130065N10Rik	1
1419356_at	3.2	Mm.29466	Klf7	1
1441428_at	3.3	Mm.131338	---	1
1440481_at	3.4	---	---	1
1441444_at	3.6	Mm.336604	---	1
1450997_at	3.7	Mm.25559	Stk17b	1
1438235_at	3.8	---	Crlf3	11
1425728_at	3.8	Mm.213453	Tgtp	11
1418612_at	4.5	Mm.10948	Slfn1	11
1436317_at	4.8	Mm.103539	D230012E17Rik	1
1416055_at	5.1	Mm.331311	Amy2	3
1451655_at	10.2	Mm.347694	Slfn8	11
1456736_x_at	11.3	Mm.370263	5230400G24Rik	1

CHAPTER 3:

TLR5 IS NOT PRIMARILY ASSOCIATED WITH SUSCEPTIBILITY TO *SALMONELLA* TYPHIMURIUM INFECTION IN MOLF/EI MICE

RATIONALE

As a member of the TLR family of pathogen recognition receptors, *Tlr5* presented itself as an excellent candidate for the recessively inherited *Salmonella* susceptibility locus, *Ity3,* on distal chromosome 1 (Kawai and Akira 2006). Previous work in our laboratory, led to the identification of numerous coding SNPs in the MOLF/Ei allele as compared to the C57BL/6J allele, as well as the observation that MOLF/Ei *Tlr5* expression in the liver is significantly lower during *Salmonella* infection when compared to C57BL/6J and 129S6/SvEvTac mice (Sebastiani et al. 2000). To further characterize the involvement of this gene in *Ity3*, MOLF/Ei and C57BL/6J coding and promoter regions were cloned and tested *in vitro*, and concurrent to the fine mapping presented in the previous Chapter, the *Ity3* congenic mice were used for *in vivo* functional characterization of the allelic forms of *Tlr5*.

Tlr5 is not primarily associated with susceptibility to *Salmonella* Typhimurium infection in MOLF/Ei mice

Vanessa Sancho-Shimizu*[1,2], Isabelle Angers*[1,2], Albert Descoteaux[3], Andrew T. Gewirtz[4] and Danielle Malo[1,2,5]

* These authors contributed equally to this work.

1 Department of Human Genetics, McGill University, Montreal, QC H3G 1A4

2 Center for the Study of Host Resistance, McGill University Health Center, Montreal, QC H3G 1A4

3 INRS-Institut Armand-Frappier, Laval, QC H7V 1B7

4 Department of Pathology and Laboratory Medicine, Emory University, Atlanta, GA 30322

5 Department of Medicine, McGill University, Montreal, QC H3G 1A4

Published in *Mammalian Genome* volume 17(5): 385-397 (2006)
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ABSTRACT

The extreme susceptibility to infection with *Salmonella* Typhimurium of wild-derived MOLF/Ei has been linked to one genomic region on chromosome 1 (*Ity3*). A member of the Toll-like receptors family, *Tlr5*, located on distal chromosome 1, was previously shown to be a candidate gene for *Ity3* based on expression studies and sequencing analysis. The candidacy of *Tlr5* as a *Salmonella*-susceptibility gene was evaluated functionally by comparing *Tlr5* C57BL/6J and MOLF/Ei alleles *in vitro* and *in vivo*. *In vitro* studies showed that the MOLF/Ei *Tlr5* allele is more transcriptionally active when the gene is removed from its natural genomic environment. This observation was supported by *in vivo* studies in B6.MOLF-*Ity3* congenic mice showing that mice homozygous for the MOLF/Ei allele at *Ity3*, including *Tlr5,* had an increased response to flagellin as measured by IL-6 and CXCL-1 secretion in the serum compared to parental MOLF/Ei mice. Despite the fact that both MOLF/Ei and B6.MOLF-*Ity/Ity3* mice are more susceptible to *Salmonella* Typhimurium infection than B6.MOLF-*Ity* mice, they exhibit a different phenotype with respect to *Tlr5* expression and Tlr5 signaling, supporting the prediction that *Tlr5* is not primarily involved in the disease phenotype underlying the *Ity3* locus in MOLF/Ei mice.

INTRODUCTION

Previous genetic analyses using a mouse model of infection with a highly virulent *Salmonella* Typhimurium serotype have demonstrated the complexity of the immune host response to infection and its polygenic nature in wild-derived MOLF/Ei mice (Sebastiani et al. 1998; Sebastiani et al. 2002). Wild-derived mice are an important source of new disease-resistance alleles and provide a broader range of genetic variation to be studied as compared to classical inbred laboratory strains due to their evolutionary divergence (Guenet 2003). MOLF/Ei mice were shown to be extremely susceptible to *Salmonella* Typhimurium infection despite the fact that they carry resistant alleles at two well-defined innate immune genes, *Slc11a1* and *Tlr4*, known to play a major role in innate resistance to infection with *Salmonella* Typhimurium (O'Brien et al. 1980; Vidal et al. 1995; Qureshi et al. 1996; Sebastiani et al. 1998). Three genomic regions named *Ity* (**I**mmunity to **Ty**phimurium locus), *Ity2* and *Ity3* were shown to influence survival time during *Salmonella* Typhimurium infection in MOLF/Ei mice, as a result of a linkage analysis carried out in a MOLF/Ei x C57BL/6J cross. A *Salmonella*-resistant phenotype was linked to *Ity* on chromosome 1 with a peak LOD score of 18.8 at *D1Mcg4*, accounting for 37% of the phenotypic variance. *D1Mcg4* is located within 6kb of *Slc11a1* and therefore this QTL was explained by the non-functional $Slc11a1^{Asp169}$ allele inherited from C57BL/6J. The two other QTLs were observed only after controlling for *Slc11a1*. Another *Salmonella*-resistant QTL was detected on chromosome 11 and named *Ity2*. It has a maximum LOD score of 7.0 at *D11Mit5* and explains 10% of the phenotypic variance. The third QTL identified, *Ity3* was mapped to distal chromosome 1. It has a maximum LOD score of 4.8 around *D1Mit100* and the MOLF/Ei allele was found to

confer susceptibility to *Salmonella* Typhimurium infection recessively. *Ity3* is estimated to be 16cM in size, located approximately 25 cM distal to *Slc11a1* and accounts for 7% of the phenotypic variance. To confirm this QTL, a second intercross was performed between MOLF/Ei and C3H/HeJ, two mouse strains carrying the wild-type functional *Slc11a1*^{Gly169} allele. In (C3H/HeJ x MOLF/Ei)F2, *Ity3* accounted for a larger proportion (25%) of the variance confirming the presence of *Ity3* (Sebastiani et al. 1998). A member of the Toll-like receptor (TLR) family, *Tlr5*, was shown to be located in the vicinity of the *Ity3* interval and was proposed as a candidate gene (Sebastiani et al. 2000).

TLRs are part of an evolutionarily conserved family of type 1 transmembrane receptors characterized by an extracellular leucine-rich repeat (LRR), a single transmembrane domain and a cytoplasmic Toll/Interleukin-1 receptor (TIR) homology domain (Slack et al. 2000). Together, the TLR family can recognize a wide range of pathogen-associated molecular patterns (PAMPs) from bacteria, fungi, protozoa and viruses. TLR5 was shown to recognize flagellin produced by many pathogenic bacteria, including *Salmonella* Typhimurium (Hayashi et al. 2001), *Pseudomonas aeruginosa* (Hayashi et al. 2001; Adamo et al. 2004), *Listeria monocytogenes* (Hayashi et al. 2001; Way 2004), *Helicobacter pylori* (Schmausser et al. 2004) and *Escherichia coli* (Donnelly and Steiner 2002). In *Salmonella*, flagellin is the structural protein subunit of the flagellum necessary for bacterial motility and is encoded by two genes *fliC* and *fljB*. *Salmonella* devoid of flagellum or mutated for both flagellin genes (*fljB*$^{-}$/*fliC*$^{-}$) are not able to mount a pro-inflammatory signaling response in the epithelial cells of the intestine (Eaves-Pyles et al. 2001; Gewirtz et al. 2001; Reed et al. 2002; Zeng et al. 2003). The response of TLR5 to flagellin is orchestrated through the MyD88-dependent pathway and culminates in the activation of the transcription factors NF-κB, p38 MAPK and JNK

(Barton and Medzhitov 2003; Akira and Takeda 2004). *Tlr5* is expressed in several tissues (Sebastiani et al. 2000) including the mucosal epithelium of the stomach (Schmausser et al. 2004), the intestine (Bambou et al. 2004) and the airway (Adamo et al. 2004). Polarized expression of TLR5 in epithelial compartments is thought to contribute to the discrimination between commensal and pathogenic bacteria by the host and to modulate mucosal immunity (Gewirtz et al. 2000; Gewirtz et al. 2001; Gewirtz et al. 2001) although a recent study reported the ability of non-pathogenic *Escherichia coli* to induce a TLR5-driven inflammatory response (Bambou et al. 2004).

TLR5 is also expressed in cells of the innate immune system including dendritic cells and monocytes in humans, and splenic macrophages and dendritic cells in mice (Kadowaki et al. 2001; McSorley et al. 2002; Means et al. 2003; Didierlaurent et al. 2004). Dendritic cells are professional antigen presenting cells that are critical initiators of immune responses against invading agents by stimulating innate immunity and activating naïve T cells (Moser and Murphy 2000). Bacterial flagellin is able to induce the migration and maturation of dendritic cells leading to T cell differentiation (McSorley et al. 2002; Means et al. 2003; Didierlaurent et al. 2004). Flagellin is also known to elicit a strong and sustained antibody response *in vivo*. In fact, most *Salmonella*-specific $CD4^+$ T lymphocytes are generated against flagellin epitopes during *Salmonella* infection (McSorley et al. 2002).

In this article, we evaluated the candidacy of *Tlr5* as the gene underlying the *Salmonella*-susceptibility locus, *Ity3* in wild-derived MOLF/Ei mice. The candidacy of *Tlr5* was based initially on the reduced level of *Tlr5* mRNA expression in the liver (a major site of *Salmonella* proliferation) of susceptible MOLF/Ei mice, and on the presence of several sequence variants within the coding sequence of the protein compared to the

C57BL/6J *Tlr5* allele (Sebastiani et al. 2000). We have investigated the impact of the MOLF/Ei *Tlr5* allele on the host response to flagellin *in vitro* using NF-κB-dependent reporter constructs and *in vivo* using *Ity3* congenic mice.

MATERIALS AND METHODS

Cell Culture: Human epithelial cell lines HeLa (ATCC number: CCL-2) and 293T (ATCC number: CRL-11268) were cultured at 37°C in 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Burlington, ON) supplemented with 10% heat-inactivated FBS (HyClone, Logan, UT) and 100 U/ml penicillin, 100µg/ml streptomycin (Invitrogen, Burlington, ON).

Mice: C57BL/6J and MOLF/Ei mice were purchased from Jackson Laboratory (Bar Harbor, ME). Congenic mice B6.MOLF-*Ity*, and B6.MOLF-*Ity/Ity3* were created by introgressing the MOLF/Ei chromosomal 1 region surrounding *Slc11a1*^{G169}*(Ity)* and/or the *Ity3* interval onto a C57BL/6J background (Figure 1). The MOLF/Ei region surrounding *Slc11a1* (*Ity*) was transferred because of the known effect of *Slc11a1* (*Ity*) in the detection of the *Ity3* QTL (Sebastiani et al. 1998). The *Ity3* region used to construct congenic mice was expanded to include the region delineated by the DNA markers *D1Mit135* and *D1Mit17,* and spans 84Mb. Mice heterozygous for the *Ity3* interval were selected for successive backcrossing to B6.MOLF-*Ity* mice to generate B6.MOLF-*Ity/Ity3*$^{B6/MOLF}$ heterozygous mice. These congenic mice at the 5th generation of backcross were intercrossed to obtain all three possible genotypes at the *Ity3* interval, MOLF/MOLF, heterozygous B6/MOLF, and B6/B6, while their *Ity* interval was fixed (MOLF/MOLF) resulting in the following congenic mice respectively, B6.MOLF-*Ity/Ity3*, B6.MOLF-*Ity/Ity3*$^{B6/MOLF}$, and B6.MOLF-*Ity*. Mice were then selected based on their *Tlr5* genotype such that B6.MOLF-*Ity/Ity3* mice are homozygous for the MOLF allele at Tlr5

(*Tlr5*$^{MOLF/MOLF}$), B6.MOLF-*Ity/ Ity3*$^{B6/MOLF}$ are heterozygous (*Tlr5* $^{B6/MOLF}$) and B6.MOLF-*Ity* mice are homozygous for the B6 allele (*Tlr5*$^{B6/B6}$). The resulting congenic mice were initially tested for susceptibility to infection with *Salmonella* Typhimurium using survival analysis as the disease phenotype to confirm the existence of *Ity3* (Sancho-Shimizu 2006). All procedures involving animals were performed in accordance with regulations of the Canadian Council of Animal Care.

Plasmid construction

Tlr5 cDNA and reporter plasmids: C57BL/6J and MOLF/Ei *Tlr5* cDNAs were initially cloned in pIRESpuro2 plasmid (Clontech, Palo Alto, CA) and then subcloned into the *EcorV* and blunted *NotI* sites of the expression vector pRc/CMV (Invitrogen, Burlington, ON). The NF-κB and C/EBPβ luciferase reporter constructs were kindly provided by Dr. J. M. Stark (Children's Hospital Medical Center, Cincinnati, OH) (Chini et al. 1998). pRL-TK plasmid which encodes for *Renilla* luciferase was purchased from Promega (Madison,WI). The reporter construct IL8-CAT spans +23 to –578 of the human IL-8 promoter and was described previously (Kunsch et al. 1994; Gewirtz et al. 2000). All plasmid constructs were prepared using Endofree Plasmid Extraction Kit (Qiagen, Mississauga, ON) and the sequence verified by sequencing using the platform ABI PRISM® 3730XL DNA Analyzer system (Applied Biosystems, Streetsville, ON).

Tlr5 Promoter: A 2.4-kb genomic fragment of the mouse *Tlr5* promoter was amplified from spleen genomic DNA of C57BL/6J and MOLF/Ei using the primers Prom-F 5'-ACAATCCCAGTGACTTAGCTTCCT-3' and Prom-R 5'-GATCCTATGGGAAAAGAGAGAATT-3'. Primer sequences were derived from the

genomic sequence upstream of the *Tlr5* open-reading frame available in the Ensembl database (Ensembl accession no. ENSMUSG00000045497). PCR products were re-amplified with the primers Prom-MluI-F 5'-AATACGCGTACAATCCCAGTGACTTAGCTTCCT -3' and Prom-XhoI-R 5'-AATCTCGAGGATCCTATGGGAAAAGAGAGAATT -3' to add restriction sites *MluI* to the 5' end and *XhoI* to the 3' end of the fragment. Re-amplified 2.4kb promoter fragments were cloned into *MluI* and *XhoI* sites of the vector pGL3-Basic (Promega, Madison,WI).

Seven fragments were derived from the 2.4kb fragments of the C57BL/6J and MOLF/Ei *Tlr5* promoter. These fragments were sub-cloned in pGL3-B vector by restriction enzyme digestion or PCR amplification (see Table 1). Enzymatic digestions were carried overnight at 37°C on the pGL3/2.4kb_B6 and pGL3/2.4kb_MOLF constructions using the restriction enzymes listed in Table 2 to shorten the length of the promoter fragment under study. Digestion products were blunted and re-ligated. Primers used to amplify the fragments are listed in Table 1 and 2 and all contain a restriction site for either *MluI* (forward primers) or *XhoI* (reverse primers) [GenBank Accesion ID DQ414410, DQ414411].

Transient transfections

Tlr5 cDNA: For all transient transfections HeLa and 293T cells ($1x10^5$cells/well) were plated in 6-well plates the day before transfection. For transient transfections with NF-κB and C/EBPβ luciferase reporters, HeLa cells were co-transfected with 0.5μg of either pRc/CMV, pCMV/Tlr5_B6 or pCMV/Tlr5_MOLF, 0.1 μg of the NF-κB or

C/EBPβ luciferase reporter plasmids and 0.05μg of pRL-TK. 293T cells were transfected with 0.1μg of the NF-κB or C/EBPβ luciferase reporter plasmids and 0.01μg of pRL-TK. Transient transfections were performed using 3.25μl of SuperFect reagent (Qiagen, Mississauga, ON) following the manufacturer's instructions. pRL-TK was included in all transfections to control for transfection efficiency. On the following day, individual wells were left untreated or were stimulated with 100ng/ml of purified flagellin for 5 h before harvesting. Luciferase activity was measured using the Dual Luciferase Reporter Assay System (Promega, Madison,WI), according to manufacturer's instructions with a Lmax™ Microplate Luminometer (Molecular Devices, Sunnyvale, CA). The cells were transfected in triplicate in at least three independent experiments using the same plasmid preparation. A single representative experiment is shown and data are expressed as ratios of average *Firefly* luciferase values over *Renilla* luciferase values ± standard deviation (SD).

For transient transfections with the pIL8-CAT reporter plasmid, HeLa cells were co-transfected with 0.5μg of either pRc/CMV, pCMV/Tlr5_B6 and/or pCMV/Tlr5_MOLF and 1μg of pIL8-CAT. 293T cells were transfected with1μg of pIL8-CAT. All transfections were performed using 3μl of SuperFect reagent (Qiagen, Mississauga, ON) following the manufacturer's instructions. On the following day, individual wells were left untreated or were stimulated with 100ng/ml of purified flagellin for 5 h before harvesting. Cells were washed twice with cold PBS and lysed in 750μl of lysis buffer. Cell extracts were centrifuged at 10,000 x *g* for 10 min at 4°C to remove cellular debris. Supernatants were recovered and assayed for total protein using the DC Protein Assay (Bio-Rad, Mississauga, ON) according to manufacturer's instructions. An

equal amount (50μg) of total protein from each lysate was assayed for chloramphenicol acetyltransferase (CAT) activity with the CAT Elisa kit (Roche, Laval, Qc) following manufacturer's instructions. Readings were done using the BioRad Microplate Reader Model 3550 (Bio-Rad, Mississauga, ON). The cells were transfected in triplicate in at least three independent experiments. A single representative experiment is shown and data are expressed as average values ± SD.

Tlr5 promoter: Transient transfections of the promoter fragments were performed in the human 293T cell line. 293T cells ($1x10^5$cells/well) were plated in 6-well plates the day before transfection and were co-transfected with 0.5μg of either promoter construct and 0.05μg of pRL-TK using 1.1μl of SuperFect reagent (Qiagen, Mississauga, ON) following the manufacturer's instructions. Cells were harvested and luciferase activity was measured using the Dual Luciferase Reporter Assay System (Promega, Madison,WI) as described for the transient transfections of *Tlr5* cDNA and luciferase reporters. The cells were transfected in triplicate in five independent experiments. A single representative experiment is shown and data are expressed as ratios of average *Firefly* luciferase values over *Renilla* luciferase values ± SD.

Flagellin Purification: Flagellin was HPLC (high-pressure liquid chromatography)-purified from *Salmonella* Typhimurium (SL3201) supernatant and purity verified as previously described (Gewirtz et al. 2001; Gewirtz et al. 2001; McSorley et al. 2002). Briefly, such purified flagellin does not contain ligands for TLRs 1-4 nor 6-10 and all its activity *in vitro* and *in vivo* is destroyed by protease treatment.

In vivo response to flagellin: C57BL/6J ($Tlr5^{B6/B6}$), MOLF/Ei ($Tlr5^{MOLF/MOLF}$) and congenic B6.MOLF-*Ity* ($Tlr5^{B6/B6}$), B6.MOLF-*Ity/Ity3*$^{B6/MOLF}$ ($Tlr5^{B6/MOLF}$) and B6.MOLF-*Ity/Ity3* ($Tlr5^{MOLF/MOLF}$) mice were injected intraperitoneally (i.p.) with 10μg of purified flagellin/20g body weight diluted in 400μl of sterile PBS. Control mice were injected i.p. with sterile PBS. Four mice of each group were sacrificed by CO_2 asphyxiation at 0, and 60 or 90 minutes post-injection and their serum was collected by cardiac puncture. IL-6 and CXCL-1 (KC) concentrations in serum samples were quantified with mouse IL-6 and mouse KC Quantikine® immunoassays (R&D Systems, Minneapolis, MN) following manufacturer's instructions on serum diluted 1/5 (IL-6) and 1/1000 (KC).

Quantification of Tlr5 mRNA levels by real-time PCR: Total RNA was extracted from livers of C57BL/6J, MOLF/Ei, B6.MOLF-*Ity/Ity3*$^{B6/MOLF}$, and B6.MOLF-*Ity/Ity3* mice at 0 and 3 days after *Salmonella* infection. The RNA samples underwent DNAse treatment using Turbo DNA-free™ from Ambion Inc. prior to reverse transcription. *Tlr5* expression was determined by sybrgreen real time PCR using the following set of primers 5'-ACATCATGGGTCCTGGCTTT -'3 and 5'-AGGATAGATTGAGAACCTGGAGGC -'3 . *Tbp* was used as a housekeeping gene control and amplified using the primers F 5'-CCCTTGTACCCTTCACCAAT-3' and 5'-ACAGCCAAGATTCACGGTAG-3'. Stratagenes's Brilliant® SYBR®Green QPCR Master Mix was used as the reagent for the PCR reactions. All samples were run in triplicate along with a standard curve of four 10-fold serial dilutions of template cDNA. The expression data are expressed in relative fold

change units using uninfected MOLF/Ei as the referent according to the following equation $2^{-((Tlr5 \text{ treatment Ct} - Tbp \text{ treatment Ct}) - (Tlr5 \text{ reference Ct} - Tbp \text{ reference Ct}))}$. The level of significance was assessed using the Student's t-test ($p<0.05$).

RESULTS

Evaluation of C57BL/6J and MOLF/Ei Tlr5 alleles in vitro: We have previously reported *Tlr5* sequence variants between C57BL/6J and MOLF/Ei mice, consisting of 16 single nucleotide polymorphisms, of which 8 were non-synonymous (Sebastiani et al. 2000). To examine whether these coding sequence variants have an impact on *Tlr5* function, we transfected transiently the *Tlr5* alleles and the reporter plasmids in HeLa cells. HeLa cells were chosen because they do not express a functional *Tlr5* gene and could not activate NF-κB in response to flagellin (Gewirtz et al. 2001). The ability of each allele to induce reporter plasmids NF-κB-Luc and C/EBPβ-Luc activity upon flagellin stimulation was investigated by transfecting HeLa cells transiently with either pCMV/Tlr5_B6 or pCMV/Tlr5_MOLF, the reporter construct and the internal control pRL-TK. The reporter constructs NF-κB-Luc and C/EBPβ-Luc were both derived from the promoter of *ICAM-1* and contain either a NF-κB or C/EBPβ (NF-IL6) binding site (Chini et al. 1998). The presence of only NF-κB or C/EBPβ sites in the reporter genes was not sufficient to detect the activation of mouse *Tlr5* by flagellin. As seen in Figure 2, HeLa cells transfected with the MOLF/Ei or C57BL/6J *Tlr5* allele did not exhibit any activation of NF-κB-Luc (Figure 2A) or C/EBPβ-Luc (Figure 2B) reporters.

We then tested a more complex promoter consisting of a 555bp fragment of the *IL8* promoter whose activation is known to be NF-κB-dependent (Kunsch et al. 1994). The ability of C57BL/6J and/or MOLF/Ei *Tlr5* alleles to induce pIL8-CAT reporter gene in response to flagellin was measured in HeLa cells (Figure 3). Induction of luciferase activity was similar in HeLa cells transfected with C57BL/6J (14 fold) or MOLF/Ei (15 fold) *Tlr5* alleles. The positive control cells, 293T, presented a 16-fold activation (Figure

3). The levels of activation of pIL8-CAT upon flagellin stimulation were similar to levels obtained with TNF stimulation (data not shown). These data suggest that coding sequence variants identified in MOLF/Ei mice do not influence the function of TLR5 *in vitro*.

Cloning and Sequence analysis of C57BL/6J and MOLF/Ei Tlr5 promoter: To further analyze the candidacy of *Tlr5*, C57BL/6J and MOLF/Ei *Tlr5* promoters were studied to determine if major rearrangements within the MOLF/Ei promoter could explain the down regulation of *Tlr5* expression in the liver of susceptible MOLF/Ei mice (Sebastiani et al. 2000). We cloned a 2.4kb fragment upstream of the first coding ATG of the promoter region of C57BL/6J and MOLF/Ei *Tlr5* genes. The 2.4kb fragments of the *Tlr5* promoter of C57BL/6J and MOLF/Ei were amplified from genomic DNA and cloned into pGL3-B vector for further sequencing. Sequence analysis of the promoter region of C57BL/6J and MOLF/Ei *Tlr5* promoter revealed the absence of an RNA polymerase II transcription consensus TATA box or consensus initiator sequences as reported for other TLRs (Rehli et al. 2000; Haehnel et al. 2002). This is usually associated with initiation of transcription from several sites rather than from one unique site. A search for consensus binding sites using the web-based program MatInspector (Quandt et al. 1995) revealed the existence of motifs recognized by transcription factors expressed solely in the lymphoid lineage (Oct-1, c-Ets-1) and those expressed in both lymphoid and myeloid lineages (PU.1). In addition, several other motifs recognized by transcription factors involved in signaling by IFNγ (STAT1, IRF-1) or IFNα/β (IRF-3 and IRF-7) have been identified. The sequence analysis of MOLF/Ei *Tlr5* promoter identified 19 sequence variants, 2 single base pair deletions and a 10-bp insertion compared to C57BL/6J

promoter. These nucleotide changes created 20 putative new transcription factor binding sites and abolished 17 motifs in the MOLF/Ei promoter when compared to the C57BL/6J *Tlr5* promoter. Based on the distribution of sequence and motif variants, 7 sub-fragments were designed and cloned in the pGL3-B vector (Figure 4). About a quarter of the novel (5/20) and deleted (4/17) predicted binding sites identified are motifs of transcription factors known to be involved in activation of pro-inflammatory genes. For instance, Pu.1, STAT1,3,5 and NF-κB binding sites are predicted to be present only in the MOLF/Ei *Tlr5* promoter and the motifs for GATA1 and IRF1,3,7 are specific to the C57BL/6J promoter.

The promoter region of MOLF/Ei Tlr5 has stronger basal activity: Deletion analysis of the C57BL/6J and MOLF/Ei *Tlr5* promoters was based on the distribution of sequence and motif variants of the MOLF/Ei promoter. Eight promoter sub-fragments were designed for both mouse strains and cloned into a pGL3-B vector (Figure 4). Each deletion mutant was transiently transfected into 293T cells and luciferase activities were normalized for transfection efficiency by co-transfection with a *Renilla* luciferase construct (pRL-TK). 293T cells were chosen because of the high transfection efficiency of this cell line (between 80-100% with SuperFect). In all five experiments performed, MOLF/Ei fragments appeared to have a stronger basal activity than the C57BL/6J allele (Figure 5). The longest fragment (2.4 kb) showed low activity for both *Tlr5* promoter alleles while the region directing maximal reporter gene expression in 293T cells is comprised in the shortest fragment IRF2 (140 bp) which is identical in C57BL/6J and MOLF/Ei. The most striking differences between MOLF/Ei and C57BL/6J *Tlr5* promoter alleles resided within the fragments SacI and PstI. The promoter activity is three times higher in MOLF/Ei compared to C57BL/6J (Figure 5). The MOLF/Ei SacI fragment

contains the largest number of novel putative transcription factor binding sites (16 new transcription factor binding sites) and contains the motif for PU.1 that was shown to be essential for the transcription of other Toll-like receptors in human monocytes (Figure 4) (Rehli et al. 2000; Roger et al. 2001; Haehnel et al. 2002; Heinz et al. 2003). We were not able to identify the minimal promoter of *Tlr5*. A similar finding was reported previously for the promoter of TLR2 (Haehnel et al. 2002). Abolishment of the transcriptional regulation of *TLR2* in human monocytes required the deletion of the whole promoter and part of the coding sequence (Haehnel et al. 2002). These data indicate that in transient transfection experiments, allele-specific cis-acting factors (DNA polymorphisms) in the flanking DNA sequence of *Tlr5* may have an impact on transcription activity.

The natural genetic background influences Tlr5 expression in MOLF/Ei mice: Although transient transfection experiments showed clearly that DNA variants in the promoter region of MOLF/Ei Tlr5 have an impact on the level of activation, these data were in contradiction with previous observations that the levels of *Tlr5* expression were low in naïve and infected MOLF/Ei mice compared to C57BL/6J mice. To test the possibility that the genetic background may modify the allelic expression of *Tlr5*, we compared liver *Tlr5* mRNA levels by real-time PCR in MOLF/Ei, C57BL/6J, and congenic mice expressing either MOLF/Ei or C57BL/6J *Tlr5* alleles (Figure 6). The congenic mice carry identical C57BL/6J background and differ only by the region on chromosome 1 surrounding *Ity3* including *Tlr5* (Figure 1). The effect of *Ity3* on disease susceptibility was previously validated in these congenic mice such that congenic mice carrying the MOLF/Ei *Ity3* interval (B6.MOLF-*Ity/Ity3*) were more susceptible to *Salmonella* infection than those that did not, B6.MOLF-*Ity/Ity3*$^{MOLF/B6}$ and B6.MOLF-*Ity*

mice (Sancho-Shimizu and Malo 2006). In naïve mice, levels of *Tlr5* mRNA were low in MOLF/Ei parental mice compared to C57BL/6J mice as previously reported (Sebastiani et al. 2000). In congenic mice independently of the origin of the *Tlr5* allele, levels of *Tlr5* mRNA were intermediate between those observed in parental strains C57BL/6J and MOLF/Ei (Figure 6). Three days after infection *Tlr5* mRNA levels decreased in C57BL/6J mice to reach the low levels observed in MOLF/Ei. In congenic mice carrying at least one MOLF/Ei allele at *Tlr5*, slightly higher *Tlr5* mRNA levels were detected (Figure 6). The observation that the expression levels of *Tlr5* in congenic mice is partly restored compared to C57BL/6J levels, suggests that MOLF/Ei specific influences down regulate the expression levels of *Tlr5* in MOLF/Ei mice.

In vivo evaluation of Tlr5 candidacy: We used congenic mice for *Ity3* to evaluate the candidacy of *Tlr5* as the gene underlying *Ity3 in vivo*. Three groups of congenic mice that were either homozygous for MOLF/Ei alleles at *Tlr5* B6.MOLF-*Ity/Ity3* ($Tlr5^{MOLF/MOLF}$), heterozygous B6.MOLF-*Ity/Ity3*$^{B6/MOLF}$ ($Tlr5^{B6/MOLF}$), or homozygous for C57BL/6J alleles B6.MOLF-*Ity* ($Tlr5^{B6/B6}$) were administered flagellin i.p. C57BL/6J and MOLF/Ei mice were used as controls ($Tlr5^{B6/B6}$ and $Tlr5^{MOLF/MOLF}$ respectively). Blood samples were collected via cardiac puncture at different time points (0, 60 and 90 minutes) after injection and the levels of the cytokines IL-6 and CXCL-1 (KC) in sera were measured as markers of flagellin stimulation using standard ELISA assays (Eaves-Pyles et al. 2001; Hayashi et al. 2001; Liaudet et al. 2002). As expected, IL-6 and CXCL-1 levels were either extremely low or not detectable in the sera of mice from all groups when left unstimulated. Upon flagellin stimulation, the levels of secretion of IL-6 and CXCL-1 were high with values ranging between 1025 and 2006 pg/ml for IL-6 and

between 50 and 369 ng/ml for CXCL-1 (Figure 7). For both IL-6 and CXCL-1, mice carrying $Tlr5^{B6/B6}$ or $Tlr5^{B6/MOLF}$ alleles presented similar levels of cytokines after flagellin exposure. Levels of IL-6 and CXCL-1 were significantly higher in congenic mice carrying $Tlr5^{MOLF/MOLF}$ mice compared to $Tlr5^{B6/B6}$ or $Tlr5^{B6/MOLF}$ animals ($p<0.05$). These results suggest that in congenic mice, MOLF/Ei Tlr5 protein presented a stronger response to flagellin as measured by IL-6 (Figure 7A) and CXCL-1 (Figure 7B) secretion in the serum compared to the C57BL/6J allele. These results contrast with the low levels of CXCL-1 observed in MOLF/Ei mice after flagellin stimulation, reinforcing the hypothesis that the genetic background influences TLR5 activity *in vivo*.

DISCUSSION

The mouse strains C57BL/6J and MOLF/Ei were found to present a similar degree of extreme susceptibility to *Salmonella* Typhimurium, a flagellated Gram-negative bacteria (Sebastiani et al. 1998). *Salmonella* susceptibility of C57BL/6J is known to be caused by a non-functional SLC11A1 protein whereas the molecular determinants of *Salmonella* susceptibility in MOLF/Ei remain unknown (Vidal et al. 1993; Sebastiani et al. 1998). QTL analyses in (C57BL/6J x MOLF/Ei)F2 progeny detected one QTL (*Ity3*) that regulates susceptibility to infection in MOLF/Ei mice (Sebastiani et al. 1998). *Ity3* covers a large genomic region rich in genes that could possibly play a role in the response of mice to infection with *Salmonella* Typhimurium. The candidacy of *Tlr5* for *Ity3* was based on the known function of TLR5 and its ability to recognize flagellin from *Salmonella* (Gewirtz et al. 2001; Hayashi et al. 2001). The identification of the molecular basis of a QTL is difficult, and the accumulation of evidence including appropriate tissue expression or polymorphisms in coding or regulatory regions are required to support QTL identity. However, the most conclusive evidence for a QTL is given by *in vitro* and *in vivo* functional studies to test the alternative alleles (Glazier et al. 2002; Abiola et al. 2003).

MOLF/Ei and C57BL6J *Tlr5* alleles were evaluated *in vitro* using human HeLa cells. HeLa cells do not express endogenous TLR5 making easier the interpretation of the results (Gewirtz et al. 2001) and were shown to have a functional IKK signalosome complex and normal NF-κB activation (Sigala et al. 2004). Gene expression during the host immune response to *Salmonella* implicates the combined effect of several signaling

pathways involving transcription factors that are members of activating protein 1 (AP-1), NF-κB and C/EBPβ families (Sundquist et al. 2004). In human cells, flagellin stimulation of TLR5 induces the transcriptional activation of the pro-inflammatory genes *ICAM-1* and *IL-8* (Liaudet et al. 2003; Maaser et al. 2004). Minimal NF-κB-Luc or C/EBPβ-Luc reporters derived from *ICAM-1* did not support the activation of C57BL/6J and MOLF/Ei mouse *Tlr5* by flagellin. In contrast, flagellin stimulation induced a strong activity of the reporter pIL8-CAT in HeLa cells transfected with either C57BL/6J or MOLF/Ei *Tlr5* alleles. pIL8-CAT reporter harbors predicted binding sites for NF-κB and C/EBP demonstrating the requirement for NF-κB and C/EBP activation in flagellin-induced TLR5 signaling in mice. Activation of human TLR5 does not appear to necessitate the cooperation between NF-κB and C/EBPβ transcription factors. In fact, flagellin stimulation of human TLR5 in 293T cells could activate the NF-κB-Luc reporter construct. Levels of luciferase activity were similar using MOLF/Ei and C57BL/6J Tlr5 constructs suggesting that the sequence variants identified in the coding sequence of MOLF/Ei *Tlr5* did not have a detectable impact on the recognition and interaction with flagellin, and the downstream signaling events *in vitro*.

A second approach we used to study the candidacy of *Tlr5* as a *Salmonella* susceptibility gene was to analyze the *Tlr5* promoter sequence and activity of C57BL/6J and MOLF/Ei promoter alleles. We reported previously that susceptible MOLF/Ei mice have markedly reduced (>75%) constitutive *Tlr5* mRNA levels in their liver compared to C57BL/6J mice and that levels of *Tlr5* expression were not modulated during infection with *Salmonella* in MOLF/Ei mice (Sebastiani et al. 2000). This observation correlates well with the data reported in the current paper showing that MOLF/Ei mice respond

poorly to flagellin *in vivo* as measured by serum levels of CXCL-1 (Figure 7). We hypothesized that major rearrangements within the *Tlr5* promoter of MOLF/Ei could explain the low level of *Tlr5* expression in MOLF/Ei liver. Based on the expression profile, the C57BL/6J *Tlr5* promoter was expected to have a stronger activity than MOLF/Ei. Although the C57BL/6J and MOLF/Ei *Tlr5* promoter differ by 37 predicted novel or deleted transcription factor binding sites, the activity of the promoters were opposite to what was expected. All promoter sub-fragments, with the exception of the shortest fragment IRF2 that is common to both alleles, had higher activity when they were derived from MOLF/Ei *Tlr5*. The presence of additional predicted transcription factor binding motifs (e.g. CREB, NF-κB or STAT1) in the MOLF/Ei *Tlr5* promoter may explain its higher activity. It is also possible that the MOLF/Ei promoter fragments used in the assay, are missing the essential repressor binding site, which is normally found in its natural genetic environment. As observed for other TLRs (*Tlr2* and *Tlr4*), *Tlr5* does not contain a TATA box upstream of its initiation codon (Rehli et al. 2000; Haehnel et al. 2002) and its transcription is most likely dependent on other elements such as some belonging to the Ets family including the transcription factor PU.1. Interestingly, only MOLF/Ei *Tlr5* promoter sequence has a predicted PU.1 binding site. However, PU.1 is expressed exclusively in myeloid and B cells and may not be a good candidate to explain the high activity of the MOLF/Ei *Tlr5* promoter in epithelial-derived cells (Klemsz et al. 1990; Hromas et al. 1993).

The current study also provides evidence that *Tlr5* transcriptional activity of MOLF/Ei allele is influenced by its genomic environmental context. In MOLF/Ei mice levels of *Tlr5* expression in the liver were low in comparison to C57BL/6J, however when the MOLF *Tlr5* allele was placed into a C57BL/6J background, B6.MOLF-*Ity/Ity3*

mice, the levels of expression in the liver were significantly higher than those detected in MOLF/Ei mice suggesting that the natural environment of the gene influenced its level of expression. The influence of the genomic environment was also detected in testing allele-specific activity of the TLR5 protein. B6.MOLF-*Ity/Ity3* congenic mice ($Tlr5^{MOLF/MOLF}$) presented the most robust response to systemic administration of flagellin compared to MOLF/Ei, or B6.MOLF-*Ity* and B6.MOLF-$Ity/Ity3^{B6/MOLF}$ showing that the genetic background influences *Tlr5* allelic function in response to flagellin. B6.MOLF-*Ity/Ity3* mice are mostly C57BL/6J and carry only specific MOLF/Ei sequences for the regions surrounding *Ity* and *Ity3* (Figure 1). Therefore, it could be envisioned that factors (e.g. transcription factors or silencers) mapping to other chromosomal regions of the MOLF/Ei genome are affecting the expression and function of *Tlr5*. Because these MOLF/Ei sequence-specific factors were not transferred along with *Ity* or *Ity3*, they cannot affect transcription and the function of *Tlr5* in the congenic mice. For instance, a transcription factor essential for *Tlr5* optimal transcription could be non-functional in MOLF/Ei mice greatly affecting *Tlr5* mRNA levels, while the C57BL/6J allele of this same transcription factor is functional in the *Ity3* congenic mice. On the other hand, an allelic-specific repressor of TLR5 activity may be more potent in MOLF/Ei compared to C57BL/6J mice.

Although sequence analysis did not reveal any mRNA instability elements in the 3'UTR of *Tlr5*, modifications of mRNA stability or any other post-transcriptional event may explain the differences observed in the levels of MOLF/Ei and C57BL/6J *Tlr5* mRNA.

Finally, constitutive levels of *Tlr5* mRNA expression and the response to flagellin in MOLF/Ei and congenic mice do not appear to correlate with *Salmonella* susceptibility. MOLF/Ei mice and B6.MOLF-*Ity/Ity3* mice are susceptible to infection with *Salmonella*

despite the observation that MOLF/Ei mice present low levels of *Tlr5* mRNA and respond poorly to flagellin *in vivo* whereas B6.MOLF-*Ity/Ity3* mice have higher levels of *Tlr5* mRNA expression and respond very strongly to flagellin *in vivo*. In humans a similar observation was reported regarding a *Tlr5* stop codon polymorphism known to abolish flagellin signaling (Hawn et al. 2003), which found no association to susceptibility to typhoid fever (Dunstan et al. 2005).

In summary, we have shown that the levels of expression and the flagellin induced TLR5 production of IL-6 and CXCL-1 in MOLF/Ei mice are clearly dependent on the natural genetic environment of the gene. Taken together, our data strongly suggest that *Tlr5* may not play a primary role in the immune response of wild-derived MOLF/Ei mice to systemic infection with *Salmonella* Typhimurium.

ACKNOWLEDGEMENTS

We thank Line Lariviere for excellent technical services and Silvia Vidal for helpful discussions. This work was supported by grants from the Canadian Institutes of Health Research (CIHR) and the Howard Hughes Medical Institute (HHMI). D. M. is a scholar of CIHR and an International Research Scholar of the HHMI.

TABLES

Table1. Description of the thirteen *Tlr5* promoter fragments sub-cloned from pGL3/2.4kb plasmids

Fragment Name	Promoter allele	Size	Restriction enzymes	Primers
pGL3/NheI_B6	C57BL/6J	2075bp	MluI &NheI	N/A
pGL3/NheI_MOLF	MOLF/Ei	2085bp	MluI &NheI	N/A
pGL3/SacI_B6	C57BL/6J	1453bp	SacI	N/A
pGL3/SacI_MOLF	MOLF/Ei	1463bp	SacI	N/A
pGL3/PstI_B6	C57BL/6J	754bp	MluI &PstI	N/A
pGL3/PstI_MOLF	MOLF/Ei	754bp	MluI &PstI	N/A
pGL3/NsiI_B6	C57BL/6J	593bp	MluI &NsiI	N/A
pGL3/NsiI_MOLF	MOLF/Ei	593bp	MluI &NsiI	N/A
pGL3/NF-κB_B6	C57BL/6J	297bp	N/A	Prom-NFκB-F Prom-XhoI-R
pGL3/NF-κB_MOLF	MOLF/Ei	297bp	N/A	Prom-NFκB-F Prom-XhoI-R
pGL3/EviI_B6	C57BL/6J	247bp	N/A	Prom-EviI-F Prom-EviI-R
pGL3/EviI_MOLF	MOLF/Ei	247bp	N/A	Prom-EviI-F Prom-XhoI-R
pGL3/IRF2[a]	C57BL/6J & MOLF/Ei	140bp	N/A	Prom-IRF2-F Prom-XhoI-R

N/A: Not Applicable

[a] Sequence of IRF2 sub-fragment is the same for C57BL/6J and MOLF/Ei

Table 2. Oligonucleotides primers designed for PCR amplification of *Tlr5* promoter sub-fragments

Primer name	Primer position	Primer sequence
Prom-NFκB-F	-297 to -280	5'GAGAGAACGCGTCTAGAACCTAGCACATGC-3'
Prom-EviI-F	-247 to -230	5'-GAGAGAACGCGTCAGCTTGGATGAAATATC-3'
Prom-IRF2-F	-140 to -123	5'-GAGAGAACGCGTGAATCTGCCAGCAGAATG-3'
Prom-XhoI-R	-1 to -24	5'-AATCTCGAGGATCCTATGGGAAAAGAGAGAATT -3'

FIGURES

Figure 1: A genetic map of the *Ity* and *Ity3* loci on mouse chromosome 1. (A) Bold lines indicate the positions of *Ity* and *Ity3* on chromosome 1 along with the candidate genes for the loci. (B) Location of microsatellite markers. (C) The recombinant chromosomes of B6.MOLF-*Ity/Ity3*, B6.MOLF-*Ity/Ity3*$^{MOLF/B6}$ and B6.MOLF-*Ity* mice. Black, white and gray intervals respectively represent homozygous C57BL/6J, homozygous MOLF/Ei and heterozygous segments respectively.

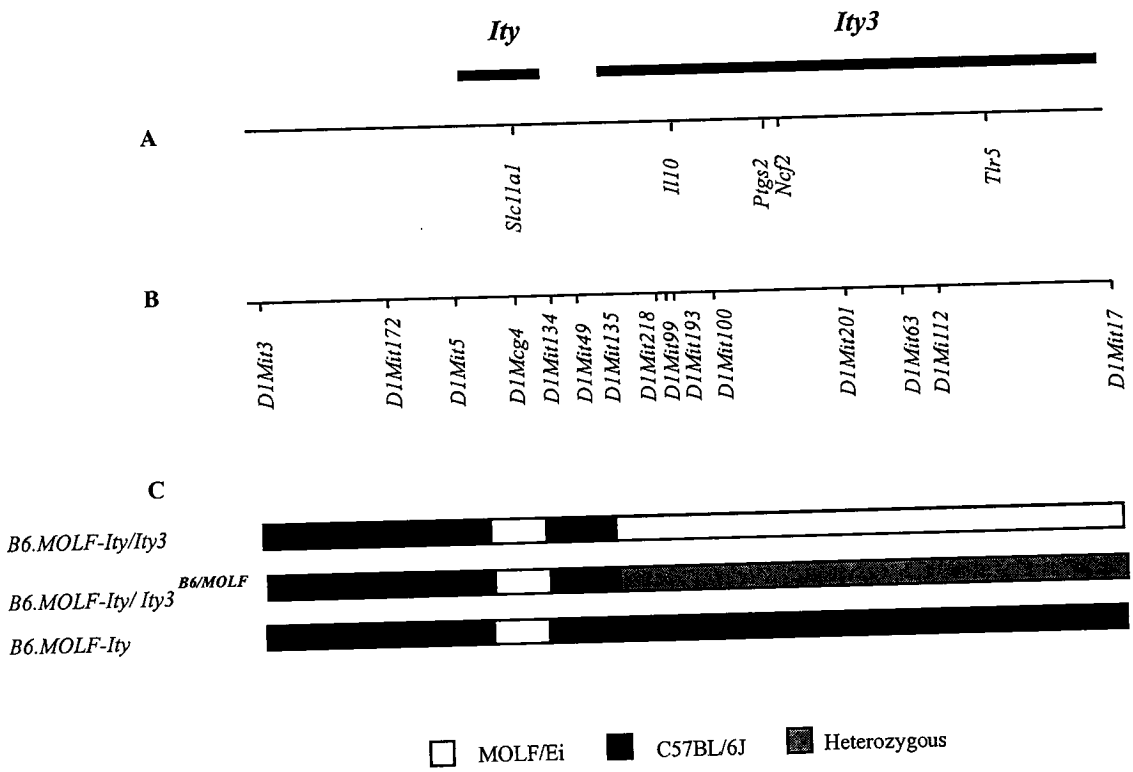
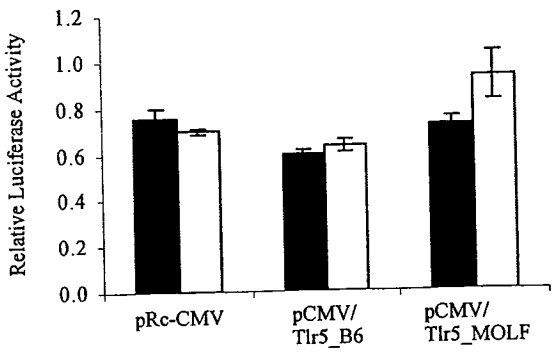

Ity
Ity3
A
Slc11a1
Il10
Pigs2
Ncf2
Tlr5
B
D1Mit3
D1Mit172
D1Mit5
D1Mcg4
D1Mit134
D1Mit49
D1Mit135
D1Mit218
D1Mit99
D1Mit193
D1Mit100
D1Mit201
D1Mit63
D1Mit112
D1Mit17
C
B6.MOLF-Ity/Ity3
B6.MOLF-Ity/ Ity3 B6/MOLF
B6.MOLF-Ity
MOLF/Ei
C57BL/6J
Heterozygous

Figure 2: Effect of TLR5 origin on activation of NF-κB and NF-IL6 reporters. (A,B) HeLa cells were transfected with a plasmid containing either C57BL/6J or MOLF/Ei Tlr5 allele, a minimal NF-κB-Luc (A) or NF-IL6-Luc (B) reporter and pRL-TK construct. Transfected cells were left unstimulated (black bars) or were stimulated 5h00 with purified flagellin (open bars). A representative experiment is shown and data are expressed as ratios of average *Firefly* luciferase values over *Renilla* luciferase values ± SD. The experiment was repeated 3 times with similar results.

A


Relative Luciferase Activity
1.2
1.0
0.8
0.6
0.4
0.2
0.0
pRc-CMV
pCMV/ Tlr5_B6
pCMV/ Tlr5_MOLF

B

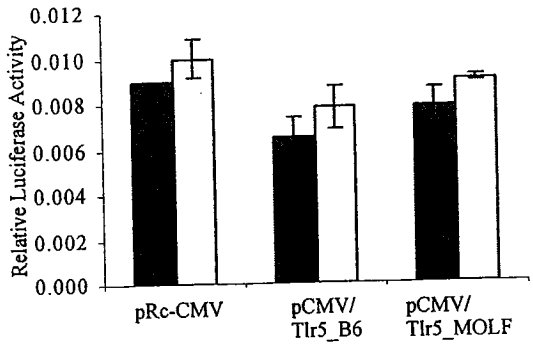

Relative Luciferase Activity
0.012
0.010
0.008
0.006
0.004
0.002
0.000
pRc-CMV
pCMV/ Tlr5_B6
pCMV/ Tlr5_MOLF

Figure 3: Effect of *Tlr5* allele and flagellin stimulation on activation of pIL8-CAT reporter. HeLa cells were transfected with a plasmid containing either C57BL/6J or MOLF/Ei *Tlr5* allele and pIL8-CAT reporter. 293T cells were transfected with reporter pIL8-CAT. Transfected cells were left unstimulated (black bars) or were stimulated 5 h with purified flagellin (open bars). A representative experiment is shown and data are expressed as average CAT values ± SD. The experiment was repeated 3 times with similar results.

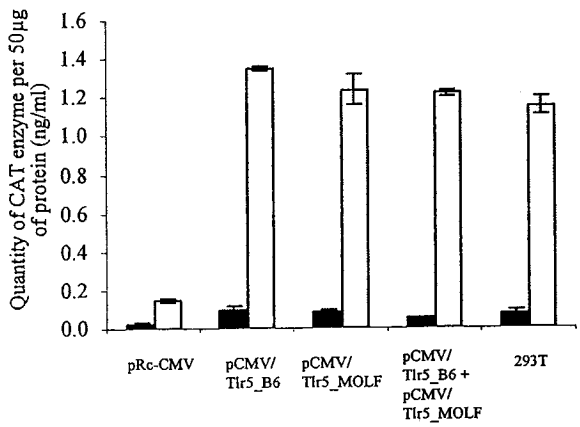

Quantity of CAT enzyme per 50µg of protein (ng/ml)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
pRc-CMV
pCMV/ Tlr5_B6
pCMV/ Tlr5_MOLF
pCMV/ Tlr5_B6 + pCMV/ Tlr5_MOLF
293T

Figure 4: Sequence variants identified between MOLF/Ei and C57BL/6J *Tlr5* promoters. (A) Nucleotide and putative transcription factor binding site changes for each fragment relative to the C57BL/6J sequence. (B) Schematic distribution of sequence variants in *Tlr5* promoter. Arrows represent the 5' border of each fragment.

A

Name	Size	Sequence Variants*	New TF sites*	Deleted TF sites*
2.4kb	2421bp (2431bp)	C-2368Del, C-2348A, C-2109T		NRF2
NheI	2075bp (2085bp)			
SacI	1453bp (1463bp)	A-1315G, G-1200A, G-1184A, -1177Ins(5XGA) G-1164A, C-1039A, A-1027G, A-1022G, G-999T, G-958C, T-938C, G-933A, T-922C, C-889A	WT, STAF, SREBP, EVI1, CLTR_CAAT, TAAC, CHR, TAX, CREB, c-Ets-1,2, Pu.1 STAT1,3,5, BCL6, Ptx1	NGFIC, GATA1, Pax5, c-Ets-1,2, CRX, IRF1,3,7, MTATA, CDP,
PstI	754bp	C-726Del, C-722T	RFX1	EGR1,2,3, NGFIC, WT, AHRARNT
NsiI	593bp	G-510C		
NF-κB	297bp	A-276G	NF-κB	
EviI	247bp	A-182G	EVI1	
IRF2	140bp			

B

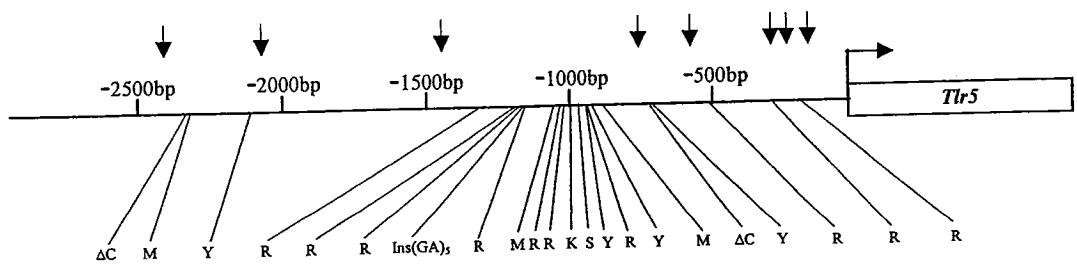


Figure 5: Basal level of C57BL/6J and MOLF/Ei *Tlr5* promoters activity. 293T cells were transfected with the 16 promoter fragments (8 per allele) and the control vector pRL-TK. The schematic representation of each sub-fragment is shown. Black and white bars represent C57BL/6J and MOLF/Ei sub-fragments respectively. Transfected cells were left unstimulated. At least five independent experiments were performed with triplicate transfections. A representative experiment is shown and data are expressed as ratios of average *Firefly* luciferase values over *Renilla* luciferase values ± SD.

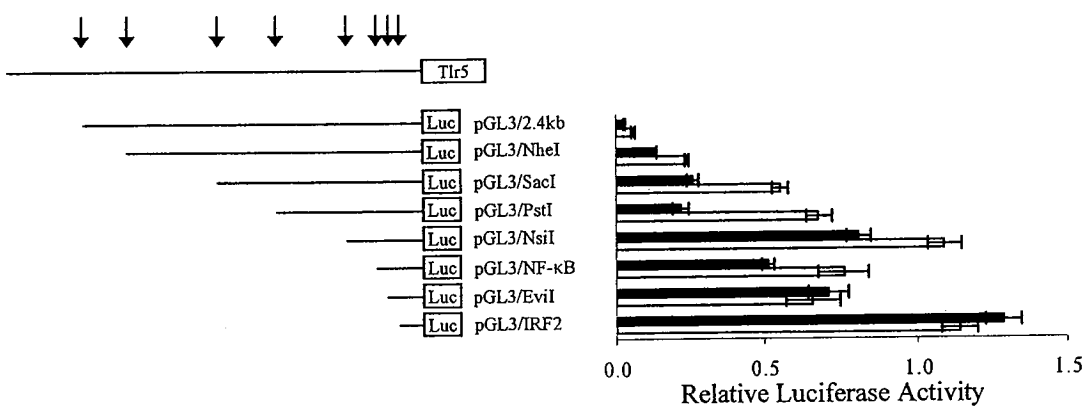

Tlr5
Luc
pGL3/2.4kb
pGL3/NheI
pGL3/SacI
pGL3/PstI
pGL3/NsiI
pGL3/NF-κB
pGL3/EviI
pGL3/IRF2
0.0
0.5
1.0
1.5
Relative Luciferase Activity

Figure 6: *Tlr5* expression in livers of control and *Salmonella* Typhimurium infected mice as measured by real-time PCR. *Tlr5* transcript levels from C57BL6/J ($Tlr5^{B6/B6}$), MOLF/Ei ($Tlr5^{MOLF/MOLF}$), B6.MOLF-*Ity/Ity3*$^{B6/MOLF}$ ($Tlr5^{B6/MOLF}$) and B6.MOLF-*Ity/Ity3* ($Tlr5^{MOLF/MOLF}$) mice were assayed with respect to the house keeping gene *Tbp*. Relative fold differences are expressed using the $2^{-\Delta\Delta Ct}$ method. Asterisks indicate a statistically significant difference ($p<0.05$).

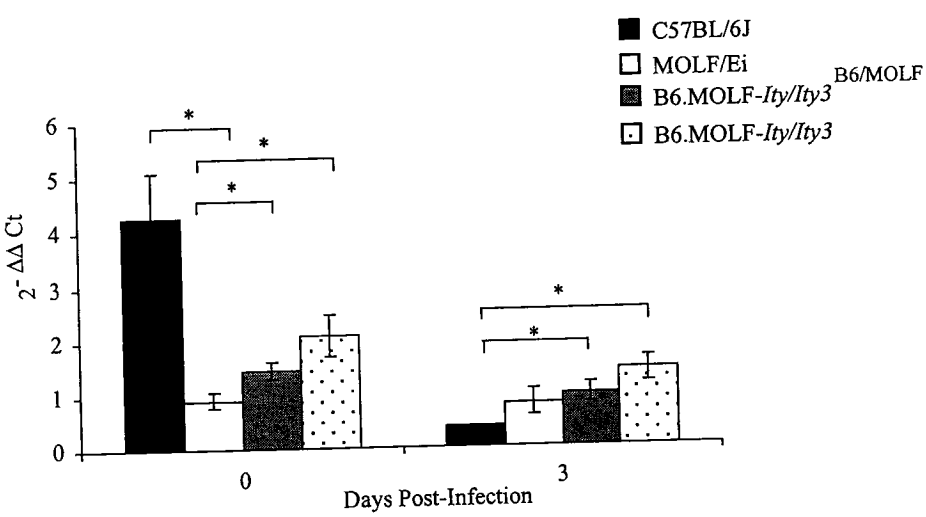
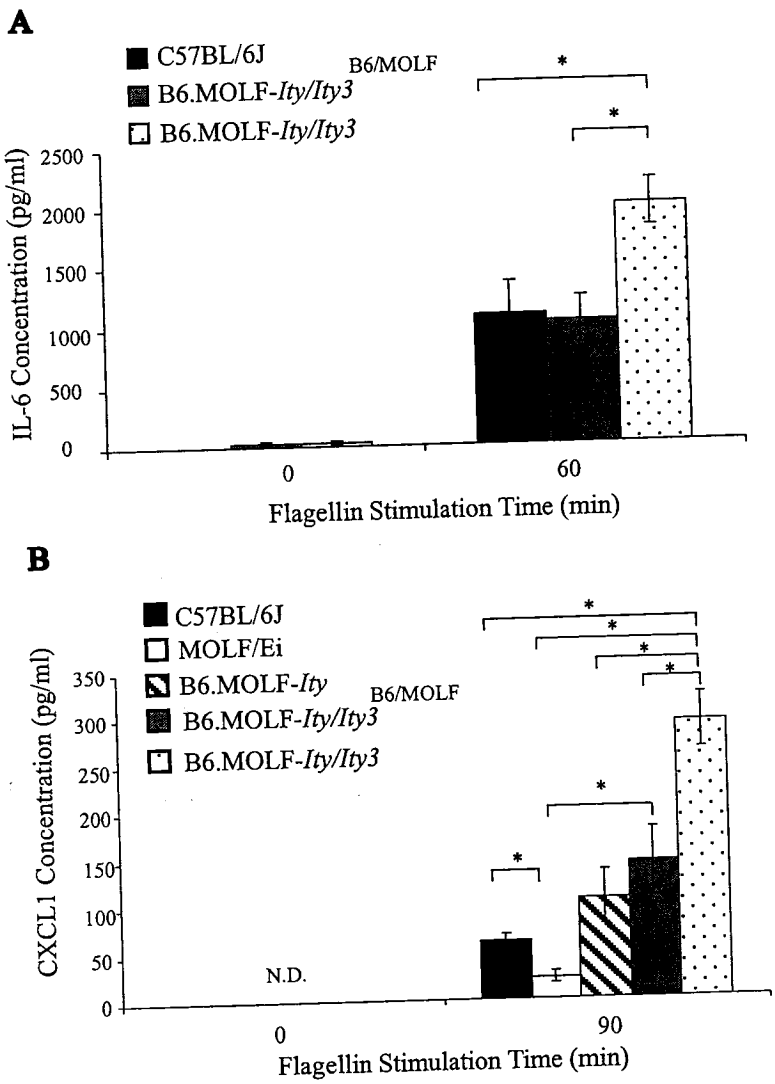

C57BL/6J
MOLF/Ei
B6.MOLF-Ity/Ity3
B6.MOLF-Ity/Ity3
B6/MOLF
2- ΔΔ Ct
0
1
2
3
4
5
6
*
*
*
*
*
0
3
Days Post-Infection

Figure 7: The effect of systemic flagellin injection on pro-inflammatory cytokine production in C57BL6/J (*Tlr5*$^{B6/B6}$), MOLF/Ei (*Tlr5*$^{MOLF/MOLF}$), B6.MOLF-*Ity* (*Tlr5*$^{B6/B6}$), B6.MOLF-*Ity/Ity3*$^{B6/MOLF}$ (*Tlr5*$^{B6/MOLF}$) and B6.MOLF-*Ity/Ity3* (*Tlr5*$^{MOLF/MOLF}$) mice. (A) Level of serum IL-6 as measured by ELISA. (B) Level of serum CXCL-1 (KC) as measured by ELISA. Four mice per group per time point were administered purified flagellin. Asterisks indicate a statistically significant difference ($p<0.05$). N.D. non detectable.


A
C57BL/6J
B6.MOLF-Ity/Ity3
B6.MOLF-Ity/Ity3
B6/MOLF
*
*
IL-6 Concentration (pg/ml)
2500
2000
1500
1000
500
0
0
60
Flagellin Stimulation Time (min)
B
C57BL/6J
MOLF/Ei
B6.MOLF-Ity
B6.MOLF-Ity/Ity3
B6.MOLF-Ity/Ity3
B6/MOLF
*
*
*
*
*
*
CXCL1 Concentration (pg/ml)
350
300
250
200
150
100
50
0
N.D.
0
90
Flagellin Stimulation Time (min)

CHAPTER 4:

SEQUENCING, EXPRESSION AND FUNCTIONAL ANALYSES SUPPORT THE CANDIDACY OF *NCF2* IN SUSCEPTIBILITY TO *SALMONELLA* TYPHIMURIUM INFECTION IN WILD-DERIVED MICE

RATIONALE

The *Ity3* interval, identified as susceptibility locus during *Salmonella* infection, is relatively large and contains numerous genes with potential relevance to the host immune response during *Salmonella* infection. In the previous chapter, *Tlr5* was eliminated as a candidate gene underlying *Ity3* based on functional *in vitro* and *in vivo* studies. Consistent with these results, the critical *Ity3* interval presented in Chapter 2, also excluded *Tlr5* based on map position. As part of the hosts antimicrobial effectors, the production of ROI's are crucial in combating infections (Vazquez-Torres and Fang 2001). Based on its map position within *Ity3*, and established role as a microbicidal host effector in *Salmonella* infections, *Ncf2* was an excellent candidate to further characterize as the gene underlying *Ity3*. This gene is of particular interest because of its known implication in chronic granulomatous disease, an immunodeficiency disorder resulting in the absence of ROI production rendering patients susceptible to recurrent infections with pathogens, including *Salmonella* (Heyworth et al. 2003).

Sequencing, expression and functional analyses support the candidacy of *Ncf2* in susceptibility to *Salmonella* Typhimurium infection in wild-derived mice

Vanessa Sancho-Shimizu[*‡] and Danielle Malo[*†‡]

[*]Department of Human Genetics, McGill University, Montreal, QC;

[†]Department of Medicine, McGill University, Montreal, QC;

[‡]Center for the Study of Host Resistance, McGill University Health Center, Montreal, QC

Published in *The Journal of Immunology,* volume 176, pp. 6954-6961 (2006)

ABSTRACT

A recessive *Salmonella* Typhimurium susceptibility locus (*Ity3*) was previously reported on distal mouse chromosome 1 using a cross between C57BL/6J and wild-derived MOLF/Ei mice. This QTL is located in a genomic region spanning 84Mb, rich in candidate genes for which a role in host resistance to *Salmonella* infection is either known or can be envisioned. Here we report the evaluation of *Ncf2* as a candidate *Salmonella* susceptibility gene for *Ity3*. *Ncf2* encodes $p67^{phox}$, a subunit of the multi-protein enzyme complex NADPH oxidase, known to be responsible for the generation of superoxides. Congenic mice carrying the *Ity3* region from MOLF/Ei, B6.*MOLF-Ity/Ity3,* were more susceptible to infection compared to control mice heterozygous at *Ity3,* B6.MOLF-*Ity/Ity3*$^{MOLF/B6}$, confirming the existence of a recessive *Salmonella* susceptibility locus on distal chromosome 1. Spleen *Ncf2* expression levels were lower in infected congenic mice homozygous for the MOLF/Ei allele at *Ity3* compared to mice heterozygous at *Ity3*. C57BL/6J and MOLF/Ei *Ncf2* sequence comparisons revealed one non-conservative amino acid change (R394Q) in the functional and highly conserved PB1 domain of the protein. Functional analysis revealed that the MOLF/Ei allele had reduced PMA- and *Salmonella*-induced superoxide induction as compared to their wild type counterparts *ex vivo*. The R394Q substitution seems to occur on an amino acid involved in electrostatic interactions with $p40^{phox}$, crucial in its activation. Moreover, a human mutation in the corresponding R395W, resulting in chronic granulomatous disease, is known to lead to reduced superoxide levels. These results support the candidacy of *Ncf2* as the gene underlying *Ity3*.

INTRODUCTION

Salmonella enterica serovar Typhimurium (*Salmonella* Typhimurium) is a facultative intracellular gram negative bacillus with a broad host range. *Salmonella* Typhimurium can infect both humans and mice causing a spectrum of disease states ranging from an asymptomatic carrier state to sepsis. There are typically two forms of clinical manifestations as a result of *Salmonella* infection, a self-limiting gastroenteritis, or enteric fever such as typhoid fever. The latter is still considered a public health issue among developing nations as there are 16 million cases reported globally every year (2001; Monack et al. 2004).

Host genetic factors are known to influence the outcome of infection. Some examples include *Slc11a1* (formerly known as *Nramp1*), *Tlr4*, *H2* complex, *IFNγ*, and *btk* (reviewed in (Roy and Malo 2002). Understanding of the pathogenesis of *Salmonella* infection to-date is largely due to the use of a well-characterized mouse model of infection. Infection of laboratory mice with *Salmonella* Typhimurium has long been used to study the systemic manifestation of disease since mice develop an illness that resembles human typhoid fever. This model consists of four distinct phases: 1) the initial clearance of the inoculum from the blood as surviving bacteria take up residence in the polymorphonuclear cells (PMNs) and macrophages of the reticuloendothelial system (RES), 2) the exponential replication phase within the RES, 3) the plateau phase where effectors of innate immunity suppress the replication of bacteria, and 4) the resolution of infection through adaptive immune mechanisms (reviewed in(Mastroeni et al. 1994)).

Herein, we will be focusing on the first 3 phases of this model which constitute the innate immune phase of infection.

The phagocyte NADPH oxidase has a crucial role in innate immunity, specifically acting to reduce molecular oxygen to superoxide. Superoxide anions give rise to numerous toxic reactive oxygen species that are used as microbicidal agents against pathogens, contributing to the respiratory burst typically seen in phagocytic cells. The complex consists of five protein subunits, two transmembrane proteins $p22^{phox}$ and the catalytic subunit $gp91^{phox}$ comprising the flavocytochrome b_{558} complex, and three cytosolic proteins, $p40^{phox}$, $p47^{phox}$ and $p67^{phox}$ in addition to a small GTPase Rac (Vignais 2002). $p67^{phox}$, encoded by *Ncf2* on mouse distal chromosome 1, is found in the cytosol at resting state, bound to $p40^{phox}$ and $p47^{phox}$ in an inactive form (Mizuki et al. 1998). Upon activation by opsonized microbes or inflammatory mediators $p67^{phox}$, along with $p40^{phox}$ and $p47^{phox}$, is translocated to the membrane, to form an active enzyme complex with flavocytochrome b_{558}, and is thought to regulate electron transfer from NADPH to flavin adenine dinucleotide (Han et al. 1998; Nisimoto et al. 1999). Once activated, superoxide is released into the phagocytic vacuole or into the extracellular space.

In fact, the importance of a functional NADPH oxidase can be seen in individuals that have mutations in the genes that encode for four of the subunits (*CYBB* for $gp91^{phox}$, *CYBA* for $p22^{phox}$, *NCF1* for $p47^{phox}$ and *NCF2* for $p67^{phox}$) that make up the complex, resulting in the rare inherited immunodeficiency, chronic granulomatous disease (CGD) (Cross et al. 2000; Heyworth et al. 2001). These patients have a total absence or very low levels of superoxides which renders them extremely susceptible to recurrent and often chronic infections with a wide range of bacterial and fungal pathogens. (Cross et al. 2000).

According to a national registry of 368 CGD patients in the U.S., *Salmonella* species accounted for the majority of bacteremia and sepsis cases in CGD patients (Winkelstein et al. 2000). Mouse models of CGD parallel the phenotypes observed in human CGD and have been successfully created by knocking out $p47^{phox}$, or $gp91^{phox}$ (Jackson et al. 1995; Pollock et al. 1995). These knockout models show increased susceptibility to *Staphylococcus aureus, Mycobacterium tuberculosis, Mycobacterium avium, Pseudomonas aeruginosa, Aspergillus fumigatus,* and *Salmonella* Typhimurium infections. (Jackson et al. 1995; Pollock et al. 1995; Cooper et al. 2000; Mastroeni et al. 2000; van Diepen et al. 2002; Sadikot et al. 2004).

We have previously reported the identification of QTLs that affect host response to *Salmonella* Typhimurium infection in the susceptible wild-derived inbred mouse MOLF/Ei (Sebastiani et al. 1998). In addition to *Slc11a1* on proximal chromosome 1 (*Ity*), two novel loci a resistance locus on chromosome 11 (*Ity2*), and a susceptibility locus on distal chromosome 1 (*Ity3*), were identified using a F_2 panel of C57BL/6J X MOLF/Ei mice. *Ity3* is the only susceptibility locus identified in MOLF/Ei, mapping just downstream of the *Ity* locus on distal chromosome 1, with a genomic interval of approximately 16cM. It was found to be inherited in a recessive fashion, accounting for 7% of the phenotypic variance with a peak LOD score of 4.8 when expressed on a *Slc11a1* wild type background (MOLF/Ei allele). Here we report our findings on the candidacy of *Ncf2* as the gene underlying the *Ity3* locus based on its essential role in innate immunity, physical map position, sequence polymorphism and functional analyses.

MATERIALS AND METHODS

Ity3 QTL Re-analysis: Eight additional markers, *D1Mit5, D1Mit135, D1Mit218, D1Mit99, D1Mit193, D1Mit201, Ncf2* (6F 5'-tgatcagtttgtcagaccagc-3' and 5R5'gctacctgagtgacaatggct-3'), and *Tlr5* (F 5'-catgtcaacagggagctttgt- 3' and R 5'-atgaagctgcctgtaacttctccc-3') were used to re-evaluate the LOD score for *Ity3*, using an additional 41 mice along with the original 191 (C57BL/6JxMOLF/Ei) F2 mice, which is (Sebastiani et al. 1998). The linkage was analysed using MapManager QTX (Manly et al. 2001), setting *D1Mcg4* as background, on the phenotype of Ln(survival) using a free regression model. The LOD score was calculated by dividing the LRS statistic by 4.6.

Construction of Congenic Mice: Inbred strains C57BL/6J (B6) and MOLF/Ei (MOLF) mice were purchased from The Jackson Laboratory. Three strains of congenic mice were generated in this study – B6.MOLF-*Ity*, B6.MOLF-*Ity/Ity3*$^{MOLF/B6}$, and B6.MOLF-*Ity/Ity3* mice, in all cases the MOLF allele represented the donor strain and the B6, the recipient strain. For the B6.MOLF-*Ity* congenics, the region surrounding *D1Mcg4*, flanked by *D1Mit3* and *D1Mit135*, was transferred from MOLF/Ei to C57BL/6J by selective backcrossing for 10 generations to C57BL/6J. After the 10th generation of backcrossing, brother sister matings were established to generate homozygous B6.MOLF-*Ity* mice, which have two copies of the resistant *Ity* locus derived from MOLF/Ei, including the wild type *Slc11a1* allele, as opposed to the mutant C57BL/6J *Slc11a1* allele. B6.MOLF-*Ity/Ity3* mice were created by introgressing the 84Mb segment of *Ity3* flanked by the markers *D1Mit135* and *D1Mit17* from MOLF/Ei to C57BL/6J though 5 successive backcrosses to B6.MOLF-*Ity* mice. The *D1Mit135-D1Mit17* interval corresponds to a 2-

LOD support interval which gives a 99% confidence interval that the locus lies within the target region. At the 5th generation of backcrossing, these mice were intercrossed to generate B6.MOLF-*Ity/Ity3* congenic mice and were genotyped for *D1Mit3, D1Mit5, D1Mcg4, D1Mit216, D1Mit218, D1Mit193, D1Mit201, D1Mit63, D1Mit17, Ncf2* (6F 5'-tgatcagtttgtcagaccagc-3' and 5R 5'gctacctgagtgacaatggct-3'), and *Tlr5* (F 5'-catgtcaacagggagctttgt- 3' and R 5'-atgaagctgcctgtaacttctccc-3'). The B6.MOLF-*Ity/Ity3* mice carry the resistant *Ity* segment from MOLF/Ei as well as the susceptible *Ity3* locus from MOLF/Ei on a C57BL/6J genetic background (Figure 1). The B6.MOLF-*Ity/Ity3* mice that were heterozygous for the *Ity3* interval, B6.MOLF-*Ity/Ity3*$^{MOLF/B6}$ were used as B6.MOLF-*Ity* controls when the B6.MOLF-*Ity* mice were unavailable. This was possible since the *Ity3* locus is inherited in a recessive fashion and should only have an impact in homozygous form (Sebastiani et al. 1998). All mice were bred and maintained in an animal care facility according to the Canadian Council on Animal Care.

Salmonella Typhimurium Infection: Six to 16 week old C57BL6/J, B6.MOLF-*Ity/Ity3* and B6.MOLF-*Ity/Ity3*$^{MOLF/B6}$ male and female mice were infected through the caudal vein with 3,000 CFUs of *Salmonella* Typhimurium strain Keller (originally a gift from Dr Hugh Robson at the Royal Victoria Hospital, Montreal, Canada). *Salmonella.* Typhimurium was prepared by growing 1ml of culture in 100ml of trypticase soy broth for approximately 90min (OD600 between 0.1 and 0.2) at 37°C, followed by plating 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6} serial dilutions in duplicate on trypticase soy agar, and placed in a 37°C incubator overnight. CFUs were enumerated the following day and the dose appropriately prepared to the desired concentration of 3,000CFU/0.2ml. The injection mixture was plated in three 10-fold serial dilutions to ensure the dosage concentration.

Infected mice were monitored at least every 12hrs, and moribund animals were sacrificed by CO_2 asphyxiation. Survival was recorded as the day of death after infection. Survival analysis was carried out using the Kaplan-Meier survival test.

Two males and two females from each strain C57BL/6J, B6.MOLF-*Ity/Ity3* and B6.MOLF-*Ity/Ity3*$^{MOLF/B6}$ were sacrificed and weighed at day 0, 1, 3 and 5 post-infection. Their spleens were aseptically removed and weighed. Half of the spleen was used for CFU analysis, placed in 2ml of 0.9% saline solution, and following homogenization, serially diluted 10-fold to be plated in duplicate on trypticase soy agar plates and placed in a 37˚C incubator overnight. CFUs were enumerated the following day and data expressed as $\log_{10}$ (CFU)/g of spleen. The other half of the spleen was snap frozen in liquid nitrogen and stored at -80˚C for RNA extraction.

DNA Extraction and Genotyping: Genomic DNA from C3H/HeSnJ, C57Br/cdJ, C57L/J, DBA/2J, RF/J, P/J, BuB/BnJ, PERA/Rk, PERC/Ei, SF/CamEi, SPRET/Ei, RBA/Dn, RBB/Dn, Sk/CamEi, ZALENDE/Ei, TIRANO/Ei, Skive/Ei, CZECHII/Ei, MOLC/Rk, MOLD/RK, MOLG/Dn, and MOLE/Rk were purchased from The Jackson Laboratory. C57BL/6J, MOLF/Ei, 129S6/SvEvTac, C3H/HeJ, B6.MOLF-*Ity*, B6.MOLF-*Ity/Ity3*, and B6.MOLF-*Ity/Ity3*$^{MOLF/B6}$ genomic DNA was extracted from tail clippings using NaOH as described by Schmitteckert et al (Schmitteckert et al. 1999). Microsatellite markers were amplified using standard PCR conditions in a final volume of 20ul. PCR products were run on Metaphor agarose (Mandel) or High Efficiency Agarose (USB) and visualized by ethidium bromide staining under a UV lamp.

Sequencing: C57BL/6J and MOLF/Ei *Ncf2* cDNA were obtained from reverse transcription of spleen RNA. *Ncf2* cDNA was amplified using two sets of primers. The first set amplified the 5' half of *Ncf2* using the primers: 5'-gcgctaggctgggaccttgaagcc-3' and 5'-ccccttctgtccattgaacatgac-3'. The second set amplified the 3'end of *Ncf2*: 5'-gtcttgaagaagggcagtgataac-3' and 5'-cacagcagagatgggtaagtcttgc-3'. The purity of the amplified fragments of *Ncf2* cDNA was verified on 1.7% agarose and treated with alkaline phosphatase. The fragments were then sequenced on polyacylamide gel using the Thermosequenase Roadiolabeled Terminator Cycle Sequencing Kit (USB Corp.), and visualized by autoradiography. Additional primers were used to sequence the gene in its entirety: 5'-ctgaggccatcagactctggaatg-3', 5'-tgcctgcaagttttccaggat-3', 5'-gagcagttggcattggcaacc-3', 5'-aatgcctgggctcggacttca-3', 5'-gcctctcatttggacggaaca-3', 5'-ccagaaatcttcagggctctg-3', 5'-atgccttacatgctcaaggtg-3', 5'-ctacagccagcttcggaacatggt-3', 5'-ctttcttcggacaggagcaga-3', 5'-ccaggtggtagcaatcttcag-3', 5'-gcattcccagagaagtctaggatc-3'. The entire coding region of *Ncf2* was read in forward and reverse orientation [GenBank accession ID DQ449939].

TaqMan Real-Time PCR: Expression of *Ncf2* in C57BL/6J, MOLF/Ei, 129S6/SvEvTac, B6.MOLF-*Ity/Ity3*$^{MOLF/B6}$ and B6.MOLF-*Ity/Ity3* was determined by Taqman real-time PCR using RotorGene (Corbett Research). cDNAs were obtained from reverse transcription of infected and control spleen RNAs. The cDNAs were amplified using the primers 5'-tcatgttcaatggacagaag-3' and 5'-tatcggattctggagaggta-3' with a probe 5'FAM-actacctggagccagttgagcttc-3'BHQ, and cycled at 95°C for 30sec, 55°C for 60sec, 72°C for 30 sec a total of 40 cycles. *18srRNA* was used as a housekeeping gene control and amplified using the primers F 5'-cacggccggtacagtgaaa-3' and 5'-

agcgagcgaccaaaggaa-3' with a probe 5'HEX-tgcgaatggctcattaaatcagtta-3'BHQ, using the following cycling conditions 95°C for 30sec, 60°C for 60sec, 72°C for 30 sec for a total of 40 cycles. Stratagenes's Brilliant® QPCR Kit Core Reagents were used for the PCR reactions. All samples were run in duplicate along with a standard curve of four 10-fold serial dilutions of template cDNA. The expression data are expressed in relative fold change units using uninfected C57BL/6J as the referent according to the following $2^{-\Delta\Delta Ct}$ equation $2^{-((Ncf2 \text{ treatment Ct} - 18srRNA \text{ treatment Ct})-(Ncf2 \text{ Reference Ct} - 18srRNA \text{ Reference Ct}))}$ (Livak and Schmittgen 2001). The level of significance was assessed using the Student's t-test ($p<0.05$).

Peritoneal Macrophage Isolation: C57BL/6J, B6.MOLF-*Ity*, B6.MOLF-*Ity/Ity3*$^{MOLF/B6}$ and B6.MOLF-*Ity/Ity3* mice were injected intaperitoneally with 1ml 3% thioglychollate and the peritoneal exudates were collected 72hrs later. The exudates were treated with red blood cell lysis buffer for 3min, washed with PBS and resuspended in RPMI-1640 10% FBS. The cells were counted and seeded in 96-well plates at a density of $2x10^5$cells/well. Adherent cells were selected for after 3hrs and the cells were then co-cultured with 0 (for *in vitro* infection), or 20u/ml IFNγ (for PMA stimulation) for 16hrs.

Chemiluminescent Lucigenin Assay: Superoxide production was determined by measuring the chemiluminescence of macrophages exposed to 25uM lucigenin (bis-N-methylacridinium, Sigma) using the LMax® Microplate Luminometer (Molecular Devices). Peritoneal macrophages described above were washed with phenol red-free RPMI twice before stimulation with 0, 100ng/ml of PMA (Sigma), and 25uM of lucigenin. The experiment was carried out in triplicate with one negative control well in

which 400U/ml of superoxide dismutase (Sigma) was added. The amount of chemiluminescence was measured at 0, 30 and 60min, reading for 15sec intervals. Time 0 was considered to be the point at which lucigenin and PMA were injected into the wells. The induction of superoxide was calculated by dividing each time point by time 0, which was considered to be the base line value. Data are represented as fold induction of superoxide over baseline values. Statistical significance was assessed using the Student's t-test ($p<0.05$).

In Vitro Salmonella Typhimurium Infection: One colony of *Salmonella* Typhimurium was grown in 5ml of trypticase soy broth overnight at 37°C at 250rpm. The following morning, the overnight culture was diluted 1:50 and grown to mid log phase (OD600=0.9), and was opsonized with 10% C57BL/6J serum for 30min on ice. Four microlitres of the opsonized *Salmonella* was added to each well of macrophages for a multiplicity of infection of 1:1. The plate of cells was centrifuged for 10min at 1000g and then incubated at 37°C 5% CO_2 for an additional 15min. The cells were then washed twice with PBS then supplemented with RPMI containing 10% inactivated FBS and 100μg/ml of gentamicin. The cells were then incubated at 37°C 5% CO_2 and assayed for superoxide with the addition of lucigenin, as described above every 30min for a period of 3hrs. Bacterial invasion was tested by lysing the cells with 1%TritonX100-PBS, and the lysates were plated for enumeration of colony forming units.

Protein Assay: Protein content was determined to verify the amount of cells in each well, using the DC Protein Assay (Bio-Rad) according to the manufacturer's guidelines. The same wells of cells used for the lucigenin assay were used for the protein

assays. These wells were washed twice with RPMI before adding 50ul of lysis buffer. 5ul of each well was sampled in duplicate for protein analysis. The standard curve was determined by using 2-fold serial dilutions of 3mg/ml of standard BSA.

RESULTS

Re-evaluation of Ity3 QTL and Creation of Ity3 Congenics: The LOD score plot for *Ity3* was re-analysed with eight novel markers and 41 additional (C57BL/6J x MOLF/Ei)F2 mice for a total of 232 progeny (Sebastiani et al. 1998)(Figure 1). The peak LOD score of 4.1 was obtained at *D1Mit218* and *D1Mit100*. In comparison to the original *Ity3* QTL, the peak LOD score of 4.8 has decreased slightly probably due to the increased detection of recombinants, however the region of interest still expands from *D1Mit135* to *D1Mit17*. B6.MOLF-*Ity/Ity3* congenics were intercrossed after 5 generations of backcrossing to B6.MOLF-*Ity* mice and consequently have a genetic background that is approximately 96.9% C57BL/6J-derived apart from the *Ity* and *Ity3* loci being transferred from MOLF/Ei. The MOLF/Ei region surrounding *Ity,* including *Slc11a1* was transferred because of the known effect of a wild-type allele at *Slc11a1* in the detection of *Ity3* (Sebastiani et al. 1998). A schematic representation of the chromosomes of B6.MOLF-*Ity/Ity3* mice is shown in Figure 1. We have generated both B6.MOLF-*Ity/Ity3* mice that are homozygous for the MOLF/Ei alleles and B6.MOLF-*Ity/Ity3*$^{MOLF/B6}$ mice that are heterozygous for the *Ity3* chromosomal region.

Ity3 Congenic Phenotype: To verify that the *Ity3* susceptibility phenotype was being successfully transferred to the B6.MOLF-*Ity/Ity3* congenics, both B6.MOLF-*Ity/Ity3* and B6.MOLF-*Ity/Ity3*$^{MOLF/B6}$ congenics, in addition to C57BL/6J mice that acted as infection controls, were infected intravenously with *Salmonella* Typhimurium (Figure 2A). B6.MOLF-*Ity/Ity3*$^{MOLF/B6}$ congenics were used in this experiment because B6.MOLF-*Ity* mice were unavailable at the time, however they should be phenotypically

identical to B6.MOLF-*Ity* mice since the *Ity3* locus is inherited in a recessive fashion (Sebastiani et al. 1998). As expected, C57BL/6J mice were extremely susceptible to infection and died within 5 days post inoculation (Vidal et al. 1995). The transfer of the MOLF/Ei *Ity* interval including the wild-type allele at *Slc11a11* into a C57BL/6J background, improved survival to infection, as seen with the B6.MOLF-*Ity/Ity3* strains. The survival analysis of these congenics demonstrate that the *Ity3* locus has also been successfully transferred since those mice that carry two copies of the "susceptible" MOLF/Ei allele, B6.MOLF-*Ity/Ity3*, succumb to infection significantly ($p<0.05$) earlier (MST = 8.4 ± 0.3 days) than those that carry only one MOLF/Ei allele at *Ity3*, B6.MOLF-*Ity/Ity3*$^{MOLF/B6}$ (MST = 10.6 ± 0.9 days).

The bacterial load in the spleens of C57BL/6J, 129S6/SvEvTac, B6.MOLF-*Ity/Ity3*, and B6.MOLF-*Ity/Ity3*$^{MOLF/B6}$ mice were assessed at 1, 3 and 5 days after infection (Figure 2B). C57BL/6J mice were found to have significantly higher CFUs in their spleens on day 3 and 5 (log 7-9 CFU/g) of infection, whereas 129S6/SvEvTac mice known to be extremely resistant to infection had relatively low CFU counts throughout infection (log 4-5 CFU/g). Both B6.MOLF-*Ity/Ity3* and B6.MOLF-*Ity/Ity3*$^{MOLF/B6}$ mice had intermediate splenic bacterial load ranging from log 4-6 CFU/g of tissue, similar to that observed previously in MOLF/Ei mice (Sebastiani et al. 2002). These results suggest that *Ity3* does not influence bacterial proliferation in MOLF/Ei and that the lower bacterial load in the spleen of B6.MOLF-*Ity/Ity3* mice compared to C57BL/6J could be explained by the presence of the MOLF/Ei *Ity* region (wild-type *Slc11a1*) (Vidal et al. 1995).

Ncf2 Sequence Analysis: Since we were able to verify that the *Ity3* QTL was impacting survival during *Salmonella* infection among our *Ity3* congenics, we sought to identify candidate genes within the *Ity3* interval (*D1Mit135* to *D1Mit17*) that may explain this effect. We considered the 84 Mb *Ity3* interval in its entirety, as it represents the 99% confidence interval where the candidate gene (s) are located. In this light, we looked at all genes that map within this region with potential relevance to *Salmonella* infection such as *Ptgs2, Ikbke* and *Tlr5*. All these genes were excluded either based on sequence analysis (*Ptgs2* and *Ikbke*) since no differences affecting the integrity of the proteins were observed within the coding regions or on a combination of expression and functional analyses (*Tlr5*) (Sancho-Shimizu et al. 2006). We focused on *Ncf2* based on its known function in related diseases in humans. The entire coding region of *Ncf2* was sequenced in both C57BL/6J and MOLF/Ei. A total of eight sequence variants were found, two in the non-coding regions of the gene, and six within the coding region (Figure 3A). The high rate of sequence variants found between C57BL/6J and MOLF/Ei is not surprising since these two strains of mice are separated by over 1 million yeas of evolution (Guenet and Bonhomme 2003). On average, there are about 6 SNPs/kb of coding sequence detected between these 2 strains of mice (V. Sancho and D. Malo, unpublished data). Of the 6 sequence variants identified in the coding region, only one, G1181A, resulted in a non-conservative amino acid change at codon 394 from an arginine to glutamine in MOLF/Ei mice (R394Q). To further determine the frequency and prevalence of the G1181A variant, DNAs from a diverse panel of 25 inbred strains of mice, were genotyped for this variant (Figure 4). The MOLF/Ei allele is present only in the *Mus musculus molossinus* family of mice (MOLF/Ei, MOLC/Rk, MOLD/Rk, MOLG/Dn, and MOLE/Rk), and in the European wild-derived strains Skive/Ei and CZECHII/Ei mice (*Mus musculus musculus*).

The R394Q variant is located within a highly conserved and functional domain of the protein known as the PB1 domain (phox Bemp1 domain) which lead us to investigate its potential impact on the integrity and function of the protein (Figure 3B).

Ncf2 Expression: The expression of *Ncf2* was analysed using real-time PCR on cDNAs of all uninfected and infected B6.MOLF-*Ity/Ity3*, B6.MOLF-*Ity/Ity3*$^{MOLF/B6}$, as well as MOLF/Ei and C57BL/6J cDNAs (Figure 5). MOLF/Ei mice have significantly reduced expression levels on day 3 post infection in comparison to the C57BL/6J mice (Figure 5A). Although not statistically significant, B6.MOLF-*Ity/Ity3* mice had generally lower transcript levels during infection than the B6.MOLF-*Ity/Ity3*$^{MOLF/B6}$ mice (Figure 5B), suggesting a trend of reduced expression in the B6.MOLF-*Ity/Ity3* congenics reflective of the MOLF/Ei allele.

Ncf2 Functional Assay: Given the facts that susceptible B6.MOLF-*Ity/Ity3* mice expressed lower levels of *Ncf2* mRNA during infection, and that the sequence variant G1181A resulted in a non-conservative amino acid change occurring in a functional and highly conserved domain of the protein, we tested the ability of the B6.MOLF-*Ity/Ity3*, B6.MOLF-*Ity/Ity3*$^{MOLF/B6}$ and B6.MOLF-*Ity* congenics that carry *Ncf2* MOLF/Ei, heterozygous and C57BL/6J alleles respectively, to produce superoxides in peritoneal derived macrophages. The macrophages were stimulated with IFN-γ for 16hrs prior to the experiment. Superoxide production was induced by the addition of 100ng/ml of PMA. The functional assay shows that at 30min and 60min after induction, C57BL/6J, B6.MOLF-*Ity/Ity3*$^{MOLF/B6}$, and B6.MOLF-*Ity* cells have significantly higher levels of superoxide induction, than the B6.MOLF-*Ity/Ity3* cells (MOLF/Ei allele) with only 9.8

fold and 8.8 fold induction levels at the respective time points (Figure 6A). Significantly reduced superoxide induction levels were consistently observed in the parent MOLF/Ei mice (data no shown).In fact the levels of induction are identical in C57BL/6J, B6.MOLF-*Ity*, and B6.MOLF-*Ity/Ity3*$^{MOLF/B6}$ cells, around 14 fold induction at 30min and 12 fold induction at 60min. These results demonstrate that B6.MOLF-*Ity/Ity3* mice have a less active (MOLF/Ei allele) *Ncf2* allele than B6.MOLF-*Ity/Ity3*$^{MOLF/B6}$, B6.MOLF-*Ity* and C57BL/6J mice carrying the C57BL/6J allele at *Ncf2*.

We carried out the same superoxide assay on peritoneal macrophages that have been infected with *Salmonella* Typhimurium *in vitro* to assess the relevance of this functional difference. Consistent with the PMA stimulated macrophages, we observed a consistent and statistically significant reduction in superoxide induction during *Salmonella* Typhimurium infection *in vitro*. The B6.MOLF-*Ity/Ity3* mice have a less active (MOLF/Ei allele) *Ncf2* allele than B6.MOLF-*Ity/Ity3*$^{MOLF/B6}$, B6.MOLF-*Ity* and C57BL/6J mice carrying the C57BL/6J allele at *Ncf2* (Figure 6B). The peak induction of superoxide occurred between 30 and 60 min after infection with *Salmonella* Typhimurium *in vitro*. The C57BL/6J mice had the highest induction levels of superoxide with a 4 fold increase, followed by B6.MOLF-*Ity/Ity3*$^{MOLF/B6}$ and B6.MOLF-*Ity*, with 1.6 and 2.2 fold respectively, and B6.MOLF-*Ity/Ity3* mice with only 1.2 fold increase over baseline.

DISCUSSION

We report in this study the successful transfer of the predicted susceptibility *Ity3* locus from MOLF/Ei mice to B6.MOLF-*Ity* mice by creating B6.MOLF-*Ity/Ity3* double congenic mice. The *Ity* locus, which maps over *Slc11a1* on chromosome 1, was transferred in addition to *Ity3* since the initial genome scan identified *Ity3* only in the presence of a wild type *Slc11a1* MOLF/Ei background (Sebastiani et al. 1998). The role of *Slc11a1*, formerly known as *Nramp1*, in host response to infection is well-characterized and is involved in suppressing the replication of various unrelated facultative intracellular pathogens such as *Salmonella* Typhimurium, *Leishmania donovani*, and *Mycobacterium bovis* BCG (Vidal et al. 1995). C57BL/6J mice are known to carry a single point mutation in this gene (G169R), rendering them extremely susceptible to infection with *Salmonella* Typhimurium (MST $\leq$ 5 days). MOLF/Ei mice carry the functional wild type allele for *Slc11a1,* which was transferred onto a C57BL/6J background in B6.MOLF-*Ity*, as well as in the B6.MOLF-*Ity/Ity3* mice. The initial genome scan results indicated that the *Ity3* locus was inherited in a recessive fashion suggesting that two copies of the MOLF/Ei *Ity3* region had to be inherited to have and impact on susceptibility to infection with *Salmonella* Typhimurium (Sebastiani et al. 1998).

Infection with *Salmonella* Typhimurium showed that B6.MOLF-*Ity/Ity3* mice are indeed more susceptible, succumbing to infection earlier than their B6.MOLF-*Ity/Ity3*$^{MOLF/B6}$ counterparts. The difference in survival between the C57BL/6J and B6.MOLF-*Ity/Ity3* mice can be attributed almost entirely due to the presence of *Ity*, which includes the wild type *Slc11a1* allele, whereas the difference in survival between

B6.MOLF-*Ity/Ity3* and B6.MOLF-*Ity/Ity3*$^{MOLF/B6}$ is specific to the *Ity3* locus. Interestingly, the bacterial load of B6.MOLF-*Ity/Ity3* and B6.MOLF-*Ity/Ity3*$^{MOLF/B6}$ mice was intermediate between those of resistant 129S6/SvEvTac and the highly susceptible C57BL/6J mice, similar to bacterial load observations made in the parental MOLF/Ei mice (Sebastiani et al. 2002).These results suggest that *Ity3* does not have an impact on the spleen bacterial load during infection and the difference observed between the congenic mice and the C57BL/6J mice is due to the effect of *Ity*, including *Slc11a1* which is known to affect intracellular survival and replication of *Salmonella* Typhimurium (Vidal et al. 1995).

Ncf2 was selected as a candidate gene for *Ity3* based on its physical map position and its known function in innate immunity. *Ncf2* encodes for p67^{phox} which is a subunit of the phagocyte NADPH oxidase. The function of the phagocyte NADPH oxidase in host defense is well established, as seen in CGD patients that present high susceptibility to bacterial and fungal infections (Heyworth et al. 2003). In human patients with CGD, mutations in the *NCF2* gene accounts for an estimated 5% of all CGD cases (Noack et al. 1999). The majority of the 17 documented human *NCF2* mutations results in no detectable superoxides production with the exception of one that was found to have reduced levels of superoxides (Noack et al. 1999).

The MOLF/Ei *Ncf2* allele carries a G to A transition at nucleotide 1181 resulting in a non-conservative amino acid change (R394Q) at position 394. This variant is a rare polymorphism and observed only in the *Mus musculus molossinus* strains as well as the European wild-derived inbred strains CZECHII/Ei and Skive/Ei, who share a common ancestor derived from the *Mus musculus* substrain (Beck et al. 2000). The R394Q variant was found to occur in a highly conserved and functional PB1 domain of *Ncf2*. This

domain, initially identified in the budding yeast protein Bem1p, was shown to mediate the formation of heterodimers between $p67^{phox}$ and $p40^{phox}$ (Ito et al. 2001) (Noda et al. 2003). The interaction between $p67^{phox}$ and $p40^{phox}$ enhances the membrane recruitment of $p67^{phox}$ and $p47^{phox}$ to the flavocytochrome b_{558} complex leading to the activation of the NADPH oxidase (Kuribayashi et al. 2002). It is possible that a substitution of a highly basic amino acid such as arginine with a polar glutamine could compromise the stability or activity of the protein.

This hypothesis is supported by the identification of a missense mutation in the corresponding amino acid (R395W) of the $p67^{phox}$ protein in two unrelated CGD patients (Noack et al. 1999; Patino et al. 1999). In addition, recombinant $p67^{phox}$ carrying the R395W mutation was found to have only 15% of normal activity in a cell-free NADPH oxidase assay, suggesting that in homozygous form, this mutation has a profound impact on the function of the protein (Patino et al. 1999). The effect of the R395W mutation on $p67^{phox}$-$p40^{phox}$ heterodimerization through PB1 domains was further demonstrated using GST pull down assays (Wilson et al. 2003). The efficiency of the heterodimerization between $p67^{phox}$ R395W PB1 and $p40^{phox}$ PB1 was reduced by about 50% compared to wild type $p67^{phox}$ (Wilson et al. 2003). The R394Q variant appears also to have an effect on the activity of phagocyte NADPH oxidase. Our functional assay indicates that the substitution of the arginine at position 394 for a glutamine results in lower PMA- and *Salmonella* infection - induced levels of superoxide. It may be possible that a glutamine residue at position 394 interferes with the dimerization with $p40^{phox}$ resulting in an overall reduction in the recruitment of the $p67^{phox}$ and $p47^{phox}$ components to the membrane complex, and in reduced production of superoxides. Lower *Ncf2* levels of expression may also participate in the decreased superoxide production observed in B6.MOLF-*Ity/Ity3*

mice. This notion is supported by complementation studies using recombinant human $p67^{phox}$ in neutrophils obtained from $p67^{phox}$-deficient CGD patients, which found that the level of $p67^{phox}$ protein expression to be the limiting factor in NADPH oxidase activation (Vergnaud et al. 2000).

However, the biological relevance of how the low expression levels of *Ncf2,* the R394Q substitution, and the reduced superoxide induction affects the host response during *Salmonella* infection still remains in question. In terms of the biological contribution, the reduction in superoxide induction is probably not impacting antimicrobial activity significantly in our model since there was no difference in spleen bacterial load observed in *Salmonella* infected B6.MOLF-*Ity/Ity3* and B6.MOLF-*Ity/Ity3*$^{MOLF/B6}$ mice, that harbor MOLF/Ei and heterozygous alleles respectively at *Ncf2*.In addition to its microbicidal activity, NADPH oxidase affects signaling capabilities of phagocytic cells. There is an emerging body of evidence indicating that reactive oxygen species (superoxide and H_2O_2) activate TLR4-dependent NF-κB gene transcription (Asehnoune et al. 2004; Park et al. 2004; Sadikot et al. 2004). The interaction of Nox4, a protein related to Nox2 ($gp91^{phox}$ of phagocytic cells), and Tlr4 triggers the production of ROS and leads to NF-κB activation in LPS stimulated monocytic cell lines (Park et al. 2004). Animals having deficient NADPH activity have impaired NF-κB activation and are more susceptible to infection with *Pseudomonas aeruginosa* (Sadikot et al. 2004). Additional evidence for interaction between TLR signaling and NADPH activity was provided recently by showing that MyD88 influences NADPH assembly and NAPDH oxidase-mediated production of superoxides (Laroux et al. 2005). The expression of several NF-κB dependent genes including *Il1*, *Il6* and *Nos2*

was shown to be down-regulated in MOLF/Ei mice compared to C57BL/6J mice during infection suggesting that the *Ity3* locus may have an impact on the expression of several pro-inflammatory genes (Sebastiani et al. 1998). To test the hypothesis that lower NADPH activity in MOLF/Ei mice may lead to improper NF-κB-dependent activation of several pro-inflammatory genes resulting in increased *Salmonella*-susceptibility of these animals, we have assayed for the presence of IL-1β, IL-1Ra, and IL-6 in the sera of infected B6.MOLF-*Ity/Ity3* and B6.MOLF-*Ity/Ity3*$^{MOLF/B6}$. We found no differences in cytokine levels between the two groups of congenic mice (data not shown) suggesting that the impaired superoxide induction in B6.MOLF-*Ity/Ity3* does not affect the expression of these cytokines. However, it was not possible to completely rule out a possible role of a defective superoxide response on improper NF-κB-activation using *in vivo* infection. Thus additional and comprehensive *in vitro* experiments will be necessary to resolve this issue.

Taken together, these findings add weight to the candidacy of *Ncf2* as the gene underlying *Ity3*, however the final proof of the candidacy of *Ncf2* will necessitate a gene-knockout interaction test (Flint et al. 2005). In addition, the resolution of the *Ity3* interval into smaller regions with the aid of recombinant sub-congenic mice will be instrumental in determining the extent of the involvement of *Ncf2* in this model of infection and the genetic architecture of the *Ity3* locus.

ACKNOWLEDGEMENTS

We thank Rosalie Wilkinson and Line Lariviere for their excellent technical assistance.

We thank Dr. Erwin Schurr, Amanda Rooyakers, Dr. Richard Stokes and Dr Albert Descoteaux for their helpful suggestions in the functional assay for superoxide detection.

FIGURES

Figure 1: LOD score plot for *Ity3* on chromosome 1. The physical position of the candidate gene *Ncf2* is shown as well as the chromosomes generated in the creation of the B6.MOLF-*Ity/Ity3* congenics. The black segments indicated the recipient C57BL/6J genotype, the white segments indicate the MOLF/Ei genotype, the hatched segments represent heterozygous intervals.

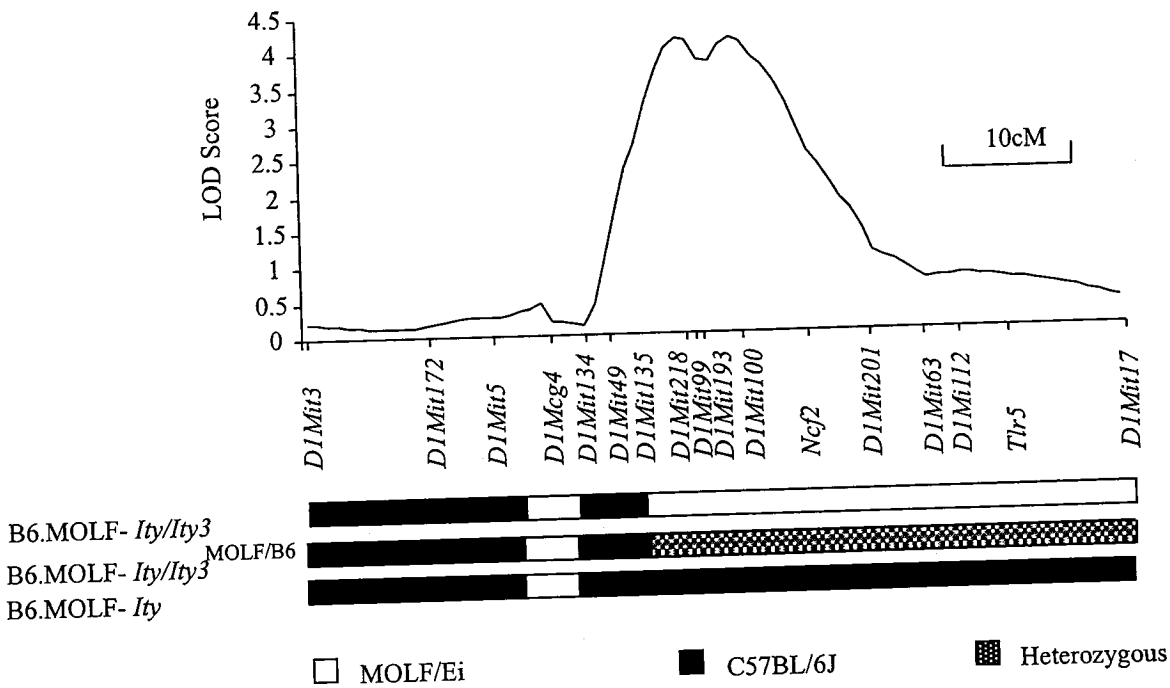
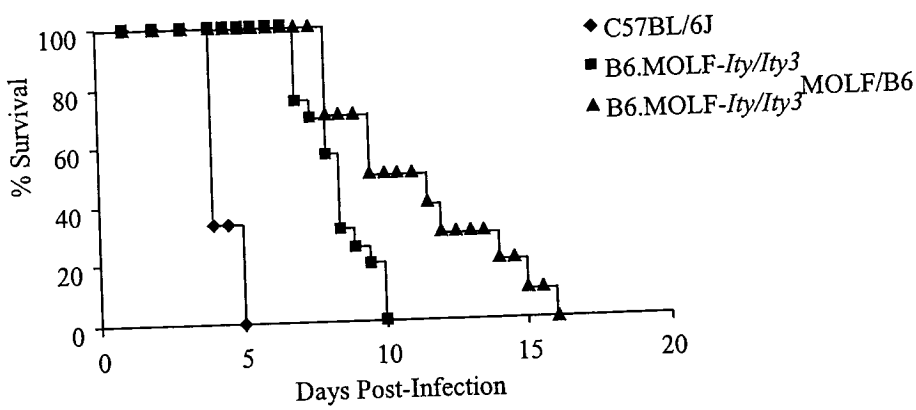

LOD Score
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
10cM
D1Mit3
D1Mit172
D1Mit5
D1Mcg4
D1Mit134
D1Mit49
D1Mit135
D1Mit218
D1Mit99
D1Mit193
D1Mit100
Ncf2
D1Mit201
D1Mit63
D1Mit112
Tlr5
D1Mit17
B6.MOLF- Ity/Ity3
MOLF/B6
B6.MOLF- Ity/Ity3
B6.MOLF- Ity
MOLF/Ei
C57BL/6J
Heterozygous

Figure 2: Survival analysis of *Ity3* congenic mice. Survival curves (A) of C57BL/6J, B6.MOLF-*Ity/Ity3*, and B6.MOLF-*Ity/Ity3*$^{MOLF/B6}$ congenic mice following intravenous challenge with 3,000 CFU of *Salmonella.* Typhimurium. The data are expressed as a percentage of total mice surviving the infection. Splenic bacterial load (B) of C57BL/6J, 129S6/SvEvTac, B6.MOLF-*Ity/Ity3*, and B6.MOLF-*Ity/Ity3*$^{MOLF/B6}$ expressed in log(CFU/g) of tissue.

A


C57BL/6J
B6.MOLF-Ity/Ity3MOLF/B6
B6.MOLF-Ity/Ity3
% Survival
100
80
60
40
20
0
0
5
10
15
20
Days Post-Infection

B

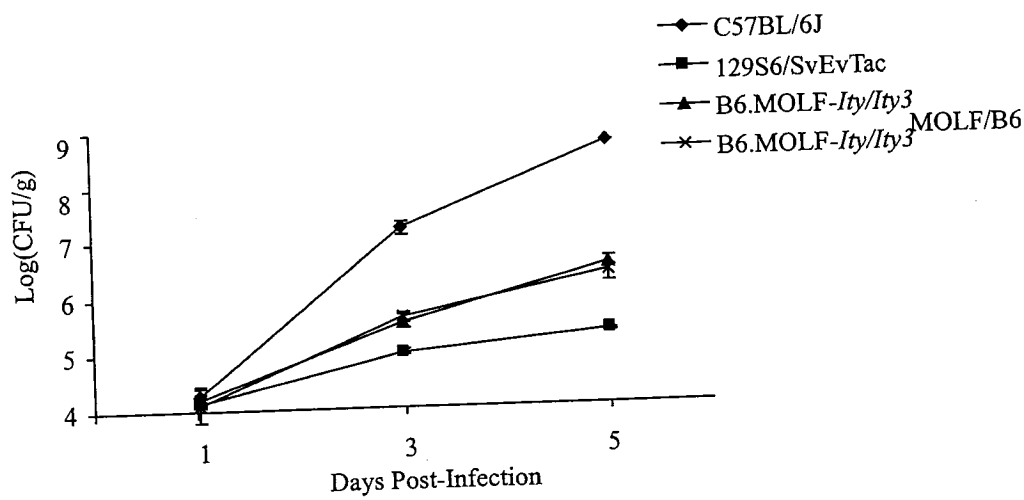

C57BL/6J
129S6/SvEvTac
B6.MOLF-Ity/Ity3
B6.MOLF-Ity/Ity3MOLF/B6
Log(CFU/g)
9
8
7
6
5
4
1
3
5
Days Post-Infection

Figure 3: *Ncf2* sequence analysis. Nucleotide sequence variants (A) between C57BL/6J and MOLF/Ei mice. The bolded letters indicate the SNP variants. Mouse NCF2 protein domain structure (B) showing the position of the non-conservative mutation occurring in the PB1 domain. Domains known to interact with other phox proteins are indicated by an arrow. TPR, tricopeptide repeat domain; SH3, SRC-homology 3 domain;PB1, Phox and Bem1p domain.

A

Nucleotide	C57BL/6J	MOLF/Ei	Amino Acid
5'UTR	-	G	-
72	GG**T**	GG**G**	^{24}Gly
666	CC**A**	CC**G**	^{222}Pro
945	TC**C**	TC**T**	^{315}Ser
1167	AC**T**	AC**C**	^{389}Thr
1181	C**G**G	C**A**G	^{394}Arg Glu
1215	TC**C**	TC**G**	^{405}Ser
3'UTR	**T**	**G**	-

B

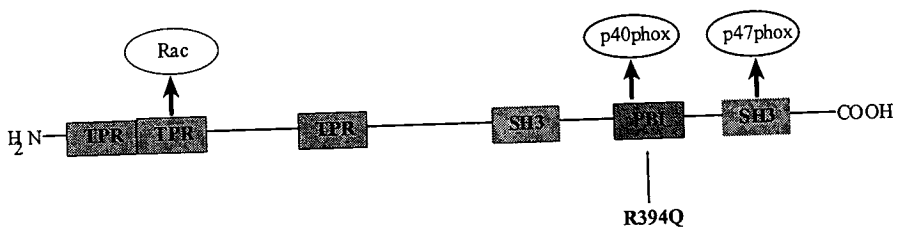
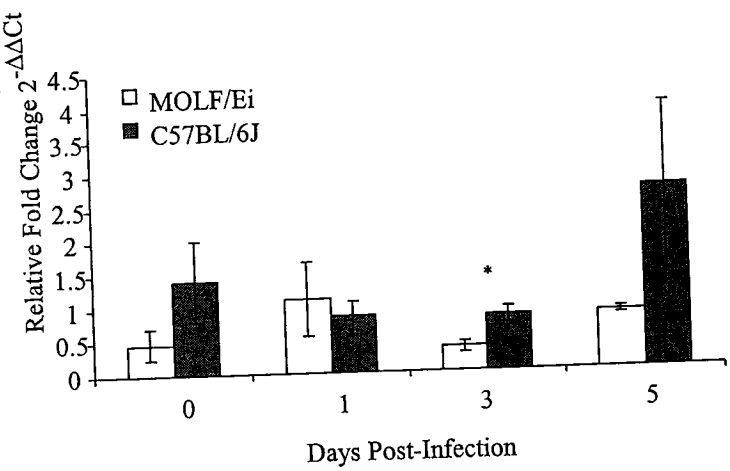


Figure 4: Distribution and frequency of *Ncf2* G1181A sequence variant. Twenty-seven inbred mouse strains were genotyped for the *Ncf2* G1181A sequence variant corresponding to the non-conservative amino acid change. Mice are grouped according to their origin. G represents the C57BL/6J allele and A represents the MOLF/Ei allele.

Origin	Mouse Strain	Nucleotide Allele
Castle's Inbred Strains	129/J	G
	C3H/HeJ	G
	C3H/HeSnJ	G
	C57BL/6J	G
	C57Br/cdJ	G
	C57L/J	G
	DBA/2J	G
	RF/J	G
	P/J	G
	CE/J	G
	BuB/BnJ	G
American Wild Derived Strains	PERA/Rk	G
	PERC/Ei	G
	SF/CamEi	G
European Wild Derived Strains	M. Spretus	G
	RBA/Dn	G
	RBB/Dn	G
	Sk/CamEi	G
	ZALENDE/Ei	G
	TIRANO/Ei	G
	Skive/Ei	A
	CZECHII/Ei	A
Asian Wild Derived Strains	MOLF/Ei	A
	MOLC/Rk	A
	MOLD/Rk	A
	MOLG/Dn	A
	MOLE/Rk	A

Figure 5: Regulation of *Ncf2* expression upon *Salmonella* infection in the spleens of (A) C57BL/6J, MOLF/Ei, and (B) B6.MOLF-*Ity/Ity3* ($Ncf2^{MOLF/MOLF}$), B6.MOLF-*Ity/Ity3*$^{MOLF/B6}$ ($Ncf2^{MOLF/B6}$) mice. Expression was evaluated by real-time PCR using C57BL/6J (A) or B6.MOLF-*Ity/Ity3*$^{MOLF/B6}$ ($Ncf2^{MOLF/B6}$) (B) uninfected cDNA as the referent. All values are expressed as $2^{-\Delta\Delta Ct}$ units, where the referent is set to 1, and the house keeping gene used was *18srRNA*. Statistical significance was assessed using the student's t-test and is indicated by an asterisk ($p<0.05$).

A


Relative Fold Change $2^{-\Delta\Delta Ct}$
MOLF/Ei
C57BL/6J
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
*
0
1
3
5
Days Post-Infection

B

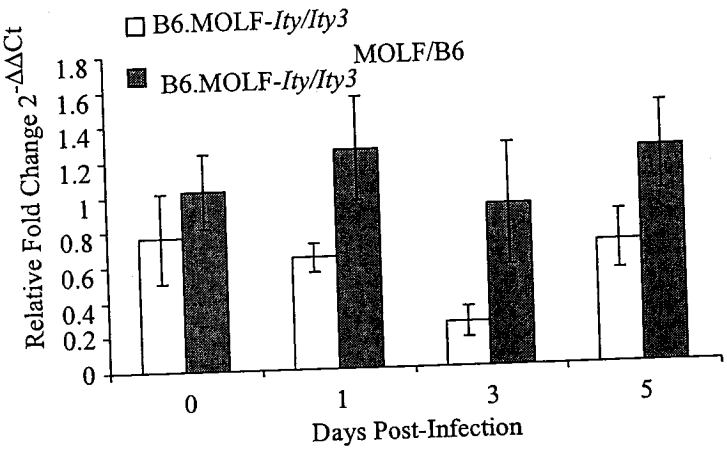
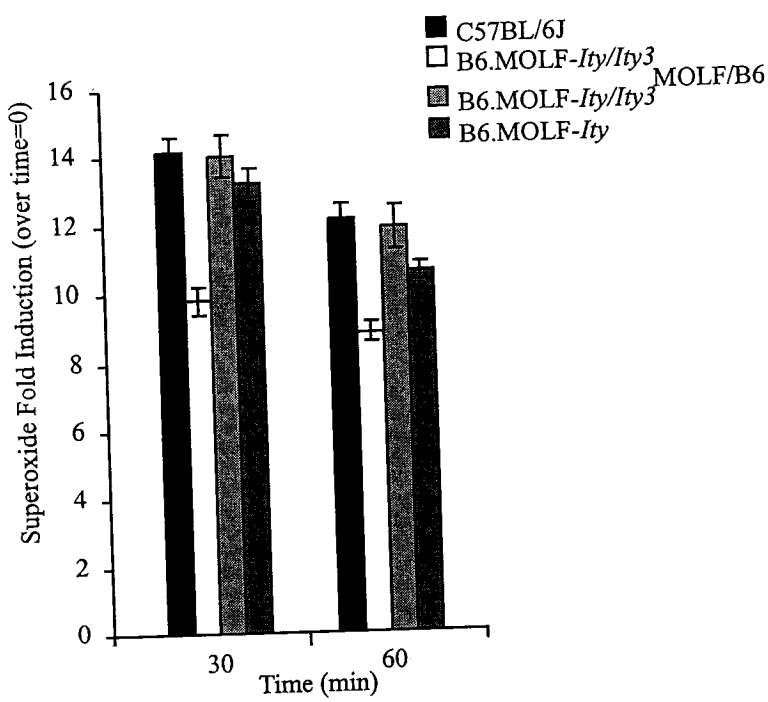
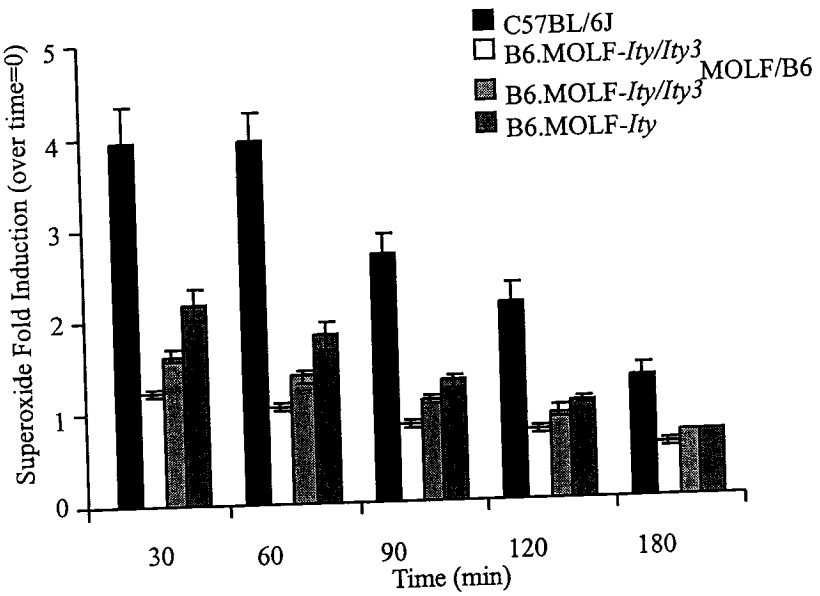

B6.MOLF-*Ity/Ity3*
MOLF/B6
B6.MOLF-*Ity/Ity3*
Relative Fold Change $2^{-\Delta\Delta Ct}$
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
1
3
5
Days Post-Infection

Figure 6: Functional assay for *Ncf2* variants. Peritoneal-derived macrophages were isolated from C57BL/6J, B6.MOLF-*Ity /Ity3* ($Ncf2^{MOLF/MOLF}$), B6.MOLF-*Ity/Ity3*$^{MOLF/B6}$ ($Ncf2^{MOLF/B6}$), and B6.MOLF-*Ity* ($Ncf2^{B6/B6}$). Macrophages were then either stimulated with 20u/ml IFN-γ and 100ng/ml PMA (A) or infected with *Salmonella* Typhimurium at a multiplicity of infection of 1:1 (B). Data are shown as fold increase in superoxide chemiluminescence over the base line at time 0. These results represent one of four (A) or one of five (B) similar assays carried out on these mice. Statistical significance was assessed using the student's t-test.

A


C57BL/6J
B6.MOLF-Ity/Ity3MOLF/B6
B6.MOLF-Ity/Ity3
B6.MOLF-Ity
Superoxide Fold Induction (over time=0)
0
2
4
6
8
10
12
14
16
30
60
Time (min)

B


C57BL/6J
B6.MOLF-Ity/Ity3MOLF/B6
B6.MOLF-Ity/Ity3
B6.MOLF-Ity
Superoxide Fold Induction (over time=0)
0
1
2
3
4
5
30
60
90
120
180
Time (min)

CHAPTER 5:

DISCUSSION

5.1 Overview

The advances made in the treatment of infectious disease over the past century have revolutionized human medicine. From the first isolation of "*Salmonella typhosa*" by Eberth in 1880, to the antibiotic treatments and vaccines available now, a substantial body of knowledge has been acquired about enteric fever (Eberth 1880; Mastroeni 2006). Although cases of typhoid fever are rarely encountered in the industrialized nations, it re-surfaces as a serious health concern when disasters strikes, and through the emergence of multidrug resistant forms (Threlfall et al. 2003; Kariuki et al. 2004; Mastroeni 2006). A considerable amount of knowledge has been gained from the use of mouse models of infection. Major genes affecting the host response to *Salmonella* infection have been identified using naturally occurring variants in mice, such as *Slc11a1* and *Tlr4* (Vidal et al. 1993; Poltorak et al. 1998; Qureshi et al. 1999). To further exploit the existence of other populations of inbred mice that may carry distinct genetic variants from that which has been largely studied, the wild-derived inbred mouse MOLF/Ei mice was herein used to identify new genetic determinants in a systemic *Salmonella* Typhimurium infection. The highly susceptible MOLF/Ei was found to contribute a resistance locus *Ity2* and a susceptibility locus *Ity3* in a (C57BL/6J X MOLF/Ei)F2 population.

5.2 Utility of Congenics in QTL Validation and Transcriptional Profiling

In Chapter 2, the previously identified QTLs, *Ity2* and *Ity3* were fine mapped using congenic mice and a prioritized list of candidates generated with the aid of expression profiling. Although some of the expression results were confounded by high

sequence variation as demonstrated by the *Slfn* family of genes in the *Ity2* congenic mice, data generated from expression profiling experiments were able to provide us with a list of target genes that are differentially regulated in the congenic mice, possibly contributing to the phenotype underlying the QTL. A study characterizing the susceptibility of wild trapped *Drosophila* to Gram negative bacterial infection, reported that most of the significant associations identified between sequence variants and susceptibility, tend to occur upstream of genes in non-coding regions either suggesting linkage disequilibrium with the causative allele in coding regions or signifying regulatory changes as the causative mechanism (Lazzaro et al. 2004). This points towards an important role of transcriptional regulation in the control of complex traits, justifying our approach. Further evidence for this is apparent in the number of other studies that have employed this approach in murine/rodent complex trait models (Rozzo et al. 2001; McBride et al. 2003; Johannesson et al. 2005; de Buhr et al. 2006).

Nevertheless, most SNPs that have been associated with a complex trait have been identified in coding regions (Glazier et al. 2002). We have not neglected the impact of coding variants that may affect *Ity2* or *Ity3*, however our efforts to compare sequences between MOLF/Ei and C57BL/6J were unrewarding in most cases, due to numerous sequence variants that were identified, none of which would potentially result in an obvious impairment of protein function (Sancho Shimizu V., Sebastiani, G. and Malo D. unpublished). In support of this notion, Thomas and Kejariwal have examined differences in coding SNPs underlying Mendelian versus complex traits and have found that unlike Mendelian trait associated coding SNPs that more often occur on highly conserved amino acids impacting the integrity of the protein, complex trait coding SNPs do not, and therefore may have more subtle effects (Thomas and Kejariwal 2004). Thus, the

observation of such "neutral" sequence variants is not uncommon in the study of complex traits hindering the identification of quantitative trait genes. In our intersubspecific cross this was likely further complicated by the fact that there are 10 fold greater number of sequence variants than an interspecific cross, making the distinction between causative and non-causative variants even more difficult (Rogner and Avner 2003).

Congenics have widely been used to validate and fine map QTLs with success (Mott 2006). Although one of the limitations of the use of congenics is that it may be a time consuming process, requiring up to 3 years to develop, it provides us with the only means of observing the impact of a QTL in isolation on a standardized genetic background. We have successfully used this technique to confirm the contribution of *Ity2* and *Ity3* in resistance and susceptibility to *Salmonella* infections. This has also enabled us to reduce the *Ity2* and *Ity3* intervals, revealing in both cases evidence for the involvement of more than one gene. In *Ity2*, there are clearly two critical intervals that are both necessary for resistance. The *Ity3* interval represented in B6.MOLF-*Ity/Ity3* mice were more susceptible than those that carry smaller intervals of *Ity3* (*RecA, RecB, RecE*) and were still more susceptible than B6.MOLF-*Ity* mice, suggestive of the participation of more than one gene. The refinement of a QTL leading to the discovery of sub-QTLs is frequent in the literature, lending to a more complex genetic architecture underlying the QTL that becomes apparent upon fine mapping of the locus (Flint and Mott 2001; Mott 2006).

5.3 Elimination of *Tlr5* as Candidate for *Ity3*

In Chapter 3, we set out to functionally characterize the MOLF/Ei and C57BL/6J alleles of *Tlr5*. Given the numerous sequence variants identified previously within both the coding and non-coding regions of the gene, as well as the reduced transcript levels in the livers of infected MOLF/Ei mice, functional analysis *in vivo* and *in vitro* were carried out (Sebastiani et al. 2000; Sancho-Shimizu et al. 2006). *In vitro* analysis of the coding sequence variants revealed that there was no difference in the capability of either allele to activate the CAT reporter gene fused to an IL-8 promoter fragment, which was shown to be NF-κB dependent and a downstream target of TLR signaling. Promoter analysis revealed the MOLF/Ei allele to be more active than that of C57BL/6J, contrary to previous findings (Sebastiani et al. 2000). This *in vitro* analysis however was carried out in a heterologous system, in which a plasmid containing the respective mouse *Tlr5* alleles were transfected into human cell lines, 293T and HeLa cells, possibly accounting for the observed differences. Hence, to further evaluate the biological significance of the gene in our model, C57BL/6J, MOLF/Ei, B6.MOLF-*Ity/Ity3*, B6.MOLF-*Ity/Ity3*$^{B6/MOLF}$, and B6.MOLF-*Ity* congenic mice (bearing homozygous MOLF/Ei, heterozygous and homozygous C57BL/6J alleles respectively) were tested for their ability to respond to flagellin stimulation. The MOLF/Ei allele had very low levels of serum cytokine production as a result of intraperitoneal flagellin stimulation, consistent with previous observations of *Tlr5* transcript levels in the liver. To our surprise, B6.MOLF-*Ity/Ity3* mice, sharing alleles at *Tlr5* with MOLF/Ei mice, had the most pronounced response to flagellin suggesting the existence of other factors in the rest of the genome, which influences response to flagellin. These results seem to suggest that the introduction of the MOLF/Ei

Tlr5 allele into a different genetic background, either in B6.MOLF-*Ity/Ity3* mice or in human epithelial 293T cells, results in upregulation of *Tlr5* activity, indicating the presence of a key repressor in the natural context of the MOLF/Ei genome. Subsequently, *Tlr5* was eliminated as a candidate for *Ity3* as the congenics did not recapitulate the phenotype observed in the parental MOLF/Ei mouse.

These results do not necessarily preclude the involvement of *Tlr5* during infection in MOLF/Ei mice, although they strongly suggest that *Tlr5* is not the genetic determinant underlying the *Ity3* locus. Recent studies have supported the notion that *Tlr5* may not be primarily involved during systemic infection with *Salmonella* Typhimurium, as intraperitoneal infection of *Salmonella* Typhimurium in *Tlr5* knockout mice, had no impact on disease outcome (Feuillet et al. 2006). Moreover, the authors suggest that *Tlr5* is involved in the detection of flagellin during infection through a synergism that exists between *Tlr4* and *Tlr5* as demonstrated by the enhanced susceptibility of *Tlr4/Tlr5* knockout mice to *Salmonella* Typhimurium infection. The lack of effect observed in the absence of *Tlr5* may be due to this redundant mechanism, either through *Tlr4* or possibly other pathways as well, that have evolved in the host. This synergy was also observed in intranasal infection with *Pseudomonas aeruginosa*, such that only *Tlr4/Tlr5* knockouts succumbed to infection more rapidly than wild type or single knockouts of either gene (Feuillet et al. 2006). In another study, Uematsu and colleagues have found *Tlr5* to be most abundantly expressed on intestinal $CD11c^{+}$ lamina propria cells, and upon evaluation of *Tlr5* knockout mice, found them to be more resistant than wild type controls to oral *Salmonella* Typhimurium infection. This unexpected result was due to the specific impairment in the transport of bacteria from the intestine to the mesenteric lymph nodes in *Tlr5* knockout mice, preventing dissemination of bacteria to the spleen and liver,

suggesting a possible new *Salmonella* virulence mechanism (Uematsu et al. 2006). Thus, these studies together suggest a more important role for *Tlr5* in mucosal immunity.

Despite being eliminated as a *Salmonella* susceptibility candidate for *Ity3*, the impact of *Tlr5* and its role in MOLF/Ei mice may warrant further analysis. Undoubtedly, MOLF/Ei possess other factors affecting *Tlr5* regulation, that are distinct from the other strains C57BL/6J and 129S6/SvEvTac, as demonstrated by reduced *Tlr5* transcript levels in the liver and diminished response to intraperitoneal flagellin (Sebastiani et al. 2000; Sancho 2006). It may be interesting to investigate what other factors are involved in the differential regulation of this gene by mapping the genetic modifiers of *Tlr5* using the established B6.MOLF-*Ity/Ity3* congenic strains. A genome scan of an F2 cross using the *Ity3* congenics and MOLF/Ei mice can be carried out, using flagellin response as a phenotype, probing for the existence of other loci affecting *Tlr5* response. The discovery of *Tlr5* regulators or interacting partners would contribute to our understanding of its role in mucosal immunity.

Furthermore, a more comprehensive characterization of TLR5 in MOLF/Ei mice could be undertaken. We have shown that *Tlr5* expression in uninfected and infected MOLF/Ei livers is lower than other strains, and that the intraperitoneal injection of flagellin does not induce serum cytokine production in these mice (Sebastiani et al. 2000; Sancho 2006). The hypothesis that MOLF/Ei mice are "naturally TLR5 deficient" may be further pursued by investigating TLR5 function in $CD11c^+$ lamina proprial cells as well as in the investigation of TLR4/TLR5 synergy discovered through systemic *Salmonella* Typhimurium and intranasal *Pseudomonas aeruginosa* infections in *Tlr4/Tlr5* knockout mice (Feuillet et al. 2006; Uematsu et al. 2006). The presence or absence of TLR5 protein levels would greatly facilitate the acceptance or rejection of this hypothesis although

TLR5 specific antibodies are currently unavailable. If they are indeed naturally deficient in TLR5, MOLF/Ei mice may be used to address *Tlr5* specific questions pertinent to mucosal immunity such as oral *Salmonella* infections as well as in other intestinal disorders including intestinal bowel disease or Crohn's disease, and possibly in lung infections with *Pseudomonas aeruginosa* (Lodes et al. 2004; Sitaraman et al. 2005; Feuillet et al. 2006) . However, we have observed cytokine production in response to flagellin in MOLF/Ei bone marrow derived macrophages (Appendix I Figure 4) suggesting that they may not necessarily be completely TLR5 deficient.

5.4 *Ncf2* as a Candidate Gene for *Ity3*

In Chapter 4, we assessed the candidacy of another gene underlying the *Ity3* locus, *Ncf2*, based on its map location and known role during infection with *Salmonella* Typhimurium (Mastroeni et al. 2000; van Diepen et al. 2002; Sancho-Shimizu and Malo 2006). As one of the NADPH oxidase subunits p67phox, encoded by *Ncf2,* is essential in the proper functioning of the enzyme, leading to the production of various microbicidal ROIs (Vignais 2002). Upon sequence analysis, one nonsynonymous sequence variant was identified in a highly conserved and functional domain of the protein present only in the *Mus musculus molossinus* strains as well as in the European wild-derived inbred strains Skive/Ei and CZECHII/Ei, leading us to believe that it may impact the function of the protein. *Ncf2* expression in the spleen of infected C57BL/6J and MOLF/Ei mice during expression did not differ significantly except for a significant reduction at day 3, and although not significant, there was a tendency towards lower expression in B6.MOLF-*Ity/Ity3* mice. Functional analysis of this sequence variant was investigated using an *ex*

vivo assay for the production of superoxide anions from peritoneal macrophages isolated from C57BL/6J, B6.MOLF-*Ity*, B6.MOLF-*Ity/Ity3*$^{MOLF/B6}$, B6.MOLF-*Ity/Ity3* mice. The results of the assay revealed a significant reduction of superoxide anion production exclusive to B6.MOLF-*Ity/Ity3* mice bearing the MOLF/Ei variant at *Ncf2* upon chemical induction, and during *Salmonella* Typhimurium infection *in vitro*. Furthermore, the existence of a mutation in the corresponding amino acid in human CGD patients makes this particular variant an attractive candidate (Noack et al. 1999; Patino et al. 1999). The biological relevance of this modest yet significant reduction in superoxide production remains elusive since we do not observe increased bacterial loads in the spleens of these mice. ROI's, specifically hydrogen peroxide and hydroxyl anions, are known to influence signaling capabilities via NF-κB, which in turn regulates cytokines and chemokines involved in inflammation, immunity, cell proliferation and apoptosis, suggesting a potential mechanism at work in our model (Gloire et al. 2006). The impact on NF-κB signaling can be examined in the *Ity3* mice to see if it has ramifications in some aspect of the diverse functions related to NF-κB function.

In order to provide additional evidence for the involvement of *Ncf2* in our model, molecular characterization of the R394Q variant, within the PB1 domain can be explored. P67^{phox} is found bound to p40^{phox} and p47^{phox} in the cytosol at resting state, and only upon activation will it translocate to the membrane where it forms an active enzyme complex with the other subunits. Interactions of p67^{phox} with p40^{phox} is mediated by the PB1 domain and has been shown to enhance recruitment of the cytosolic complex, p67phox and p40^{phox}, to the membrane (Kuribayashi et al. 2002). Wilson et al have investigated the role of the human mutation R395W in p67^{phox} and demonstrated its reduced capacity to heterodimerize with its partner, p40^{phox} in a cell free assay (Wilson et al. 2003).

Impairment in $p67^{phox}$ - $p40^{phox}$ heterodimerization can lead to reduced translocation and activation of the NADPH oxidase enzyme, and reduced superoxide production. A similar experiment using the R394Q variant may elucidate the molecular defect of the variant. To investigate whether the translocation and proper assembly is affected, co-localization of $p67^{phox}$ with the membrane subunit $gp91^{phox}$ using confocal microscopy may be carried out to further validate this hypothesis, as was recently described in a model of *Leishmania donovani* infection (Lodge et al. 2006).

It may also be interesting to see whether the other strains that carry this non-conservative SNP at *Ncf2*, CZECHII/Ei, SKIVE/Ei, MOLD/Rk, MOLG/DnJ (MOLC/Rk and MOLE/Rk are extinct) have similar superoxide induction in response to PMA and *in vitro Salmonella* Typhimurium infection. Moreover, their survival to systemic *Salmonella* Typhimurium infection would be of particular interest, especially in MOLD/Rk and MOLG/Dn mice as they are very closely related to MOLF/Ei mice, however since multiple genes are known to affect survival (*Slc11a1, Tlr4, Btk*, etc...) this may complicate the interpretation of the results.

5.5 Limitations and Implications of Study

5.5.1 From QTL to Quantitative Trait Gene Identification

Together from this thesis we have validated and identified critical intervals of *Ity2* and *Ity3* that contribute to *Salmonella* response in MOLF/Ei mice, highlighted candidates based on their expression difference, and assessed the candidacy of *Tlr5*, and *Ncf2* as genes underlying *Ity3* (Sancho-Shimizu et al. 2006; Sancho-Shimizu and Malo 2006). From this, we are left with a picture in which *Ncf2* emerges as an excellent candidate for

the *Salmonella* susceptibility locus on distal chromosome 1, *Ity3*, as it is within its critical interval. Evidence for two critical intervals underlying *Ity2*, and the recombinant congenic-specific differential susceptibility in *Ity3* congenics, both suggest the involvement of more than one gene. Although the road to actual gene identification is still far, progress towards this end has been demonstrated in this dissertation. A significant limitation of the QTL mapping approach lies in the identification of the causative gene, well demonstrated by the very few genes, under 1% of all QTLs, that have been successfully identified (Flint et al. 2005). There are several documented disadvantages to QTL mapping that makes gene identification challenging. QTL mapping is a relatively coarse mapping strategy, and once at the point of fine mapping, a different picture emerges. This is primarily due to the fact that QTLs map a "genetic effect" and not necessarily a gene. The "genetic effect" may be one but can be due to multiple genes underlying the QTL interval, revealing a hidden complexity of the QTL genetic architecture (Darvasi and Soller 1997; Flint and Mott 2001). Epistatic interactions between loci also contribute to this complexity, which is not well represented in QTL mapping studies complicating the interpretation of results obtained from fine mapping (Flint and Mott 2001; Carlborg and Haley 2004). Hence, the difficulties encountered are not unique to this particular study per se but is a point of concern in most QTL mapping studies.

The use of two susceptible parental strains C57BL/6J and MOLF/Ei in the initial F2 cross has revealed the presence of *Ity2* and *Ity3*, of which only the latter was found to contribute to susceptibility. The phenotypic variance that *Ity3* accounted for was 7% in this highly susceptible mouse and we have been able to demonstrate its effect through the construction of B6.MOLF-*Ity/Ity3* mice. It would be naïve to assume that the extreme

susceptibility that MOLF/Ei display is due to this one locus, and thus there must be the existence of other susceptibility loci in MOLF/Ei that was probably masked due to the segregation of a major gene affecting *Salmonella* survival, *Slc11a1*, accounting for 37% of the phenotypic variance. The answer may lie in the suggestive QTLs identified in the initial study, in which 2 other MOLF/Ei derived loci on chromosome 10 and 13 were found to contribute to susceptibility (Sebastiani et al. 1998). Although, this cross was initiated with the aim of identifying modifiers of *Slc11a1*, no significant interaction was identified with *Ity2* and was undetermined with *Ity3* due to small sample size (Sebastiani et al. 1998). A complete analysis of epistasis between all loci using available statistical software, will be informative to gain a better understanding of the mode of action of the loci involved (Chase 1997; Broman et al. 2003). A genome scan using a fully informative cross such as the (129S6 x MOLF/Ei)F2, would possibly reveal more susceptibility loci. The confirmation of the MOLF/Ei-derived resistant *Ity2* locus in the (129S6 x MOLF/Ei)F2 provides us with a glimpse into the highly complex genetic architecture of MOLF/Ei immune response, since the highly susceptible MOLF/Ei has a strong positive influence on survival over and above the resistant 129S6 allele. In addition, the inherent complexity of the immune response as measured by survival is clearly demonstrated by the heterosis observed in the (MOLF/Ei X C57BL/6J)F1 hybrids, and in the identification of both resistance and susceptibility loci in the two F2 populations tested, contributed by the highly susceptible MOLF/Ei.

5.5.2 The Use of Wild-derived Mice

The advantage of using wild-derived inbred strains of mice lies primarily in their genetic diversity as compared to classical inbred strains of mice. The one million years of evolution and geographical separation between the two strains have allowed them to accumulate sequence variants on the order of 1 every 100bp as opposed to 1 every 1000bp among classical inbred strains (Wade et al. 2002; Ideraabdullah et al. 2004). This high sequence variability is comparable to the difference between chimps and humans, a significant divergence in evolutionary history. As most *Salmonella* susceptibility loci have been identified using the more commonly used, classical inbred strains of mice such as C57BL/6J, C3H/HeJ, 129S6/SvEvTac, A/J among many others, the use of wild-derived inbred strains was carried out with the notion that they would harbor novel genetic variants not captured in the classical inbred strains.

5.5.2.1 Breeding and Handling Difficulties

MOLF/Ei mice are inbred strains however they have only been maintained in captivity for 36-44 generations (October 6 2005 update Jax®Mice Database; http://jaxmice.jax.org/strain/000550.html) as compared to C57BL/6J mice that have been inbred for over 226 generations (November 16 2005 update Jax®Mice Database; http://jaxmice.jax.org/strain/000664.html), making breeding and handling of these "characteristically hyperactive" mice challenging at best. Breeding with MOLF/Ei mice can be frustrating as many breeding pairs are unsuccessful, and those that produced progeny have relatively small litter sizes (Sancho-Shimizu V. and Malo D. unpublished). Thus, breeding can be extremely laborious and time consuming, as was the experience in

the generation of the *Ity2* and *Ity3* congenic strains in this study. The generation of congenics using 10 backcross generations, typically takes 3yrs, and although this was more or less achieved for the *Ity2* congenics, the *Ity3* congenics took 4yrs to establish due to breeding difficulty. In fact, one year and a half was spent on the unsuccessful attempt at the initial *Ity3* congenic cross in which progeny were backcrossed to MOLF/Ei mice so that the "resistant" *Ity3* locus derived from C57BL/6J may be introduced into the MOLF/Ei background. To complicate matters, cases of hydrocephaly characterized by the appearance of domed skulls due to a build up of the cerebrospinal fluid in the ventricles of the brain causing cerebral atrophy and death, appeared in all breeding pairs (Crews et al. 2004). In most cases, the severity of the hydrocephaly peaked at 4-8 weeks at which point the animal had to be sacrificed. According to the Jackson Laboratories website (http://jaxmice.jax.org/library/notes/490f.html) hydrocephaly rates in C57BL/6J is 0.029%, however rates were significantly higher in the *Ity2* congenics. The *Ity2* congenic although bred relatively well initially, encountered a population bottleneck where breeders stopped producing litters, at which point the incidence of hydrocephaly increased up to 25%, bringing about the demise of the B6.MOLF-*Ity/Ity2* and B6.MOLF-*Ity/Ity2.RecD* congenics. Thus the two most relevant *Ity2* congenics used in our study in Chapter 2 to restrict this resistance QTL on chromosome 11, have gone extinct, making it difficult to further identify the functional roles of candidates within this region. This may have occurred due to incompatibilities in this particular region between the two genomes. Evidence for this comes from another group that reportedly had problems in generating chromosome 11 consomics using a European wild-derived strain, PWD/Ph on a C57BL/6J recipient background (Forejt J, Complex Trait Consortium 2005 personal communication). However, as the same QTL was discovered in the (129S6 x

MOLF/Ei)F2 cross, congenics may be reconstructed with 129 mice employing the speed congenic approach to save time (Wakeland et al. 1997). Although a great reservoir for novel genetic variants that may affect disease outcome, the practical use of MOLF/Ei mice, and wild-derived inbred strains in general is a limitation to keep in mind.

5.5.2.2 The Genetic Diversity

The genetic diversity was the primary reason that MOLF/Ei were used to identify genes underlying susceptibility to *Salmonella*. This is particularly problematic in an intersubspecific cross that we have undertaken, due to 10 fold greater number of sequence variants, making the distinction between causative and non-causative variants difficult (Rogner and Avner 2003). However, through the course of investigation it has become increasingly clear that these two strains may represent two genomes that may be too divergent for this type of comparison herein employed. The trans effects or genetic background effects of MOLF/Ei seem to play a significant role in the expression and manifestation of phenotypes as illustrated by our observation of *Tlr5* expression and function observed in MOLF/Ei versus B6.MOLF-*Ity/Ity3* mice. This suggests that the C57BL/6J genetic background or other contributing genes are significantly different from that of MOLF/Ei when it comes to the regulation of this particular gene. In support of this, we were unable to recapitulate the parental phenotype even through the generation of triple congenic mice carrying all three QTLs on a C57BL/6J background (Sancho-Shimizu V. and Malo D. unpublished) indicating that other factors in the MOLF/Ei genome are necessary for the complete manifestation of the phenotype. The failure to reconstitute the parental phenotype in a triple congenic is not too surprising as the genetic analysis of the (C57BL/6J X MOLF/Ei)F2 cross predicted that all three QTLs account for

55% of the phenotypic variance, however other studies have been able to accomplish this as was the case in systemic lupus erythematosus susceptibility (Morel et al. 1997; Sebastiani et al. 1998; Morel et al. 2000). It should be noted that these loci were identified in the genetically heterogeneous F2 population, such that loci identified in such a genomic context may not have the same effect as in the natural setting of the parental genome, possibly with the exception of major gene effects. This phenomenon has been elegantly demonstrated in the recent identification of the genes underlying high temperature growth QTLs between different strains of *Saccharomyces cerevisiae* (Sinha et al. 2006). In this study, the genes identified to regulate high temperature growth through an intercross, did not have the same predicted effect in different genetic backgrounds, including the original parental strains, highlighting the importance of genetic background on the phenotype as well as the complex nature of quantitative studies (Sinha et al. 2006). Thus the current QTL mapping approach employed herein undermines the impact of genetic background or is otherwise not sensitive enough to detect the numerous other modest effects due to genetic interactions necessary for the manifestation of a particular phenotype.

Genes rarely work in isolation. Genes are usually part of a network or pathway whose proper coordinated interaction leads to the manifestation of the phenotype. One can imagine that the mere introduction of one locus derived from an evolutionary distinct subspecies, in an F2 population or in congenics, into the network of interactions of another can lead to substantially different phenotypic effects. An analogy used by biostatistician and mouse geneticist Gary Churchill, is that it is like placing a Porsche engine into a Volkswagen car, the car will run but not like a Porsche (International Mouse Genome Conference 2005 Strasbourg, France). Although MOLF/Ei and C57BL/6J are

both mice, they are constructed differently through evolutionary selection and although the individual component may "fit", in totality the resulting phenotype differs.

Over evolutionary time, favorable allelic combinations have been selected for and co-inherited, resulting in the genomically and phenotypically distinct C57BL/6J and MOLF/Ei. The concept of the co-inheritance of alleles is not new and has been revisited in the recent years (Dobzhansky 1970; Nei 2003; Petkov et al. 2005). The co-inheritance of alleles in nature would be under positive selection as shown in a recent study of natural genetic variation among immune related genes in a wild population of *Drosophila* (Lazzaro et al. 2004). Clearly there are other factors specific to the MOLF/Ei genetic background that are co-inherited in our model accounting for its susceptibility that is not detected for reasons discussed earlier in this Chapter. However it is hard to believe that the progenitors of MOLF/Ei mice in the wild were selected to be highly susceptible to *Salmonella*, which is a natural rodent pathogen, unless it was associated with a survival advantage to another more relevant phenotype affecting their fitness and/or survival. In fact, O'Brien and colleagues have examined the response to subcutaneous *Salmonella* Typhimurium infections in different mice captured in the wild, including Japanese *Mus musculus molossinus* mice and found that they were resistant based on splenic bacterial load in comparison to susceptible C57BL/6J mice (O'Brien et al. 1986). This report is somewhat consistent with our results since MOLF/Ei have significantly lower bacterial loads than C57BL/6J mice however they die one day after C57BL/6J mice, making them susceptible based on survival (Sebastiani et al. 1998). The O'Brien et al study did not follow survival accounting for differences in the way they defined resistant and susceptible strains when compared with our study. A strain survey of the existing *Mus musculus molossinus* strains (MOLG/Dn, MOLD/Rk) would be of interest as it may

reveal whether the apparent susceptibility in MOLF/Ei arose during inbreeding or was ancestral in origin.

5.5.3 The Phenotype: Survival

The phenotype used in the study, survival, is complex. It represents the merging of a multitude of different factors which lead to death. In fact, mice known to succumb to infection within a week of infection die due to defective *Slc11al*, or *Tlr4* function, which already demonstrates the variety of reasons leading to the end phenotype of survival in different immunological pathways. The use of a less complex phenotype would perhaps make the identification of genetic contributors easier. Pinpointing a specific pathway or mechanism of the immune response may be a starting point in unraveling the complex interplay of the numerous effectors of the host immune response during *Salmonella* infection.

5.5.3.1 Summary of MOLF/Ei Phenotype

Systemic infection of MOLF/Ei mice with *Salmonella* Typhimurium, results in death by day 6 post infection with 1 x 10^7 CFU/g of spleen and liver (Sebastiani et al. 1998; Sebastiani et al. 2002). Other mice that are highly susceptible to infection include C3H/HeJ succumbing to infection on day 6 due to a mutation in *Tlr4*, with comparable bacterial loads of 1 x 10^6 CFU/g liver (Qureshi et al. 1996; Poltorak et al. 1998; Qureshi et al. 1999). C57BL/6J mice are similar in their extreme susceptibility succumbing to infection at day 5 however they have on the order of 100 fold higher CFUs due to a point mutation in *Slc11a1*(Vidal et al. 1993). However, in comparison to the fully resistant

129S6/SvEvTac, MOLF/Ei mice do show higher bacterial burden in the RES, suggesting that they are capable of suppressing bacterial replication to a certain degree, probably due to the functional *Slc11a1* allele they harbor in contrast to C57BL/6J mice. The reason for their demise may lie in their increased sensitivity to the bacteria possibly due to an inappropriate immune response.

With the aim of gaining a deeper understanding of the MOLF/Ei immune system, the immunological phenotype of MOLF/Ei mice was further analyzed in comparison to the highly susceptible C57BL/6J and resistant 129S6/SvEvTac (Sebastiani et al. 2002). In general, MOLF/Ei had a phenotype that was intermediate between the two strains, reflected by their bacterial load in the RES and in their patterns of lesions in histopathological analysis. Their LPS response detected via cytokine secretion was normal, although the LPS mitogenic response was lower than that of C57BL/6J. Higher IL-1α and β transcripts were found in the spleen and liver later in the infection. Although *Nos2* transcript levels (mapping to the *Ity2* region) in the spleen during infection were similar to resistant 129S6 mice, the ability of peritoneal macrophages to produce nitric oxides was diminished as compared to C57BL/6J and 129S6/SvEvTac. Evaluation of the systemic infection on the immune response in the brain revealed increased IκBα and TLR2 expression in the central nervous system (CNS), genes that are known to be regulated by LPS and IL-1 in the brain (Lacroix et al. 1998; Laflamme et al. 1999; Laflamme and Rivest 2001; Sebastiani et al. 2002). It is possible that the increase in IL-1 cytokines plays a pivotal role in the immune response of MOLF/Ei mice during *Salmonella* infection, leading to their demise.

5.5.3.2 MOLF/Ei - Congenic Phenotype Correlations

The success of phenotype dissection is well illustrated in the mapping of systemic lupus erythematosus susceptibility in NZM2410 mice which has led to the identification of numerous loci, each of which contribute distinct phenotypes as assessed by congenics, when re-assembled in a single congenic, manifest the original parental phenotype (Morel et al. 1997; Morel et al. 2000). Despite the fact that we were able to reconstitute either resistance or susceptibility respectively in the *Ity2* and *Ity3* congenics, efforts to pinpoint a parallel causative phenotype that may hint at a particular affected pathway in the congenics has led to further questions. In Chapters 2 and 4, bacterial load as measured in the RES organs showed no correlation with survival in *Ity2* and *Ity3* congenics suggesting that these loci do not affect bacterial load.

The search for a subphenotype for survival was aggressively pursued in the B6.MOLF-*Ity/Ity3* congenics in search for a phenotype that could be attributed to this susceptibility QTL. Thus, responses that were similar between the parental MOLF/Ei and B6.MOLF-*Ity/Ity3* mice, but were different from B6.MOLF-*Ity*, were sought. Since certain cytokines especially IL-1, were found to be different in MOLF/Ei mice, levels of serum IL-6, IL-1β, IL-1Ra during infection were assessed in the congenic mice. No difference between B6.MOLF-*Ity/Ity3* and B6.MOLF-*Ity/Ity3*$^{MOLF/B6}$ mice were observed, suggesting that *Ity3* does not affect this particular trait (Appendix I Figure 1) (Sebastiani et al. 2002)). Hematological analysis of blood specimens during infection revealed no significant similarities with the exception of a severe neutrophilia at day 5 post infection in MOLF/Ei and B6.MOLF-*Ity/Ity3* mice (Appendix I Figure 2). In addition, the ability of bone marrow derived macrophages and splenocytes to respond to various PAMPs, and the mitogenic response using splenocytes comprised primarily of lymphocytes, was

assessed (Appendix I Figures 3 and 4). There were no correlations between MOLF/Ei and B6.MOLF-*Ity/Ity3* mice in the spleen mitogenic response or in cytokine production from stimulated splenocytes. MOLF/Ei mice did have a diminished mitogenic response to LPS and imiquimod, lower production of IL-6 and TNF in response to CpG, and higher levels of TNF were detected with LPS (Appendix I Figure 3). The reduced response to LPS as measured by mitogenic response but robust cytokine response to LPS, was consistent with our previous study, pointing out a distinct B-cell proliferation profile in these mice (Sebastiani et al. 2002). Cytokine production in bone marrow derived macrophages found a potential correlation in diminished IL-6 response to lipoteichoic acid (LTA), and reduced TNF in response to CpG stimulation in MOLF/Ei and B6.MOLF-*Ity/Ity3* mice (Appendix I Figure 4A,B). MOLF/Ei macrophages had a unique cytokine profile in response to most PAMPs, including increased levels of IL-6 and TNF to zymosan and no response to poly-IC, implicating altered TLR2/TLR6 and TLR3 responses. In general, MOLF/Ei macrophages had higher cytokine levels as measured by IL-6, and lower TNF levels than most other mice. Most striking, was the robust IL-1β production in response to LPS, LTA, imiquimod, flagellin and heat killed *Salmonella* detected only in MOLF/Ei mice, consistent with previous observations (Appendix I Figure 4C)(Sebastiani et al. 2002). These preliminary results indicate that signaling through TLR1/2 (LTA) and TLR9 (CpG) as well as the increase in neutrophils in the blood at day 5 of infection, may be phenotypes that *Ity3* may affect, and should be pursued.

The observation that MOLF/Ei almost always demonstrates a distinct and unique profile, indicates that there must be other factors involved in the MOLF/Ei immune response that is not represented by *Ity3*, which warrants further investigation. Although efforts to identify other subphenotypes were largely unsuccessful, the pursuit of other

subphenotypes should be continued as we have only examined several aspects of the immune response. The production of other cytokines relevant to *Salmonella* infection such as IFNγ, IL-18, IL-12, IL-10, chemokine levels, the development of fever, bacteremia, DC function, T cells function, CNS immune response are just a few other phenotypes that can be further exploited. The dissection of the phenotype would greatly facilitate our understanding of the host immune response in MOLF/Ei mice. We have attempted to tease out the complexity of the host response to *Salmonella* infection in MOLF/Ei mice, by identifying components of the immune response that may be affected. The identification of the gene(s) underlying *Ity3* will provide us with one piece of the complex puzzle that is the host immune response.

5.6 Conclusion

The genetic dissection of the immune response to *Salmonella* Typhimurium infection has illuminated our understanding of the pathogenesis of systemic typhoid fever. The immune system represents a complex array of networks, whose coordinated expression and interaction requiring the involvement of multiple cell subsets is necessary to combat invading pathogens. With the aim of identifying other genetic factors affecting this process, *Ity2* and *Ity3* were identified in MOLF/Ei mice. Further fine mapping using a series of recombinant congenics carrying even smaller intervals for the *Ity3* region in combination with the assessment of the new candidates within the interval will hopefully lead to the identification of the gene(s) underlying this QTL. *Ncf2*, is clearly an excellent candidate and warrants further investigation although conclusive evidence would only come from a gene knockout interaction test (Flint et al. 2005). Further investigation of

Ity2 using the candidates identified as well as their validation in the development of a new line of congenics using 129S6 mice, will hopefully reveal the mechanisms underlying this resistance QTL. Finally results from a genome scan of the informative cross 129S6 x MOLF/Ei will hopefully lead to the identification of additional susceptibility loci that may help explain the extreme susceptibility observed in these wild-derived mice. The work presented in this dissertation has demonstrated the highly complex nature of the immune response to infection and revealed the amount of genetic and phenotypic variation that sets the wild-derived inbred strain MOLF/Ei apart from the classical inbred strains of mice.

CLAIMS TO ORIGINALITY

1. Development of congenic strains of mice for the *Ity* interval on chromosome 1, B6.MOLF-*Ity*; creation of double congenics B6.MOLF-*Ity/Ity2* carrying *Ity* as well as the *Ity2* region on chromosome 11; and B6.MOLF-*Ity/Ity3* harboring *Ity* and the *Ity3* interval on distal chromosome 1.
2. Validation of the existence of the resistance QTL *Ity2* and the susceptibility QTL *Ity3* in the context of a wild-type *Slc11a1* (*Ity*) in the MOLF/Ei mice using the congenic mice generated, as well as in the new informative (129S6 X MOLF/Ei)F2 cross.
3. Demonstration that a wild-type *Slc11a1 (Ity)* affects survival time as well as bacterial load during systemic infection with *Salmonella* Typhimurium infection, as expected. *Ity2* and *Ity3* were shown to influence survival however, neither loci was shown to affect bacterial load in the spleen and liver.
4. Identification of minimal intervals for both *Ity2* and *Ity3*, effectively reducing the candidate interval, and thus the number of candidate genes to be considered in future analysis.
5. Development a custom QTL specific oligonucleotide microarray, representing 375 transcripts that map within the *Ity3* interval.
6. Proposition of a list of prioritized candidate genes for *Ity2* and *Ity3* as a result of congenic and expression profiling analyses.
7. Functional characterization of the impact of both MOLF/Ei and C57BL/6J *Tlr5* alleles, eliminating it as a candidate for the *Ity3* locus.

8. Identification of a non-synonymous sequence variant in the coding region of MOLF/Ei *Ncf2,* shared only by other members of the *Mus musculus molossinus* subspecies as well as two European wild-derived strains CZECHII/Ei and Skive/Ei, occurring in a highly conserved and functional domain of the protein.
9. Functional characterization of the impact of the *Ncf2* sequence variant, leading to diminished superoxide production upon PMA stimulation and *Salmonella* infection *in vitro*, highlighting its' excellent candidacy for the *Ity3* locus.

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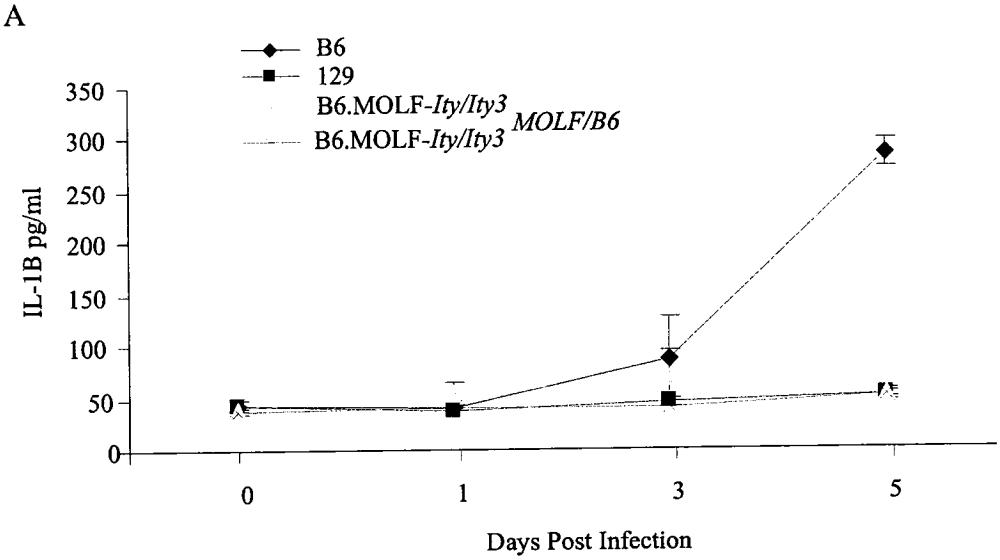
APPENDIX I:

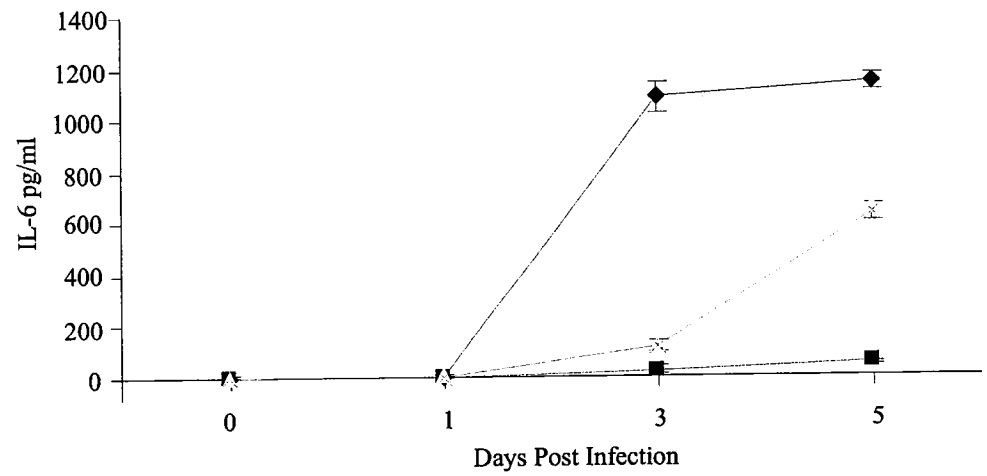
SUPPLEMENTAL FIGURES

Figure 1: Serum cytokine levels during systemic infection with *Salmonella* Typhimurium in 129S6/SvEvTac, C57BL/6J, B6.MOLF-*Ity/Ity3*, B6.MOLF-*Ity/Ity3*$^{MOLF/B6}$ mice. Blood samples were collected by cardiac puncture from uninfected and infected mice at days 1, 3 and 5 after intravenous infection of 1000CFU of *Salmonella* Typhimurium, and were left to coagulate overnight at 4°C. Sera were harvested and cytokine levels assessed using the Quantikine® ELISA mouse IL-6, IL-1β, IL-1Ra kit (R&D Systems). Four mice were used for each timepoint in each group, and a duplicate sample of each mouse was assessed in the ELISA. Cytokine profiles for IL-6 (A), IL-1β (B), and IL-1Ra (C) are shown.

A


B6
129
B6.MOLF-Ity/Ity3
B6.MOLF-Ity/Ity3 MOLF/B6
IL-1B pg/ml
350
300
250
200
150
100
50
0
0
1
3
5
Days Post Infection

B


IL-6 pg/ml
1400
1200
1000
800
600
400
200
0
0
1
3
5
Days Post Infection

C

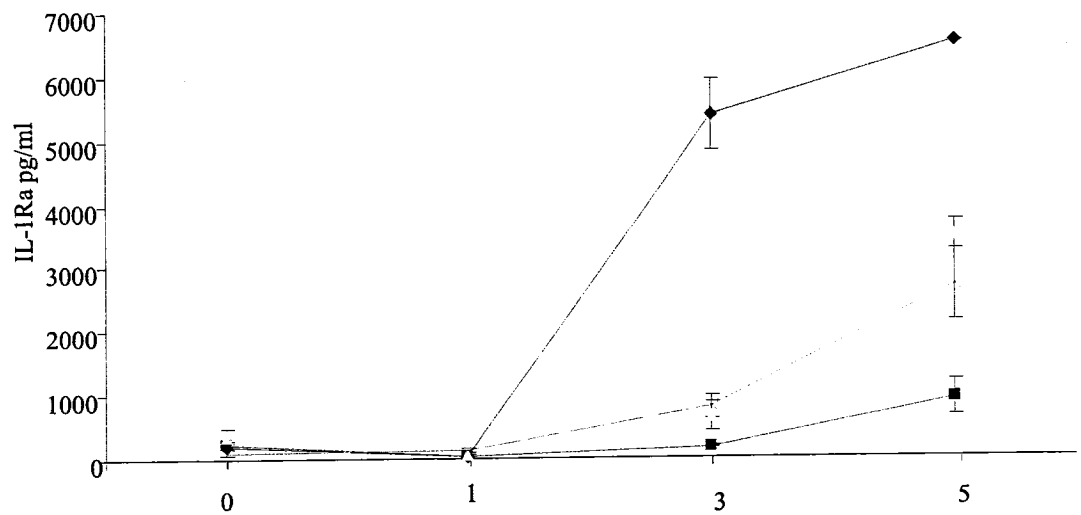

IL-1Ra pg/ml
7000
6000
5000
4000
3000
2000
1000
0
0
1
3
5

Figure 2: Hematological analysis of blood samples in C57BL/6J, MOLF/Ei, B6.MOLF-*Ity* and B6.MOLF-*Ity/Ity3* mice. Mice were infected with 1000CFUs of *Salmonella* Typhimurium intravenously, and blood samples collected by cardiac puncture at days 0, 5 and 7 post-infection. Blood samples were collected in Microvette tubes coated with EDTA tripotassium salt (Sarstedt) to prevent coagulation. Complete blood count with differentials and hematocrits were assessed at the McGill University Animal Resource Center. Up to four mice per group per timepoint were used. Hematocrits (A), red blood cell counts (B), neutrophil percentage (C), monocyte percentage (D), lymphocyte percentage (E), and platelet counts (F) are shown.

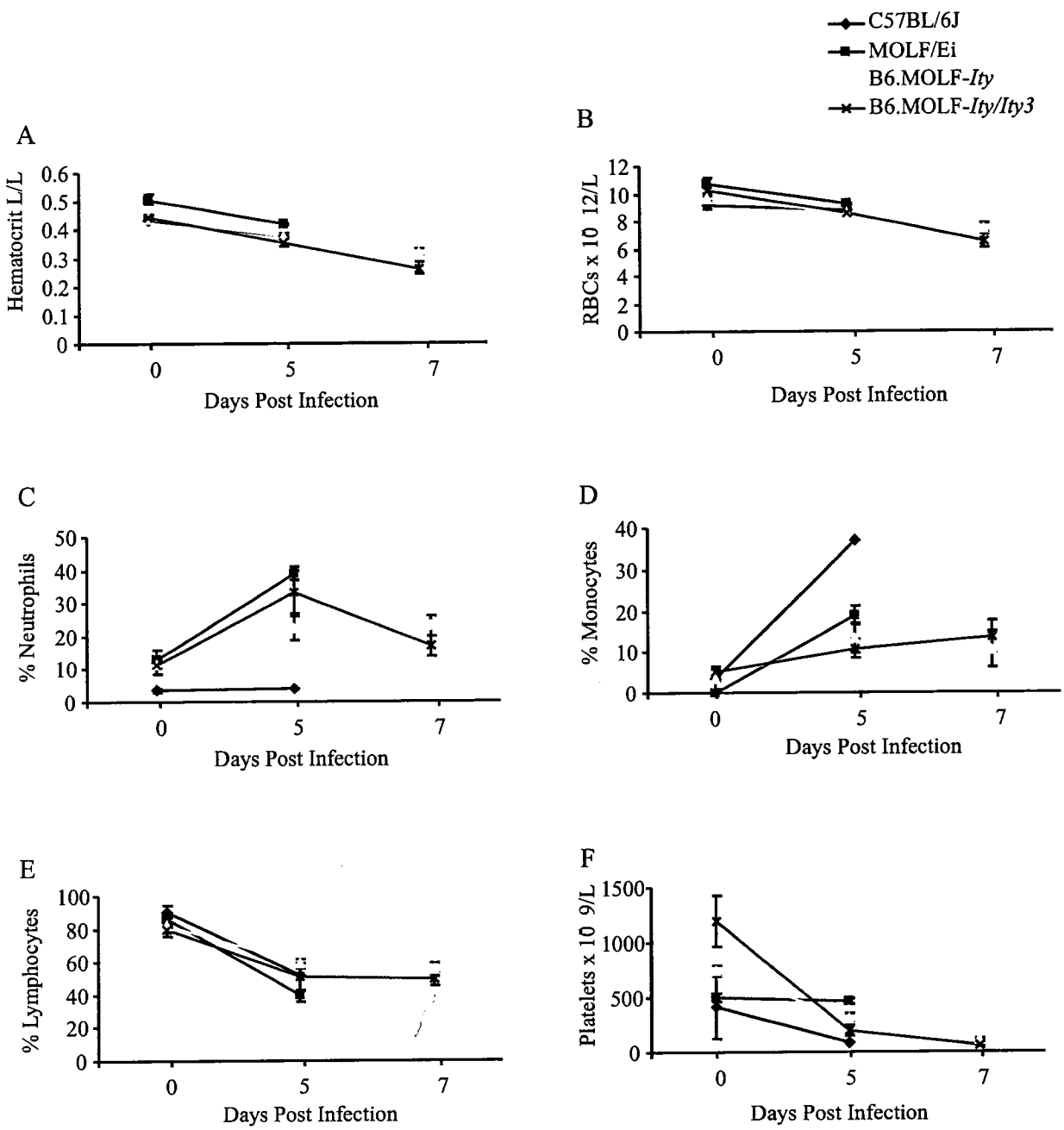

C57BL/6J
MOLF/Ei
B6.MOLF-Ity
B6.MOLF-Ity/Ity3
A
Hematocrit L/L
0.6
0.5
0.4
0.3
0.2
0.1
0
0
5
7
Days Post Infection
B
RBCs x 10 12/L
12
10
8
6
4
2
0
0
5
7
Days Post Infection
C
% Neutrophils
50
40
30
20
10
0
0
5
7
Days Post Infection
D
% Monocytes
40
30
20
10
0
0
5
7
Days Post Infection
E
% Lymphocytes
100
80
60
40
20
0
0
5
7
Days Post Infection
F
Platelets x 10 9/L
1500
1000
500
0
0
5
7
Days Post Infection

Figure 3: Splenocyte mitogenic response (A) and cytokine production as measured by IL-6 (B) and TNF (C) in response to various PAMPs in C57BL/6J, MOLF/Ei, B6.MOLF-*Ity*, and B6.MOLF-*Ity/Ity3* mice. Splenocytes were harvested and cultured, and spleen cell mitogenic response assessed as previously described (Sebastiani et al. 2002). Spleen cells for mitogenic response were stimulated using 1μM CpG (AlphaDNA) for TLR9 signaling, 100μg/ml Poly-IC (Amersham) for TLR3, 10μg/ml LPS (Sigma) for TLR4, 10μg/ml zymosan (Sigma) for TLR2/TLR6, 1.5μg/ml conA (Sigma) as positive control for mitogenic response, 10ug/ml imiquimod (Invivogen) for TLR7/8, 100ng/ml flagellin (gift from Dr. Gewirtz, Emory University, Atlanta) for TLR5. Two mice per group were used and are represented as separate bars on the graphs. Spleen cells used for cytokine assessment were also stimulated with 10μg/ml LTA (Sigma) for TLR1/TLR2 signaling. Cytokine levels were determined using the BD OptEIA™ Set Mouse IL-6 and TNF (BD Biosciences). Cell supernatants were harvested 24hrs after stimulation.

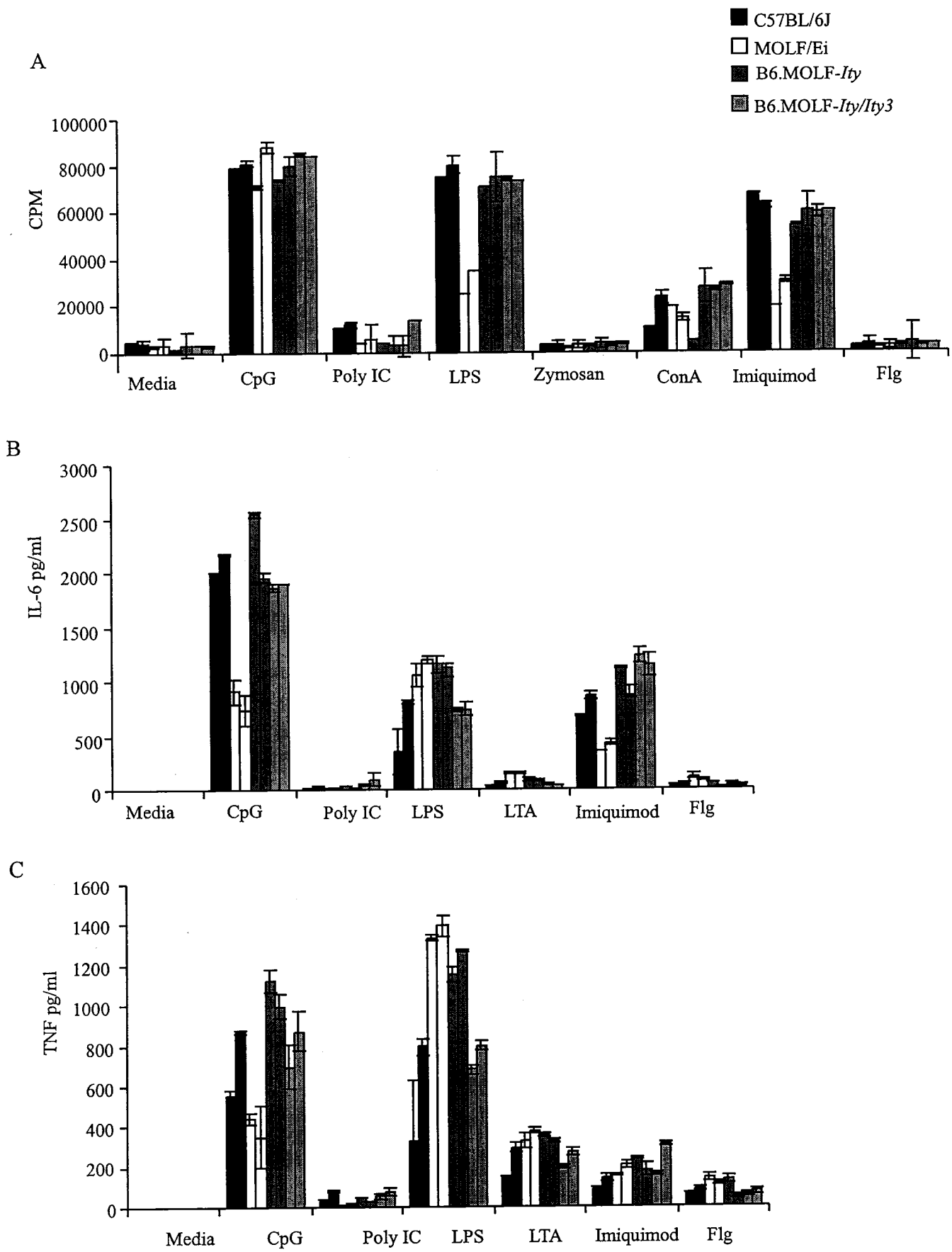
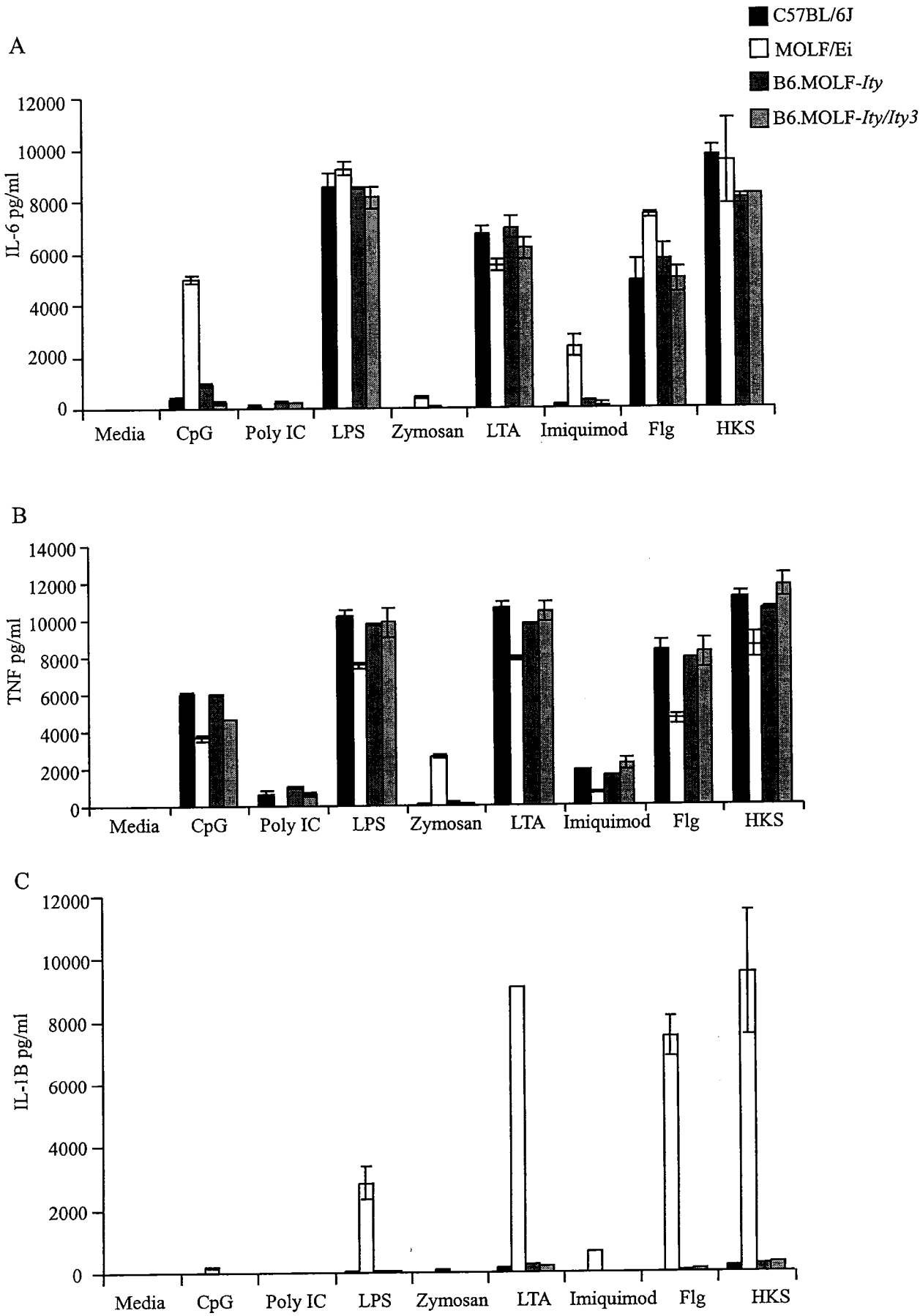

A
C57BL/6J
MOLF/Ei
B6.MOLF-Ity
B6.MOLF-Ity/Ity3
CPM
100000
80000
60000
40000
20000
0
Media
CpG
Poly IC
LPS
Zymosan
ConA
Imiquimod
Flg
B
IL-6 pg/ml
3000
2500
2000
1500
1000
500
0
Media
CpG
Poly IC
LPS
LTA
Imiquimod
Flg
C
TNF pg/ml
1600
1400
1200
1000
800
600
400
200
0
Media
CpG
Poly IC
LPS
LTA
Imiquimod
Flg

Figure 4: Cytokine response as measured by IL-6 (A), TNF (B), and IL-1β (C) in C57BL/6J, MOLF/Ei, B6.MOLF-*Ity*, and B6.MOLF-*Ity/Ity3* bone marrow derived macrophages stimulated with various PAMPs. Bone marrow derived macrophages were prepared as described in Salez et al (Salez et al. 2001). Macrophages were stimulated using the same concentrations as in Figure 3, with the exception of LPS (10ng/ml). BD OptEIA™ Set Mouse IL-1β kit (BD Biosciences) was used for IL-1β. Bone marrows from two mice were pooled for each group. Cell supernatants were harvested 6hrs after stimulation for IL-6 and TNF, and 24hrs after stimulation for IL-1β.


A
C57BL/6J
MOLF/Ei
B6.MOLF-Ity
B6.MOLF-Ity/Ity3
IL-6 pg/ml
12000
10000
8000
6000
4000
2000
0
Media
CpG
Poly IC
LPS
Zymosan
LTA
Imiquimod
Flg
HKS
B
TNF pg/ml
14000
12000
10000
8000
6000
4000
2000
0
Media
CpG
Poly IC
LPS
Zymosan
LTA
Imiquimod
Flg
HKS
C
IL-1B pg/ml
12000
10000
8000
6000
4000
2000
0
Media
CpG
Poly IC
LPS
Zymosan
LTA
Imiquimod
Flg
HKS

APPENDIX II:

PUBLICATIONS

Papers

Vanessa Sancho Shimizu, Serge Mostowy, Line Lariviere, Noemie Riendeau, Marcel Behr, Danielle Malo. (2006) Molecular genetic analysis of two loci (*Ity2* and *Ity3*) involved in the host response to *Salmonella* Typhimurium infection in wild-derived mice. (Manuscript in Preparation)

Vanessa Sancho Shimizu and Danielle Malo. (2006) Sequencing, expression and functional analyses support the candidacy of *Ncf2* in susceptibility to *Salmonella* Typhimurium infection in Wild-derived mice. Journal of Immunology 176(11):6954-6961.

Vanessa Sancho Shimizu*, Isabelle Angers*, Albert Descoteaux, Andrew Gerwitz, and Danielle Malo (2006). *Tlr5* is not primarily associated with susceptibility to *Salmonella* Typhimurium infection in MOLF/Ei mice. Mammalian Genome 17(5):385-397.

* Authors contributed equally to the work.

Giovanna Sebastiani, Veronique Blais, **Vanessa Sancho**, Stefanie N. Vogel, Mary M. Stevenson, Philippe Gros, Jean-Martin Lapointe, Serge Rivest, and Danielle Malo (2002) Host Immune Response of Wild-Derived Mice during *Salmonella enterica* Serovar Typhimurium Infection. Infection and Immunity 70(4): 1997-2009.

Published Abstracts

Posters

Vanessa Sancho and Danielle Malo. May 15-19, 2006. *Ncf2*, gène de prédisposition à l'infection à salmonelles chez la souris sauvage MOLF/Ei.. 74 Congres de l'Acfas. Montreal, Canada.

Vanessa Sancho Shimizu*, Isabelle Angers*, Albert Descoteaux, Andrew Gerwitz, and Danielle Malo (2006). The Effect of Genetic Background in an Intersubspecific Mouse Cross: the Candidacy or *Tlr5* in *Salmonella* Susceptibility of MOLF/Ei mice. 5th Annual Complex Trait Consortium. Chapel Hill, NC. * Authors contributed equally to the work.

Vanessa Sancho and Danielle Malo. June 26-29, 2005. Candidacy of *Ncf2* in *Salmonella* Susceptibility of Wild-derived Mice. 4th Annual Complex Trait Consortium. Groningen, Netherlands.

Vanessa Sancho and Danielle Malo. February 13-16, 2005. Candidate Gene Analysis of *Ncf2* in Salmonella Susceptibility of Wild-derived Mice. Canadian Bacterial Disease Network/CMCI Annual General Meeting. Banff, Canada.

Vanessa Sancho and Danielle Malo. July 18-23, 2004. The Candidacy of *Ncf2* as the Underlying Gene in *Salmonella* Susceptibility of Wild-derived Mice. 12th International Congress of immunology and 4th Annual Conference of FOCIS. Montreal, QC.

Vanessa Sancho and Danielle Malo. July 6-9, 2004. Fine Mapping of Quantitative Trait Loci linked to *Salmonella* Immunity in Wild-derived Mice. Complex Trait Consortium 2004, 3rd Annual Conference. Bar Harbor, Maine.

Vanessa Sancho, Isabelle Angers and Danielle Malo. June 22-25, 2004. Howard Hughes Medical Institute 2004 Meeting of International Research Scholars. Estonia.

Vanessa Sancho, Isabelle Angers and Danielle Malo. January 8-13, 2004. Fine Mapping and Candidate Gene Analysis of Complex Immunity to *Salmonella* Typhimurium in Wild-Derived Mice. Keystone Symposia Meeting – Natural Variation and Quantitative Genetics in Model Organisms. Breckenridge, Colorado USA.

Vanessa Sancho, Genevieve Lacroix, Danielle Malo. November 9-12, 2003. Fine Mapping of QTL's underlying Immunity to *Salmonella* Typhimurium in Wild-strain Mice. 17th International Mouse Genome Conference. Braunschweig, Germany.

Vanessa Sancho, Danielle Malo. April 24-26, 2003. Candidate Genes Underlying Immunity to *Salmonella* Typhimurium in Wild-strain Mice. Canadian Bacterial Disease Network/CMCI Annual General Meeting. Calgary, Canada.

Giovanna Sebastiani, Judith Caron, Vanessa Sancho, Danielle Malo. June 20-23, 2001. Genetics of susceptibility to infection with *Salmonella* in mice. Howard Hughes Medical Institute 2001 Meeting of International Research Scholars. Vancouver, Canada.

Invited Presentations

Vanessa Sancho and Danielle Malo. June 13-14, 2005. Candidate Gene Analysis of *Ncf2* in *Salmonella* Susceptibility of Wild-derived Mice. Fifth Annual Quebec Parasitology Symposium. Montreal, Canada.

Vanessa Sancho and Danielle Malo. February 13-16, 2005. Candidate Gene Analysis of *Ncf2* in Salmonella Susceptibility of Wild-derived Mice. Canadian Bacterial Disease Network/CMCI Annual General Meeting. Banff, Canada.

Vanessa Sancho and Danielle Malo. July 18-23, 2004. The Candidacy of *Ncf2* as the Underlying Gene in *Salmonella* Susceptibility of Wild-derived Mice. 12th International Congress of immunology and 4th Annual Conference of FOCIS. Montreal, QC.

APPENDIX III:

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Von: Vanessa Sancho Shimizu [mailto:vanessa.sancho@mail.mcgill.ca]
Gesendet: Dienstag, 6. Juni 2006 16:36
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Vanessa

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Von: Vanessa Sancho Shimizu [mailto:vanessa.sancho@mail.mcgill.ca]
Gesendet: Dienstag, 23. Mai 2006 16:17
An: Sturm, Rosita, Springer DE
Betreff: Permission to include article in PhD Thesis

Dear Dr Sturm

I have been referred to you by Dr Louise Tinsley. I am a PhD candidate enrolled at McGill University and am in the process of writing up my thesis. I would like to include an article that has been published in your journal, Mammalian Genome, namely:

Isabelle Angers, Vanessa Sancho Shimizu, Albert Descoteaux, Andrew Gerwitz, and Danielle Malo (2006). Tlr5 is not primarily associated with susceptibility to Salmonella Typhimurium infection in MOLF/Ei mice. Mammalian Genome. 2006 May;17(5):385-97

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Thank you

Vanessa Sancho Shimizu