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FACULTÉ DES ÉTUDES SUPÉRIEURES
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FACULTY OF GRADUATE AND
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M.Sc. (Microbiology and Immunology)

GRADE / DEGREE

Department of Biochemistry Microbiology and Immunology

FACULTÉ, ÉCOLE, DÉPARTEMENT / FACULTY, SCHOOL, DEPARTMENT

The Role of Type-1 Interferons During *Salmonella Typhimurium* Infection

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The role of type-I interferons during *Salmonella typhimurium* infection

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Thesis submitted to the Faculty and Postdoctoral Studies
in partial fulfillment of the requirements for the
M.Sc. degree in Microbiology and Immunology
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Your file *Votre référence*
ISBN: 978-0-494-69097-0
Our file *Notre référence*
ISBN: 978-0-494-69097-0

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Acknowledgements

I would like to begin by thanking my supervisor, Dr. Subash Sad. Having had the tremendous opportunity of working in your lab I have gained invaluable experience both technically and academically. For this I am truly grateful. Special thanks are due to Renu Dudani, Sue Maclean, Jeffrey Nuyens, Nirmal Robinson and Marsha Russell for their technical expertise and assistance. And to all my lab mates, those that were there from the beginning and those that I've just met, thank you for helping me through the highs and lows of research with insightful comments and lively lunch discussions. The range of ideas that can come out of a 12' X 12' windowless room is simply phenomenal! Your support and friendship will never be forgotten. Finally, I would like to thank my family for their continued support through all of my endeavours. For always being there, and knowing just what I need to hear, thanks Mom, Dad, K and Ri.

Table of Contents

Abstract.....	5
List of Abbreviations.....	6
List of Figures.....	8
1. Introduction.....	10
1.1 <i>Salmonella</i> Pathogenesis	
i) Overview.....	11
ii) Virulence: Invasion and Survival.....	12
iii) Nramp1 expression: innate resistance to ST infection.....	12
iv) Inflammation and Innate Immunity.....	13
1.2 Type-I Interferons	
i) Overview.....	19
ii) Role of IFN-I in Bacterial Infections.....	20
2. Rationale, Hypothesis and Objectives	
2.1 Rationale.....	23
2.2 Hypothesis.....	26
2.3 Objectives.....	27
3. Materials and Methods	
3.1 Mice.....	28
3.2 Bacteria and Infection Model	
i) <i>in vivo</i>	28
ii) <i>in vitro</i> infection of bone marrow-derived macrophages.....	29
iii) <i>in vitro</i> infection of bone marrow-derived dendritic cells.....	29
3.3 Assessment of Survival.....	30
3.4 Assessment of Bacterial Burden	
i) Whole organ.....	30
ii) Intracellular burden.....	31
3.5 Flow Cytometry.....	31
3.6 Cell Purification.....	32
3.7 Cytokine Analysis	
i) TNF α / β Bioassay.....	33
ii) NO $_2^-$ Greiss Reagent System.....	33
iii) Cytokine ELISAs.....	33
iv) Mouse Proteome Array.....	34
v) qRT-PCR.....	35
3.8 Cytotoxicity Assays	
i) MTT Cytotoxicity Bioassay.....	36
ii) Neutral Red Cytotoxicity Bioassay.....	37
iii) Cellular Imaging.....	37
3.9 Type-I Interferon Neutralization.....	37
4. Results	
4.1 Inflammatory mediators are crucial for innate host survival.....	39
4.2 CpG treatment renders normally resistant mice susceptible to ST infection.....	42
4.3 Effects of IFN-I signalling on host survival.....	45

4.4 IFN-I signalling regulates the host inflammatory response to ST infection.....	47
4.5 IFN-I signalling influences innate cell population kinetics in the spleen.....	54
4.6 IFN-I receptor-deficiency enhance intracellular ST burden control.....	57
4.7 ST-induced IFN-I compromises the survival of BMMs but not BMDCs	63
4.8 IFN- β and not IFN- α enhances ST-induced M ϕ death.....	68
5. Discussion	
5.1 Prelude.....	70
5.2 Early inflammation is crucial for host survival to ST infection.....	71
5.3 IFN-I signalling exacerbates ST infection.....	74
5.4 Ablation of IFN-I signalling reduces ST-induced inflammation.....	77
5.5 IFN-I signalling regulates macrophage survival.....	78
5.6 IFN- β regulates macrophage death during ST infection.....	80
6. Conclusion.....	81
Model.....	85
7. References.....	86
8. Curriculum Vitae.....	94

Abstract

Type-I interferons (IFN-I) play a key protective role during viral infections, however, their role during bacterial infections remains unclear. We evaluated the influence of IFN-I signalling during infection of mice with the facultative intracellular bacterium, *Salmonella typhimurium* (ST). Wild-type (WT) C57BL/6J mice succumbed to infection by day 7 and death was accelerated in mice deficient in key innate immune mediators (IFN- γ , TNF- α , iNOS-2). Surprisingly, IFN-I receptor-deficient (IFN-I R^{-/-}) mice survived ST infection up to 35 days. Despite enhanced inflammation, WT mice displayed uncontrolled ST burden and lower macrophage numbers in the spleen, compared with IFN-I R^{-/-} mice. *In vitro*, IFN-I-deficient macrophages expressed reduced levels of TNF- α and NO₂⁻ in response to ST and displayed prolonged survival in comparison with WT macrophages. Since macrophages play key protective roles during intracellular bacterial infections, our results indicate that IFN-I signalling during ST infection promotes the elimination of macrophages resulting in poor pathogen control.

List of Abbreviations

APC	Antigen presenting cells
B cell	B lymphocyte
BHI	Brain Heart Infusion
BLC	B lymphocyte chemoattractant
BMDC	Bone marrow-derived dendritic cell
BMM	Bone marrow-derived macrophage
cDNA	complementary deoxyribonucleic acid
DC	Dendritic cell
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
GM-CSF	Granulocyte-macrophage colony stimulating factor
IFN-α	Interferon alpha
IFN-β	Interferon beta
IFN-γ	Interferon gamma
IFN-I	Type-I interferons
IFN-I R	Type-I interferon receptor
IL-6	Interleukin 6
IL-10	Interleukin 10
IL-12	Interleukin 12
iNOS2	Inducible nitric oxide synthase
i.v.	Intravenous
i.p.	Intraperitoneal
IP-10	10 kDa interferon gamma-induced protein
ISG	Interferon stimulated gene
LM	<i>Listeria monocytogenes</i>
LPS	Lipopolysaccharide
Mϕ	Macrophage
M cells	Microfold cells
MCP	Monocyte chemotactic protein
M-CSF	Macrophage colony stimulating factor
MIG	Monokine induced by gamma interferon
MLN	Mesenteric lymph nodes
MTb	<i>Mycobacterium tuberculosis</i>
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NK	Natural killer cell
NKT	Natural killer T lymphocyte
NO	Nitric oxide
NO$_2^-$	Nitrite ion
NOD	Nucleotide binding oligomerization domain
NRAMP	Natural resistance associated macrophage protein
PAMP	Pathogen associated molecular pattern
PBS	Phosphate buffered saline

PCR	Polymerase chain reaction
p.i.	Post-infection
PMSF	Phenylmethanesulphonylfluoride
PP	Peyer's patches
PRR	Pattern recognition receptor
qRT-PCR	Quantitative real-time polymerase chain reaction
R8	RPMI+8%FCS
RES	Reticuloendothelial system
RLR	Rig-like receptors
RNA	Ribonucleic acid
RNI	Reactive nitrogen intermediates
ROI	Reactive oxygen intermediates
SCV	<i>Salmonella</i> containing vacuole
SPI-1, -2	<i>Salmonella</i> pathogenicity island-1, -2
ST	<i>Salmonella typhimurium</i>
T cell	T lymphocyte
TLR	Toll-like receptors
TNF-α	Tumour necrosis factor alpha
TTSS	Type-III secretion system
WT	Wild-type (C57BL/6 mouse or cell)

List of Figures

Fig. 1. T cell activation is delayed following ST infection, when compared with LM.....	24
Fig. 2. C57BL/6 mice succumb to ST infection prior to T cell activation.....	25
Fig. 3. Innate mediators affect host survival during ST infection.....	40
Fig. 4. Survival of mice is not modulated significantly by the absence of MyD88- independent signalling.....	43
Fig. 5. CpG treatment renders normally resistant mice susceptible to ST.....	44
Fig. 6. Effects of IFN-I on host survival and bacterial burden during ST infection.....	46
Fig. 7. Effects of IFN-I on serum chemokine/ cytokine profile following ST infection.....	49
Fig. 8. Effects of IFN-I on chemokine/ cytokine expression in the spleen following ST infection.....	50
Fig. 9. Cytokine expression by M ϕ s during ST infection <i>in vitro</i>	53
Fig. 10. Cytokine expression by DCs during ST infection <i>in vitro</i>	55
Fig. 11. Kinetic changes in NK-T, NK, Neutrophil and DC populations following ST infection.....	56
Fig. 12. Kinetic changes in M ϕ populations in the spleen following ST infection.....	59
Fig. 13. Reduced ST burden in the M ϕ s of IFN-I R-deficient mice.....	60
Fig. 14. Reduced ST burden in M ϕ s of IFN-I R-deficient mice in the peritoneum.....	61
Fig. 15. Reduced ST burden in DCs of IFN-I R-deficient mice.....	62
Fig. 16. IFN-I receptor engagement enhances M ϕ susceptibility to ST-induced cell death.....	65
Fig. 17. IFN-I R-deficiency enhances M ϕ survival following ST infection.....	66

Fig. 18. DCs from WT and IFN-I R-deficient mice are equally susceptible to ST-induced cell death.....67

Fig. 19. IFN- β contributes to macrophage death during infection with ST.....69

Fig. 20. Model depicting cellular events in the presence or absence of IFN-I signalling that ultimately lead to disparity in host survival.....85

1. Introduction

Salmonella spp. are gram negative facultative intracellular bacteria belonging to the family Enterobacteriaceae. Approximately 2500 serotypes have been identified to date, each differentiated by unique expression of extracellular structures (1). Although some serotypes are host-specific pathogens, a large number of *Salmonella enterica* serotypes cause salmonellosis and gastroenteritis in a range of host species. Gastroenteritis is a major global concern; poor sanitary conditions and food handling and an increase in food automation have led to outbreaks in developing and developed nations alike. *Salmonella enterica* serovars *typhi*, *paratyphi A*, *paratyphi B*, and *paratyphi C* are primate-specific pathogens and are the aetiological agents of human typhoid fever (2, 3). Typhoid fever, endemic in developing nations, is a severe systemic disease caused by the ingestion of contaminated food or water. Typhoid fever affects more than 16 million people world-wide and is responsible for more than 600,000 deaths annually (4). Two vaccines are currently licensed for use against typhoid fever, however, they display only moderate efficacy, offering protection to 50-70% of vaccinated individuals in their target groups (children aged >5yr and young adults) (4). A need remains to find more effective vaccines against typhoid fever. *Salmonella enterica* serovar *typhimurium* (ST) can infect a variety of host species each displaying unique symptoms and severity of infection. In humans, ST causes mild gastroenteritis, in mice however, it causes a severe typhoid-like disease in which ST disseminates from the gut into systemic compartments. Thus, ST infection in mice mimics Typhoid infection in humans and serves as a good model to study systemic *Salmonella* pathogenesis (3).

1.1 *Salmonella* Pathogenesis

1.1 i) Overview

Salmonella spp. are generally transmitted via the oral route through the ingestion of contaminated food or water. *Salmonella* gains access to the host reticuloendothelial system (RES) via Peyer's patches (PP) in the small intestine. These specialized lymphoid structures line the lamina propria of the ileum and contain specialized enterocytes called micro-fold cells (M-cells) on their luminal surfaces that continually sample the intestinal lumen. *Salmonella* preferentially invades M-cells (5) via its type three secretion system (TTSS), although it has been shown to gain access to the RES by infecting resident dendritic cells that also sample the intestinal lumen. Once inside the PPs, *Salmonella* are taken up by resident phagocytes (predominantly macrophages, M_{ϕ} s) which traffic to mesenteric lymph nodes (MLN). *Salmonella* travels through the lymph to the circulatory system quickly disseminating systemically (3, 6). In the blood stream opsonisation and complement activation initiate bacterial clearance through phagocytic uptake by M_{ϕ} s in the spleen and liver, however not all bacteria are killed (6, 7). Unique invasion and virulence mechanisms allow *Salmonella* to transform non-phagocytic cells into phagocytes and enhance intracellular survival and replication thus enabling evasion of host immune mechanisms resulting in chronic infection. Genetically determined innate resistance or susceptibility largely dictates the fate of the host (8). In fatal infections, uncontrolled ST replication results in bacteraemia, endotoxic shock and rapid host death (8, 9). Conversely, in sublethal ST infections macrophages sufficiently control bacterial levels and subsequently an effective, albeit delayed, T cell response eliminates the remaining infection and induces memory for protection against subsequent ST infections (10).

1.1 ii) Virulence: Invasion and Survival

As previously discussed, *Salmonella* spp. have evolved a number of mechanisms to enhance invasion and survival within infected hosts. Genes encoding a variety of virulence proteins are clustered in *Salmonella* pathogenicity islands-1 and -2 (SPI-1, -2). These clusters encode both structural and effector proteins that mediate specific virulence mechanisms (7). The TTSS is a needle-like structure which is used as a means for ST to inject effector proteins into host cells (3, 8). Intestinal epithelial invasion is largely SPI-1-mediated including *Salmonella*'s inherent ability to transform non-phagocytic cells into phagocytes by inducing membrane ruffling and bacterial-mediated endocytosis (3, 11). Following invasion, effector proteins encoded by SPI-2 prevent phagosomal-lysosomal fusion (12), effectively evading host antimicrobial peptides contained within the lysosome. Additionally, *Salmonella* sp. can modify the lipid A portion of their LPS, thus downregulating its inflammatory potential. These and other genes encoded by SPI-2 ultimately promote both the intracellular survival and proliferation of *Salmonella* sp. (13).

1.1 iii) Nramp1 expression: innate resistance to ST infection

Host susceptibility to ST infection varies starkly among different strains of mice (14). Whereas innately susceptible (eg. C57BL/6, BALB/C) mice succumb to ST infection within the first 7 days, resistant mice (eg. 129SvJ, CBA) are able to mount innate and adaptive responses that effectively curb bacterial replication enabling host survival (15, 16). Innate resistance to ST has been associated with the presence of the natural resistance associated macrophage protein 1, Nramp1, which is expressed exclusively in M ϕ s, monocytes and polymorphonuclear leukocytes (14, 15). The *Nramp1* gene, located on

mouse chromosome 1, encodes an integral membrane protein localized in the endosome which is recruited to the phagosomal membrane immediately after ST uptake. The *Nramp1* protein regulates divalent cation exchange, actively pumping divalent cations from the phagosome into the cytoplasm limiting ST from accessing important metabolic cofactors (6, 8, 9, 15, 22, 23). The *Nramp1* gene displays a dominant inheritance pattern and it confers resistance to other intracellular pathogens including *M. bovis* and *Leishmania donovani* (16-18). The rate of ST uptake by resident $M_{\Phi}s$ is unaffected by *Nramp1* expression, however it was demonstrated to mediate enhanced intracellular killing by phagocytes contributing to host resistance (19-21). Immediate intracellular killing by $M_{\Phi}s$ in the spleen and liver, reduces ST burden in the blood within the first two hours following infection. Immediate clearance was shown to be *Nramp1*-independent since *Nramp1* expression is delayed until 24h post-infection (24). Thus *Nramp* regulates M_{Φ} function following initial clearance, enhancing resistance by mediating long-term intracellular killing by $M_{\Phi}s$.

1.1 iv) Inflammation and innate immunity

The innate response to ST is initiated immediately following pathogen recognition at the intestinal epithelial barrier following oral infection. Host cells express a variety of pathogen recognition receptors (PRRs) that recognize specific, conserved, pathogen associated molecular patterns, (PAMPs). There are three known families of PRRs: the toll-like receptors (TLRs), the rig-like receptors (RLRs) and the intracellular nucleotide binding oligomerization domain receptors (NODs). The TLR family, consisting of as of yet, 13 members in mice (25), are all membrane-bound, located at either the cell or endosomal

membranes (26). TLRs are particularly important for host recognition of ST PAMPs; TLR-4, -5 and -9 recognize lipopolysaccharide (LPS), flagellin and unmethylated DNA (CpG), respectively. Of these TLR-PAMP pairs, the interaction between LPS and TLR-4 elicits the most potent inflammatory response (27). TLR-4 stimulation results in signalling that occurs via two distinct pathways; the MyD88-dependent and –independent pathways. The MyD88-dependent pathway requires the adaptor protein, MyD88, responsible for the recruitment of the IL-1 receptor-associated kinase (IRAK) which subsequently associates with TRAF6. From here, two divergent signalling cascades result in the activation of the transcription factors NF κ B and JNK (26, 28). The MyD88-independent pathway requires the adaptor protein TRIF/TICAM-1 resulting in the activation of the transcription factor IRF3 (26, 29). The MyD88-independent pathway leads primarily to the production of interferon- β (IFN- β) and will be discussed in later sections (25, 29, 30). Conversely, the MyD88-dependent signalling pathway results in the transcription of a variety of cytokines and chemokines characteristic of ST infection (eg. IL-12, TNF- α , IFN- γ , IL-6) (31, 32). All TLRs are not ubiquitously expressed on every cell type, thus the variety and quantity of the chemokine/ cytokine profile produced following ST infection varies by cell type and location (26).

The early host response to ST infection is characterized by massive and rapid cellular infiltration to the site of infection, most notably by neutrophils and M ϕ s, following the production of massive amounts of chemokines (eg. The MCP and MIP families of chemokines) after TLR signalling (31). Cytokine/ chemokine expression is initiated upon host recognition of ST by epithelial cells or splenocytes following oral or intravenous infection, respectively.

In both oral and systemic models of ST infection, neutrophils have shown to predominate as the first cellular infiltrate following initial stimulation by ST. Models of neutropenic mice (33) and neutrophil depletion highlight the important early and protective roles mediated by neutrophils following ST infection. The antimicrobial actions mediated by neutrophils are simulated upon activation by IL-18. Neutrophils are phagocytes that have been shown to mediate initial stages of pathogen clearance, harbouring an abundance of antimicrobial peptides and reactive oxygen and nitrogen intermediates (ROI and RNI, respectively) (34). Additionally, they have the capacity to regulate pathogen trafficking and have immunomodulatory properties that generally result in the polarization of a Th1 response (characterized by massive IL-12 and IFN- γ production) following infections with intracellular pathogens (33, 34). Neutrophils are thus important not only for mediating initial pathogen clearance, but also in initiating inflammation by the release of pre-stored IL-12, an important activator of subsequent cellular infiltrates including $M_{\Phi}s$.

It has been well established that $M_{\Phi}s$ play a key role in early immune responses to ST invasion (35). Depletion studies highlight an important role for $M_{\Phi}s$ in the early response to ST infection (35-37); where M_{Φ} -depleted mice showed enhanced susceptibility to ST challenge. Studies of the innate response to ST have largely focused on M_{Φ} populations, due to the fact that they have been demonstrated to be the primary cell type responsible for mediating ST clearance and harbour the majority of ST residing intracellularly (38). Phagocytosis by $M_{\Phi}s$ normally results in the fusion of phagosomal and lysosomal compartments which initiates controlled intracellular pathogen clearance mediated by a number of antimicrobial effectors contained within the lysosome. ROIs, RNIs and enzymes for bacterial destruction, for example lysozyme, are characteristic

mediators of pathogen clearance (7). The ability of ST to reside within macrophages has been shown in both resistant and susceptible hosts and is largely due to ST virulence factors encoded by SPI-2. As previously discussed, SPI-2 virulence genes encode effector proteins that interrupt phagosomal-lysosomal fusion within the M_{Φ} (12) effectively evading clearance and enhancing the ability of ST to replicate within host cells (7). In addition to their phagocytic role, M_{Φ} s play an important role in the balancing of pro- and anti-inflammatory mediators during ST infection. M_{Φ} s are activated by a number of cytokines including TNF- α and IFN- γ , and produce a large number of proinflammatory cytokines in response to their interactions with ST. IFN- γ , TNF- α , IL-1, IL-12 and IL-18 expression are upregulated following ST interaction, and are involved in the massive inflammatory response characteristic of systemic ST infections (31, 32) In addition to its role in M_{Φ} activation, IFN- γ has also been shown to directly inhibit bacterial replication (39). Interestingly, type-I interferons α/β (IFN-I) are also induced following ST infection (40, 41); traditionally viewed as an important antiviral defence, the role of IFN-I during non-viral infections remains unclear, and will be discussed further in section 1.2.

In addition to primary effectors like neutrophils and M_{Φ} s, dendritic cells (DCs), natural killer cells (NKs) and natural killer-T cells (NKTs) have also been shown to mediate important roles in the host response to ST (42, 43). NKs have very important, indirect effects on the innate response to ST. As the primary cellular source of IFN- γ , they are largely responsible for M_{Φ} activation (44). Strong crosstalk and reciprocal activation between NKs (IL-12/IL18 produced by M_{Φ} s) and M_{Φ} s (IFN- γ produced by NKs) enhances M_{Φ} -mediated ST clearance (42). The role of NKTs during ST infection however, is less clear. NKT cells are also thought to contribute to enhanced IFN- γ production following

oral ST infection; thus, contributing to M_{ϕ} activation (43). However, others suggest that NKs and NKTs are secondary to M_{ϕ} s and neutrophils which are the primary IFN- γ producers during primary ST infections (45). Regardless of the extent to which they produce IFN- γ , it is clear that NKs and NKTs play primarily an immunostimulatory role during ST infection inducing M_{ϕ} activation, and likely enhancing early inflammation (42, 45).

Mature DCs are known to highly influence the host adaptive response to ST in ST-resistant mice through their capacity for antigen presentation (9, 46). Maturation occurs via two distinct mechanisms; directly, by bacterial-mediated PAMP-PRR interactions, or indirectly by cytokine activation produced following PAMP-PRR interactions by infected DCs or other cell types. The resultant increase in expression of the costimulatory molecules CD80 and CD86 on the surface of DCs is absolutely required for T cell priming (46). Innate DC function includes bacterial uptake at the initial stages of ST invasion. Where invasive ST is found in both M_{ϕ} s and DCs in the lamina propria, DCs are the only cell type known to constantly sample the intestinal lumen by extending cellular processes (dendrites) between epithelial cells and engulfing ST within the lumen (46, 47). Effective phagocytosis of ST results in the production of the proinflammatory cytokines IL-1, IL-6 and IL-12 (48). Thus, they are the primary cell involved in trafficking non-invasive strains to the MLN (47) and are likely to contribute significantly to inflammation characteristic of ST infections.

ST is a facultative intracellular bacterium and while it can inhabit intracellular environments (49), where it successfully evades a number of host responses, it has also been shown to survive extracellularly (50). It is thus important to consider other innate

responses important for mediating protection against extracellular pathogens including B cell antibody-mediated responses. While B cells have shown to be important for ST clearance in resistant mice, they seem to be less important in mediating protection in susceptible mice. In resistant mice, passive resistance was shown to be conferred via serum transfer from mice vaccinated with killed ST. Interestingly, however both serum and T cell transfer were required to confer protection in ST-susceptible mice (51). Additionally, antibody mediated protection seems to be mostly important for mediating phagocytosis prior to systemic dissemination during intra-gastric and intra-peritoneal infections (52). Studies have shown that although B cells are involved in mediating ST clearance via FcR-mediated phagocytosis and opsonisation of ST, host protection requires additional adaptive mechanisms (6).

A complex interaction of innate effector cells, chemokines and cytokines contribute to the host response and inflammation characteristic of ST. Inflammation following ST infection has shown to be both protective and detrimental to hosts. In resistant mice, innate inflammatory mediators enable ST clearance and effectively initiate subsequent adaptive responses. Conversely, uncontrolled inflammation is a major contributor to the lethal outcome in susceptible mice. Lethal systemic models of ST infection result in host death when high bacterial burdens ($>10^8$) are reached in the liver and spleen (9). Death is thought to be largely due to endotoxic shock. This has been shown using a model where *Salmonella* expressing mutant LPS molecules can grow to normally lethal titers (10^9) without inducing host death in susceptible mice (9). LPS (endotoxin) is highly expressed on the surface of ST and is recognized by TLR-4 on host cells. The recognition of LPS by TLR-4 induces the production of massive quantities of pro-inflammatory cytokines

including IL-1, IL-12, IL-6 IFN- γ and TNF- α (9, 53). Inflammatory mediators activate innate effectors and mediate efficient ST clearance. Consistent with this, it has been demonstrated that the administration of antibodies against IL-12 or against TNF- α results in exacerbated ST infection in resistant mice (53, 54). However, inflammation if left unchecked, results in host cell and tissue damage, and ultimately host death (25). Tight regulation of inflammatory mediators is required for efficient pathogen protection, while maintaining host integrity. Anti-inflammatory mediators, such as IL-10, expressed upon PRR-PAMP signalling, are responsible for dampening the inflammatory response, preventing host tissue damage. Dysregulation of inflammation following ST infection is likely to be the main cause of death in ST-susceptible mice.

1.2 Type I interferons (IFN-I)

1.2 i) Overview

Type-I interferons (IFN-I) were defined in the late 1950s as host proteins induced upon viral infection, capable of interfering with viral replication (55). Since then, IFN-I have been extensively studied. In mice, IFN-I are a family of proteins consisting of numerous IFN- α subtypes and a single IFN- β . All members of the IFN-I family signal through a common receptor, the type-1 interferon α/β receptor (IFN-I R), expressed on a wide variety of cell types (56). The production of IFN-I occurs following pathogen recognition by PRRs on host cells. As discussed previously, PRRs recognize specific PAMPs and initiate intracellular signalling cascades resulting in the production of a variety of chemokines and cytokines. The same families of PRRs that induce the production of cytokines/ chemokines, TLRs, NODs, and RLRs, are also capable of inducing IFN-I

production following viral infections (57, 58). Initial signalling leads to the activation of transcription factors that regulate IFN-I transcription: c-Jun/ATF2, NFκB and IRF1. While the c-Jun/ATF1 and NFκB regulate large families of gene expression, including a variety of chemokines and cytokines in addition to IFN-I, IRF1 is primarily responsible for the transcription of IFN-β (59, 60). Following its production, IFN-β acts in both an autocrine and a paracrine fashion signalling through the IFN-I R on infected and neighbouring cells. IFN-I R signals through the JAK/STAT pathway ultimately resulting in the transcription of interferon stimulated genes (ISGs) and IFN-I subtypes by IRF3 and IRF7, respectively (59). Many ISGs directly interfere with viral replication by inhibiting mechanisms of translation, or editing or degrading viral RNA. Other ISGs include a number of cytokines and chemokines with immunomodulatory roles in innate and adaptive immunity. For example, IL-12 and IL-18 enhance NK survival and the chemokines IP-10 and RANTES are M_φ and memory T cell chemoattractants, respectively (60, 61). Numerous ISGs are involved in regulating the cell cycle, and in many cases IFN-I production results in apoptosis of virally infected cells effectively limiting pathogen spread (60-62). IFN-I, ISGs and their effects have been largely studied in the context of their antiviral functions; whether they mediate similar effects during non-viral infections was only recently addressed.

1.2 ii) Role of IFN-I in bacterial infections

Disparate results are accumulating regarding the role of this cytokine during bacterial infections. In the host response to infection by several extracellular bacteria (Group B streptococci, *S. pneumoniae* and *Escherichia coli*) IFN-I appear to be protective

(63) however, the reverse is true for the intracellular pathogens *Listeria monocytogenes* (LM) and *Mycobacterium tuberculosis* (MTb) (64-67). Interestingly, the source of IFN-I also influences the host response. With LM infection, endogenous IFN- β production leads to enhanced host susceptibility (64, 65) whereas exogenous administration of recombinant IFN- β proved to be protective (68). The detrimental effects of IFN-I production on host survival during LM infection have recently been established. IFN-I R^{-/-} mice displayed enhanced survival (64-66), which correlated with increased expression of pro-inflammatory mediators (65), IL-12 and IFN- γ , enhanced bacterial clearance and enhanced M ϕ and T cell survival (66, 69, 70). In addition to its pro-inflammatory properties, IFN-I is a potent inducer of nitric oxide (NO) which mediates a variety of cytostatic, cytotoxic and inflammatory events following infection (71). With respect to LM infection, it has been associated with both necrotic and apoptotic cell death (66, 69, 70). While the role of IFN-I during LM infection has been addressed, the role of IFN-I during other bacterial infections is not clear. Since pathogens vary in virulence, cell wall structure, intracellular life-style, and the type and magnitude of the inflammation induced, all of these factors may influence the role that IFN-I play during infection with other bacterial species. The evidence that three extracellular bacteria, group B streptococci, *S. pneumoniae* and *E.coli* (two of which are gram positive and one gram negative) all indicate protective roles for IFN-I on host survival (63) supports the idea that it is the cellular location of the pathogen and not surface structure that is more important for determining the protective capacity of IFN-I during bacterial infections. New insights into this idea will prove interesting given the paradoxical role of IFN-I in mediating host protection during bacterial and viral infections. Interestingly, despite knowing little about the cellular and/or cytoplasmic receptors

responsible for initiating cellular signalling, the pathways leading to LM- and MTb-induced IFN-I production mimic those induced following viral recognition (67, 72). To date our knowledge is limited to a single study that demonstrated IFN-I production following LM infection is both TLR and NOD2-independent (72).

Numerous *in vitro* studies have shown receptor-antagonist combinations that elicit an IFN-I response following signalling by bacterial PAMPS; this is true for members of the NOD, RLR and TLR families (60). Of particular interest to ST infection, TLR-4 and its antagonist LPS produce large amounts of IFN-I following signalling (29, 73). Signalling through TLR-4 follows either MyD88-dependent or –independent pathways resulting in the activation of NFκB and NFκB and IRF3, respectively (29). Since IRF-3 is largely *IFN-β*-specific, it is likely that the MyD88-independent signalling mechanism contributes to most, if not all of the IFN-I production following TLR-4 stimulation by LPS (25, 26, 29).

Since susceptibility to ST is determined during the innate phase of the host response, early innate responses are crucial for determining host outcome. Elucidation of the role of specific innate mediators, including IFN-I, during ST infection will therefore provide insight into the innate response that is so crucial for determining host resistance.

2. Rationale, Hypothesis and Objectives

2.1 Rationale

It is well established that T cell responses are crucial for mediating long-term protection to the facultative intracellular pathogen, ST (6). However, susceptibility to ST occurs rapidly within the first seven days, and is under genetic control. Previous work in our lab has shown that ST induces a delayed T cell response in ST-resistant animals, reaching peak activation at day 15 post infection (Fig. 1). Susceptible mice succumb to ST infection within the first seven days, prior to the onset of T cell activation (Fig. 2). This observation shifts the focus into investigating the earlier innate responses that could regulate such a detrimental outcome.

Susceptibility to ST is thought to be due, in large part to LPS-mediated endotoxic shock. TLR-4 recognition of LPS elicits an immediate, massive inflammatory response with limited control. IFN-I, expressed during bacterial and viral infections typically results in the enhanced inflammation (40, 74) and death of M ϕ s and T cells (66, 69).

Type-I interferons play important but disparate roles in host defence against bacterial pathogens, enhancing host clearance of a group of extracellular pathogens (63) while exacerbating infection in the case of the intracellular pathogens LM and MTb (64-66). The suggestion that type-I interferons play distinct roles during bacterial infections based on the preferred residence of the pathogen within the host is new and thus is limited by the small number of studies that have addressed the question directly. Examination of the role of IFN-I during infection with the facultative intracellular pathogen, ST, may provide insight into the seemingly conflicting roles of IFN-I signalling during bacterial infections.

Fig. 1. T cell activation is delayed following ST infection, when compared with LM. B6.129F1 mice were infected with 10^3 ST or 10^3 LM i.v.. Days 1, 2, 3 and 6 p.i. spleens were aseptically removed and homogenized to a single cell suspension. To enumerate CFUs, ten-fold serial dilutions in 0.9% saline were plated on BHI agar and incubated at 37 °C overnight (A). On days 0, 1, 3 and 6 spleens were aseptically removed and homogenized to a single cell suspension. Splenocytes were incubated with fluorescently labelled antibodies against cell surface markers of interest. T cell ($CD3^+$) activation was assessed by flow cytometry. Circles and numbers within each panel indicate the percentage of T cells that display an activated phenotype ($CD62L^{low} CD44^{high}$) at each time point following LM or ST infection (B).

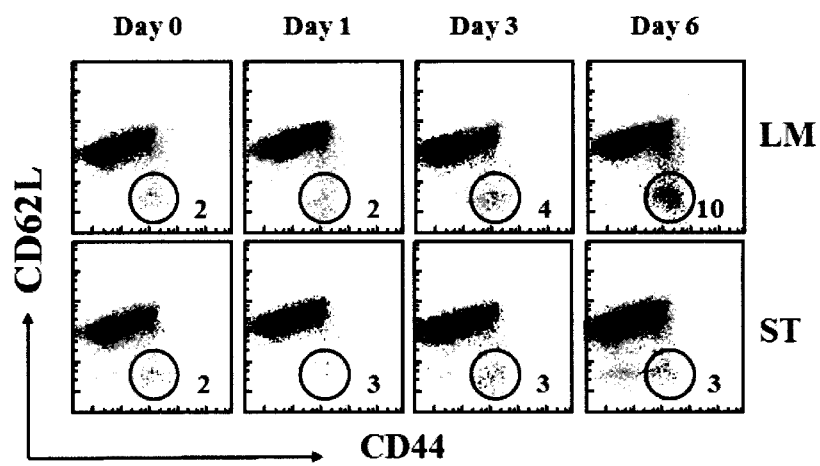
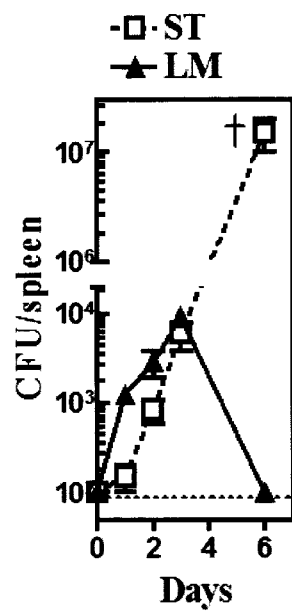
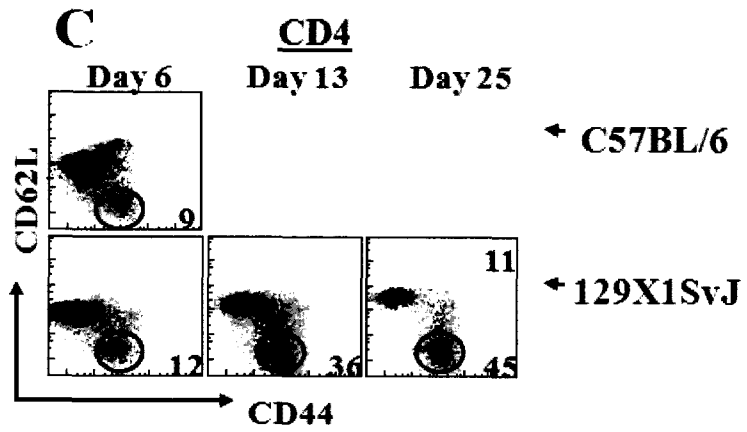
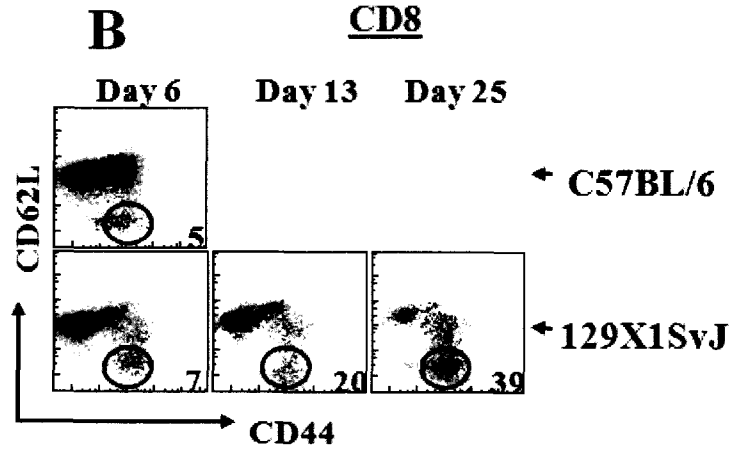
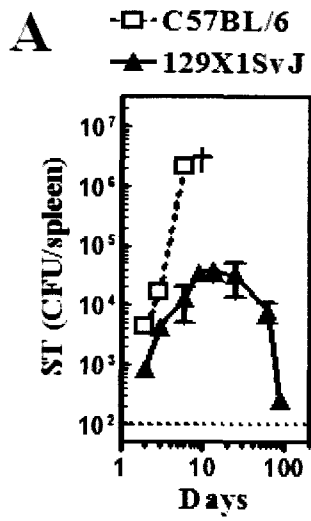


Fig. 2. C57BL/6 mice succumb to infection prior to T cell activation. C57BL/6 and 129X1SvJ mice were infected with 10^3 ST i.v.. At various time points following infection spleens were aseptically removed and homogenized to a single cell suspension. To enumerate CFUs, ten-fold serial dilutions in 0.9% saline were plated on BHI agar and incubated at 37 °C overnight (A). On days 6, 13 and 25 p.i., spleens were aseptically removed and homogenized to a single cell suspension. Splenocytes were incubated with fluorescently labelled antibodies against cell surface markers of interest. CD4⁺ (upper panels) and CD8⁺ (lower panels) T cell activation was assessed by flow cytometry. Circles and numbers within each panel indicate the percentage of cells that display an activated phenotype (CD62L^{low} CD44^{high}) at each time point following ST infection (B).



In this study susceptibility to ST infection is defined by host lethality. Resistance is defined as the ability of hosts to mount effective innate and adaptive responses, resulting in ST clearance and host survival. Thus, mice displaying ‘enhanced resistance’, although not ‘resistant’ to ST infection, effectively delay the lethality of infection.

2.2 Hypothesis:

Type-I interferons are potent pro-inflammatory cytokines produced by host cells significantly following viral infection, and are responsible for mediating cell death in a variety of host cells, including M_{ϕ} s and T cells (66, 69, 70). While this is an important mechanism for host protection in preventing the spread of viral infections, the elimination of M_{ϕ} s, important for phagocytic uptake and subsequent ST clearance, limits the capacity of hosts to control bacterial infection.

ST pathogenesis in susceptible wild-type (WT) C57BL/6 mice is characterized by massive and uncontrolled inflammation in the spleen, resulting in host death by day 7 post-infection. **I propose that IFN-I mediates enhanced susceptibility to ST infection by its inherent ability to limit the activation of key cells, facilitate cell death in macrophages and initiate uncontrolled inflammation ultimately enhancing host pathology.**

2.3 Objectives:

1) To examine the role of inflammatory mediators on host survival during ST infection.

- i) Evaluate the role of key inflammatory mediators in mediating host survival against ST.
- ii) Determine the effects of enhancing innate immune responses during ST infection using CpG.

2) To examine the role of IFN-I in the host response to ST infection.

- i) Evaluate the influence of IFN-I on host survival, inflammation and bacterial burden.
- ii) Evaluate the mechanism by which IFN-I modulates the host response to ST infection.

3. Materials and Methods

3.1 Mice:

6-8week old female C57BL/6, B6.129S7-ifngtm1Ts (IFN γ ^{-/-}), B6.129P2-Nos2^{tm1Lau} (Nos2^{-/-}), B6.129S-Tnfrsfla^{tm1Tmx}Tnfrsflb^{tm1lmx} (TNF α ^{-/-}), B6.129-II6^{Tm1Kopf} (IL-6^{-/-}), B6.129S6-IL10^{tm1Flv} (IL-10^{-/-}) and C57BL/6J-Ticam1^{Lps2} (TRIF^{-/-}) mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA). IFN-I R^{-/-} mice on the SvJ background were backcrossed on the C57BL/6 background for 14 generations and bred in-house at the animal care facility at NRC (Ottawa, ON, CA). The original IFN-I R^{-/-} breeding pair were a gift from Dr. Kaja Murali-Krishna. B6.129 mice were bred in-house at the animal care facility at NRC (Ottawa, ON, CA) by mating female 1291XSvJ mice with male C57BL/6 mice. All mice were housed under specific pathogen-free conditions in the animal care facility at NRC (Ottawa, ON, CA) in accordance with the regulations of the Canadian Council on Animal Care.

3.2 Bacteria and Infection Model:

Salmonella typhimurium SL1344 (ST) was grown in liquid brain-heart infusion (BHI) medium (Difco Laboratories). Bacteria were harvested at mid-log phase (O.D._{600nm} = 0.8) and frozen at -80°C in 20% glycerol in BHI. CFUs of frozen stocks were determined by performing 10-fold serial dilutions in 0.9% NaCl and spread onto BHI agar plates.

- i) ***in vivo* infection:** Frozen bacterial stocks were thawed immediately prior to use and were diluted to the desired infectious dose of 10² bacteria/200ul in 0.9% NaCl. All

mice were infected either intravenously (i.v.) via the lateral tail vein, or intraperitoneally (i.p.) where indicated.

ii) ***in vitro* infection of bone marrow-derived macrophages:** Bone marrow was harvested from both C57BL/6 and IFN-I R^{-/-} mice. Bone marrow-derived macrophages (BMMs) were cultured in RPMI+8%FCS (R8) media supplemented with 10ng/ml M-CSF. Cells were gently washed on days 2 and 4 to remove non-adherent cells. Adherent cells remained in culture in M-CSF/R8. On day 8 BMMs were washed and incubated in PBS for 10min at 37°C to remove the adherent BMMs and were subsequently seeded onto a 96-well U-bottom plate at a concentration of 10⁵ cells/well. ST stocks were thawed immediately prior to use, washed and resuspended in R8 at 10⁶ ST/100ul R8. BMMs were treated with either 100ul of the ST suspension (10 MOI) or R8 alone (control). Plates were centrifuged for 10min at 1600rpm and incubated at 37°C for 20min. Cells were centrifuged, washed and resuspended in 50ug/ml gentamycin in R8 for 2h to eliminate extracellular bacteria. Subsequently cells were centrifuged, washed and resuspended in 10ug/ml gentamycin in R8 and incubated at 37°C. At various time points following infection, cells were assessed for intracellular bacterial burden or cell viability (Neutral Red Dye Cytotoxicity Bioassay) and supernatants were stored at -70°C for subsequent cytokine ELISAs and TNFα bioassays.

iii) ***in vitro* infection of bone marrow-derived dendritic cells:** Bone marrow was harvested from both C57BL/6 and IFN-I R^{-/-} mice. Bone marrow-derived dendritic cells (BMDCs) were cultured in R8 media supplemented with 5ng/ml GM-CSF. Cells were gently washed on days 2 and 4 to remove non-adherent cells. Adherent

cells remained in culture in GM-CSF/R8. On day 6 BMDCs were washed and subsequently seeded onto a 96-well U-bottom plate at a concentration of 10^5 cells/well. ST stocks were thawed immediately prior to use, washed and resuspended in R8 at 10^6 ST/100ul R8. BMDCs were treated with either 100ul of the ST suspension (10 MOI) or R8 alone (control). Plates were centrifuged for 10min at 1600rpm and incubated at 37°C for 20min. Cells were centrifuged, washed and resuspended in 50ug/ml gentamycin in R8 for 2h to eliminate extracellular bacteria. Subsequently cells were centrifuged, washed and resuspended in 10ug/ml gentamycin in R8 and incubated at 37°C . At various time points following infection, cells were assessed for intracellular bacterial burden or cell viability (Neutral Red Dye Cytotoxicity Bioassay) and supernatants were stored at -70°C for subsequent cytokine ELISAs and TNF α bioassays.

3.3 Assessment of Survival:

Mice weights prior to infection were recorded. Subsequently, a variety of knockout mice (all on the C57BL/6 background) and WT controls were infected with 10^2 ST i.v. Mouse weights were monitored daily and mice were euthanized by CO₂ asphyxiation and cervical dislocation when a drop in body weight of $>15\%$ was observed, or at the first sign of distress (ex. Erector pili, hunching, decreased mobility).

3.4 Assessment of Bacterial Burden:

- i) **Whole Organ:** Mice were euthanized by CO₂ asphyxiation followed by cervical dislocation and spleens and livers were removed aseptically. Spleens were

homogenized between the frosted ends of two glass slides in 10mL RPMI, whereas livers were homogenized using a motorized homogenizer in 10mL 0.9% NaCl. Ten-fold serial dilutions of the tissue homogenate were diluted in 0.9% NaCl and 100ul samples from each were plated on BHI agar. Bacterial colonies were counted after 24h incubation at 37°C. Where burden was assessed for spleen supernatant and cell fractions separately, following whole spleen CFU, the spleen suspension was centrifuged at 1600rpm for 10min. The supernatant was removed, vortexed and the cellular pellet was resuspended in 10mL RPMI. Ten-fold serial dilutions were performed separately for both fractions in 0.9% NaCl and plated on BHI agar. CFU assessment was again determined following incubation at 37°C for 24h.

- ii) **Intracellular Burden:** Intracellular burden was assessed in BMM and BMDC cultures; supernatants were removed and cells were lysed using lysis buffer (1% tritonX, 0.1% SDS in PBS, pH 7.2). Cell lysates were plated on BHI agar and colonies were counted after 24h incubation at 37°C. Additionally, CFUs were assessed by a similar protocol in purified cell samples (F4/80⁺ and, separately, CD11c⁺ cell fractions).

3.5 Flow Cytometry:

Cell populations in the spleen were monitored using the BD FACSCanto™ flow cytometer. At various time points following infection mice were euthanized by CO₂ asphyxiation followed by cervical dislocation. Spleens were aseptically removed and smushed between the frosted ends of two glass slides. Cell suspensions were strained and resuspended in PBS/1%BSA. Cells were incubated with Fc block and subsequently

combinations of antibodies of interest. All antibodies (NK1.1, CD3, F4/80, CD11b, CD11c, Gr-1, CD8, CD4, CD44 and CD62L) were purchased from BD Biosciences (Mississauga, ON, CA). Cells were washed and resuspended in 0.5% fixative and acquired immediately. All analysis was performed using BD FACSDiVa™ software.

3.6 Cell Purification:

Age matched C57BL/6 and IFN-I R^{-/-} mice were infected with 10² ST i.v. At various time points following infection, mice were euthanized using CO₂ asphyxiation followed by cervical dislocation and spleens were aseptically removed. In some cases mice were infected with 10² ST i.p. and cell separation was performed from peritoneal lavage samples. F4/80⁺ and CD11c⁺ cell purification was performed using the Stem Cell PE selection kit and the CD11c⁺ selection kit respectively, as described by the manufacturer's instruction (STEMCELL Technologies, Vancouver, CA). In brief, spleens were homogenized in dissociation medium and rocked at room temperature for 30min. Spleens were further homogenized by passing the suspension through a 16 gauge needle. Suspensions were incubated with 10mM EDTA and subsequently labeled with α-F4/80-PE (BD Biosciences), or α-CD11c antibody followed by incubation with PE selection cocktail and magnetic nanoparticles. Cell suspensions were placed in StemCell magnets and magnetic separations was achieved by pouring off the supernatant. Purified cells were resuspended in separation buffer. Cell counts and CFUs were performed; purity was assessed by flow cytometry (BD Biosciences, FACSCanto™, Analysed using FACSDiva™ Software)

3.7 Cytokine Analysis

- i) ***TNF Bioassay:*** WeHi cells (TNF α – susceptible cells) were seeded at 5×10^3 cells/well on a 96-well flat bottom plate. The standard curve concentration was standardized and two standard curves with two-fold dilutions of TNF α (Endogen, Woburn, USA) were used for comparison. Cell supernatants from BMM and BMDCs were diluted two-fold 8 times. Standards and unknown dilutions were incubated with the WeHi cells at 37°C overnight. Cell viability was assessed at 24h using the MTT cytotoxicity bioassay.
- ii) ***NO₂⁻ Greiss Reagent System:*** BMM and BMDC cell culture supernatants were removed at various time points following *in vitro* infection. Supernatants were diluted two-fold four times and plated on a 96 well plate. Serial dilutions of NaNO₂ beginning with 25uM NaNO₂ were used for a standard curve. Greiss reagent 1 (1% sulphanimide in 2.5% H₃PO₄) and Greiss reagent 2 (0.1% naphthylethylenediamine dihydrochloride in 2.5% H₃PO₄) were added to the standards and the samples in order. Absorbance was measured on a plate reader set to 570nm. Analysis was performed using SoftMax[®] Pro software.
- iii) ***Cytokine ELISAs:*** Blood was obtained by cardiac puncture under anesthesia, before euthanasia of mice and was collected in microtainer serum separator tubes (BD Biosciences, Mississauga, Canada). Blood samples were allowed to clot at 4°C and subsequently, serum was separated by high-speed ultra-centrifugation, and stored at -70°C. Levels of serum IL-6 and IL-12p40/p70 were assayed by sandwich

ELISA. IL-6 (clone MP5-20F3) and IL-12p40/70 (clone 551219) antibody pairs were purchased from BD Biosciences, Mississauga, Canada. Cytokine standards were purchased from ID Labs (London, Canada). Duplicate standard curves encompassing several doubling dilutions of the standard were included on each plate. All serum samples were assayed at the same time to minimize day-to-day error in cytokine detection. The detection limits for cytokines in the ELISAs were 200 pg/ml for IL6, 100 pg/ml for IL12p40/70. Cytokine ELISAs were also performed on BMM and BMDC cultures in addition to serum samples. Absorbance measurements were taken on a plate reader at 450nm with a 570nm reference wavelength and were analyzed using SoftMax[®]Pro software.

iv) ***Mouse proteome array:*** Blood was obtained by cardiac puncture under anesthesia, before euthanasia of mice and collected in microtainer serum separator tubes (BD biosciences). Blood samples were allowed to clot at 4°C, and the serum was subsequently separated by high-speed ultra-centrifugation, and stored at -70°C. Relative levels of 40 different mouse cytokines/chemokines were detected using the Proteome Profiler[™] Mouse Cytokine Array Panel A Array Kit from R&D systems (Minneapolis, USA). Each array kit consists of 4 nitrocellulose membranes each consisting of duplicate spots of 40 different anti-cytokine antibodies, a detection antibody cocktail, array buffers (buffer number 4, 5 and 6) and wash buffer concentrate. Membranes were processed using the manufacturer's protocol and treated with SuperSignal[™] West Femto chemiluminiscent detection reagent for 2-3 minutes. The photons emitted by the membranes were then captured by a

Flouorochem 8900 (Alpha Innotech) imager. Spot densitometry analysis was completed using AlphaEase™ imaging software.

- v) ***qRT-PCR***: Spleens from infected and non-infected WT and IFN-I R^{-/-} mice were aseptically removed immediately following CO₂ asphyxiation and cervical dislocation. Spleens were immediately snap frozen in a dry ice/ 100% ethanol bath. Spleens were cut into small sections, placed in 1ml of lysis buffer and were homogenized further in a MiniBeadbeater3110BX (BioSpec Products) with 0.5mm and 0.1mm glass beads (BioSpec Products). Total RNA was extracted using the Qiagen RNeasy mini kit according to the manufacturer's instructions. Following extraction, total RNA was treated with RNase-free DNase I (Roche, Applied Science) for 30 min at 37°C. DNase was removed and 2-5µg of total RNA was used for cDNA synthesis using AncT primers (Sigma-Aldrich). RNA was made linear at 65°C for 5 min and cDNA was synthesized in a 40-µl reaction volume containing: 1.5 µl of AncT primers (100 pM/µl), 8 µl 5 x first-strand buffer, 4 µl of DTT (100 mM), 5 µl of dNTP (5 mM), 1 µl of RNase OUT (40 U/µl), 2 µl of Superscript II (200 U/µl) (Invitrogen Life Technologies), and 15 µl of RNA template. Reverse transcription was performed in a Thermo Cycler 9700 (Applied Biosystems) at 42°C for 15 min and 45°C for 2 h. Identical samples not treated with Superscript II were also prepared as controls to measure DNA contamination. The remaining RNA template was hydrolyzed with 1 M NaOH at 65°C for 5 min and neutralized with 1 M HCl. cDNA was purified using Microcon YM-30 centrifugal filter units (Millipore). The number of amplicons was measured by quantitative real-time PCR using gene-specific primers and quantitative PCR SYBR green supermix (ABgene).

Gene-specific primers were designed using Primer Express 2.0; β actin was used as an internal reference control. Ten-fold dilutions of cDNA were used as template to generate the standard curve for each primer-template set (1x, 1/10x, 1/100x, 1/1000x). This standard curve was run together with triplicate reactions of the uncharacterized samples. PCR was performed in sealed tubes in a 96-well microtiter plate in an ABI Prism 7000 thermocycler (Applied Biosystems). The 25- μ l reaction consisted of 12.5 μ l of quantitative PCR SYBR green supermix, 2.5 μ l of primer mix (1.5 pM/ μ l each), and 10 μ l of template. Thermal conditions were as follows: activation at 95°C for 15 min, followed by 40 cycles of denaturation at 95°C for 15 sec, annealing at 60°C for 1 min, and extension at 72°C for 1 min. Fluorescence was measured during the annealing step and plotted against the amplification cycle. Relative quantitative analysis of the data was extrapolated from the standard curve. Primer efficiencies were 98–100%.

3.8 Cytotoxicity Assays

i) MTT Cytotoxicity Bioassay

Following *in vitro* ST infection of BMDCs or BMMs, cell supernatants were aspirated and 5mg/ml MTT in PBS was added to the culture wells. Plates were incubated 37°C for 4 hours. The MTT solution was discarded and 150ul of isopropanol/HCl was added to each well. Thorough mixing solubilized the dye and absorption at 570nm (650nm reference) was measured on a plate reader. Analysis was performed using SoftMax[®] Pro software.

ii) Neutral Red Dye Cytotoxicity Bioassay

Neutral Red Dye cytotoxicity kit (Xenometrix AG, Switzerland) was used to assess cell viability of BMM and BMDC following *in vitro* infection. The kit was used according to the manufacturer's instructions. In brief, culture media was aspirated and replaced with a 1:200 dilution of NR labeling solution in R8 (dilution ensured optimal dye uptake with no extracellular crystal formation). Cells were incubated at 37°C for 4 hours and subsequently inspected under the microscope to confirm dye uptake by the cells. The labeling solution was aspirated and cells were washed with fixative solution (0.1% CaCl₂ in 0.5% Formaldehyde) for 1 minute. Finally, the fixative solution was removed and the dye solubilized using the solubilization solution (1% acetic acid in 50% ethanol). The colorimetric reading was measured in a plate reader at 540nm with a reference wavelength of 690nm. Analysis was performed using SoftMax[®]Pro software.

iii) Cellular Imaging

To observe BMM morphology following ST infection BMMs (infected with ST *in vitro*) were imaged on the Olympus IX81 inverted fluorescence microscope (100X) at 24, 48 and 72 hr post-infection.

3.9 Type-I interferon neutralization:

BMM and BMDC cell suspensions derived from IFN-I R^{-/-} and C57BL/6 mice were seeded onto a 96-well U-bottom plate at a concentration of 10⁵ cells/well. ST stocks were thawed immediately prior to use, washed and resuspended in R8 at 10⁶ ST/100ul R8. 100ul was added to the appropriate wells for an infectious dose of 10 MOI. Plates were

centrifuged for 10min at 1600rpm and incubated at 37°C for 20min. Cells were centrifuged, washed and resuspended in 50ug/ml gentamycin in R8 for 2h to eliminate extracellular bacteria. Subsequently cells were centrifuged, washed and resuspended in 10ug/ml gentamycin in R8 alone or containing one of the three following treatments: i) 10ug/ml α -IFN- α , ii) 10ug/ml α -IFN- β , or iii) 10ug/ml α -IFN- α and 10ug/ml α -IFN- β . Cells were incubated at 37°C for 48h at which point cell viability was assessed by the Neutral Red Dye Cytotoxicity Bioassay. Both monoclonal antibodies used for this assay were purchased from PBL Interferon Source (Piscataway, NJ, USA).

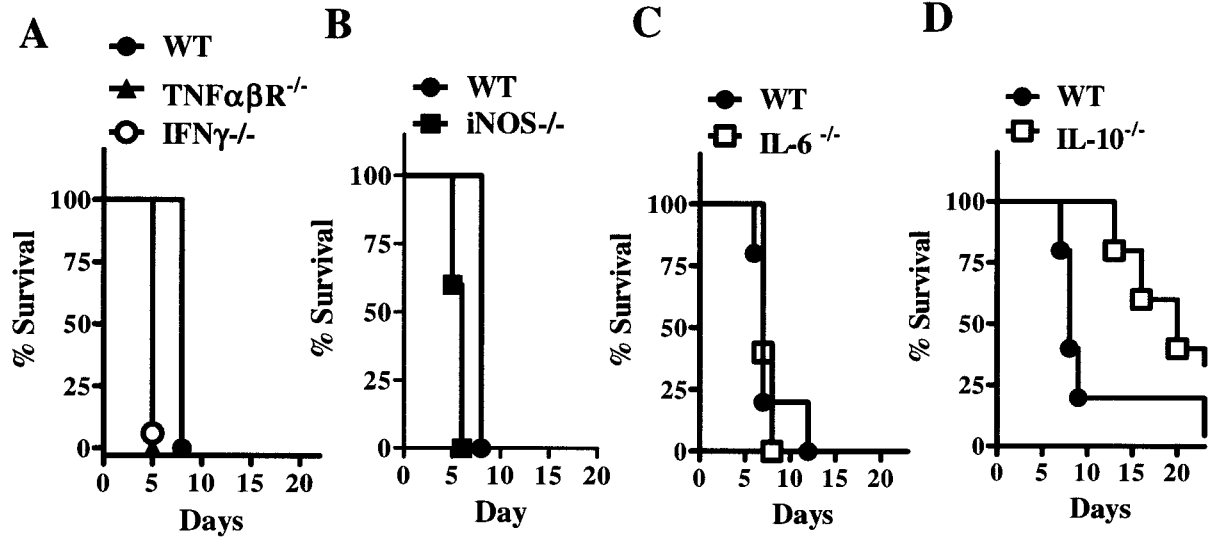
4. Results

4.1 Inflammatory mediators are crucial for innate host survival

Previous work in our lab has shown that even in mice resistant to ST infection, T cell responses are greatly delayed relative to other intracellular pathogens such as *Listeria monocytogenes* (Fig. 1) indicating that although acquired immunity may participate later on, it is the innate immune compartment that plays a key role in controlling ST early during infection. Innate immunity is crucial for effectively mediating rapid host protection since susceptible mice succumb to ST infection prior to the activation of T cell responses (Fig. 2, B and C) and is associated with uncontrolled bacterial replication within the spleen (Fig. 2, A).

Since the innate immune response is comprised of numerous inflammatory mediators and cell types, experiments were outlined to delineate the cells and cytokines involved in mediating resistance against ST. TNF α and IFN γ play key roles in facilitating survival against a number of pathogens. To evaluate their role in controlling pathogenesis in ST-susceptible mice, survival was evaluated following ST infection in C57BL/6 mice deficient of the TNF α / β receptor (TNF α / β R $^{-/-}$) or in IFN γ production (IFN γ $^{-/-}$) (Fig. 3, A). Mice were infected with 10^2 ST i.v. and the weights of individual mice were monitored daily. Mice that displayed a loss in body weight of greater than 15% were euthanized. Not surprisingly, mice deficient in either TNF α / β or IFN γ signalling displayed enhanced susceptibility to ST infection when compared with C57BL/6 (WT) controls. Whereas WT mice survived ST infection up to day 7 post-infection (p.i.), all TNF α / β R $^{-/-}$ and IFN γ $^{-/-}$ mice succumbed to ST infection by day 5 p.i. (Fig. 3, A). Similarly, mice deficient in the inducible nitric oxide synthase (iNOS $^{-/-}$) displayed enhanced susceptibility to ST infection

Fig. 3. Innate mediators affect host survival during ST infection. Mice that are deficient in key inflammatory mediators (iNOS2^{-/-}, TNF- α / β R^{-/-}, IFN- γ ^{-/-}, IL-6^{-/-}, IL-10^{-/-}) were infected with 10² ST i.v. Normal wild-type C57BL/6 mice served as controls. Survival was assessed by monitoring the weights of individual mice daily. Where a >15% drop in body weight was observed, mice were euthanized; the end point indicated; n=5.



compared with their WT counterparts. *iNOS*^{-/-} mice succumbed to ST infection by day 6 p.i.; one day prior to WT mice (Fig. 3, B). Comparison of survival rates revealed no significant difference among any of the groups, given the rapidity with which WT mice succumb to ST infection.

IL-6 is rapidly induced following ST infection (31, 32); however it had no influence on host survival in susceptible mice. IL-6 deficient mice (*IL-6*^{-/-}) displayed similar survival to ST infection when compared with WT mice (Fig. 3, C). In contrast, mice deficient of the anti-inflammatory cytokine IL-10 (*IL-10*^{-/-}) displayed significantly enhanced survival when compared with WT mice, surviving a minimum of 7 days longer than WT mice before displaying susceptibility to ST infection (Fig. 3, D). These results indicate that several inflammatory mediators (*IFN* γ , *TNF* α/β , and nitrite ions) appear to control ST pathogenesis in susceptible mice. The anti-inflammatory cytokine IL-10, however, had the most pronounced effect on host survival.

Cytokines are predominantly produced following recognition of pathogen associated molecular patterns (PAMPs) by pathogen recognition receptors (PRRs) resulting in signalling via the MyD88-dependent and -independent pathways. I therefore examined the involvement of the MyD88-independent signalling pathway in mediating host protection by evaluating the survival of mice deficient in the MyD88-independent signalling adapter molecule TRIF (*TRIF*^{-/-}). WT and *TRIF*^{-/-} mice were infected with 10² ST i.v. and the weights of individual mice were monitored daily. Mice displaying greater than 15% loss of body weight were euthanized; the endpoint indicated. *TRIF*^{-/-} mice displayed a trend towards increased host survival following ST infection (Fig. 4)

suggesting that innate mediators produced as a consequence of MyD88-independent signalling influence host protection during ST infection.

4.2 CpG treatment renders normally resistant mice susceptible to ST infection

Since mediators of the innate immune response are important for host defence, I evaluated the influence of amplification of the innate immune response on ST pathogenesis. As CpG has been widely used to amplify innate immunity, the influence of amplification of the innate immune response by CpG on host susceptibility following ST challenge was evaluated. Since ST-susceptible C57BL/6 mice succumb to infection so rapidly, the effects of CpG on ST pathogenesis in resistant mice (F1 hybrids of ST-resistant 129X1SvJ and ST-susceptible C57BL/6 mice) were delineated. B6.129F1 mice were infected with 10^3 ST (i.v.) and concomitantly treated with either 50 μ g CpG intraperitoneally (i.p.) or PBS (control). The weights of individual mice were monitored daily and mice were euthanized when a drop in body weight greater than 15% was observed. Surprisingly, CpG treatment rendered B6.129F1 mice susceptible to ST infection by 20 days p.i. (Fig. 5, A). Prophylactic CpG treatment (three days prior to ST infection) of B6.129F1 resulted in similar mortality (data not shown) indicating that CpG treatment enhances susceptibility to ST infection independently of the dosing schedule.

To evaluate the influence of CpG on ST pathogenesis, ST burden in the spleen was assessed over the course of infection. Mice were euthanized on days 3, 7 and 15 p.i.; spleens aseptically removed and homogenized. Serial dilutions of spleen homogenates were plated on BHI agar plates and CFUs were enumerated after incubation at 37°C for 24hr (Fig. 5, B). Increased susceptibility of CpG treated mice to ST infection was

Fig. 4. Survival of mice is not modulated significantly by the absence of MyD88-independent signalling. Mice deficient in the MyD88-independent adapter signalling molecule TRIF (TRIF^{-/-}) and WT mice were infected with 10² ST i.v. Survival was assessed by monitoring the weights of individual mice daily. Where a >15% drop in body mass was observed, mice were euthanized; the end point indicated; n=5.

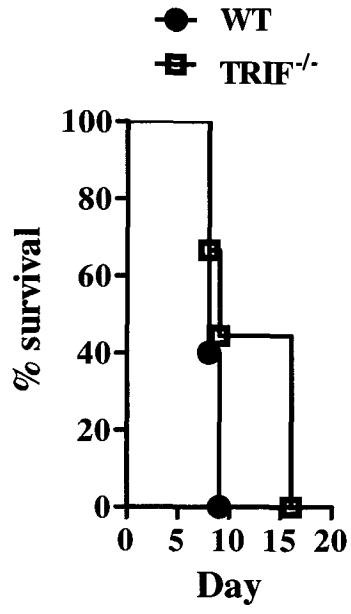
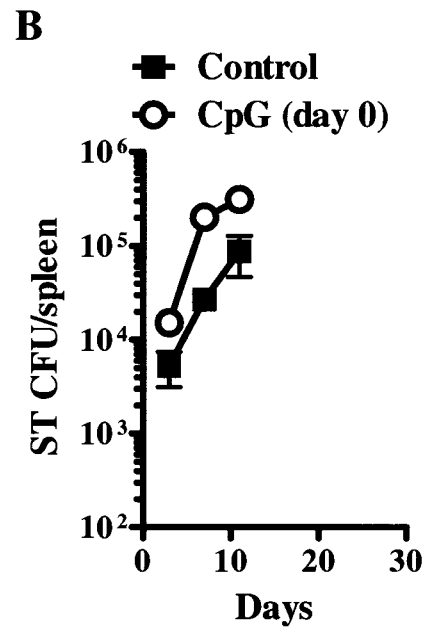
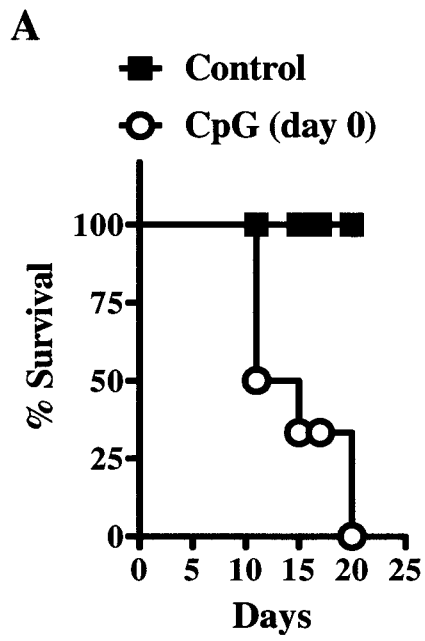


Fig. 5. CpG treatment renders normally resistant mice susceptible to ST. B6.129F1 mice were infected with 10^3 ST i.v. Concomitantly, mice received CpG treatment (50 $\mu\text{g}/\text{mouse}$ given intraperitoneally) or PBS (Control). Survival was assessed by monitoring the weights of individual mice daily. Where a $>15\%$ drop in body weight was observed, mice were euthanized; the end point indicated. Data are representative of two independent experiments, $n=5$ (A). ST burden in the spleen was assessed at day 3, 7 and 11 p.i. At each time point spleens were aseptically removed and homogenized to a single cell suspension. To enumerate bacterial burden, ten-fold serial dilutions in 0.9% saline were plated on BHI agar plates and incubated at 37°C overnight. Data represents mean CFU values $\pm\text{SEM}$ and are representative of two independent experiments; $n=3$ (B).



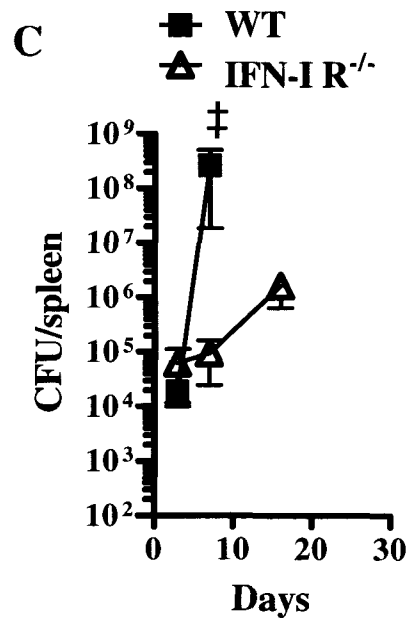
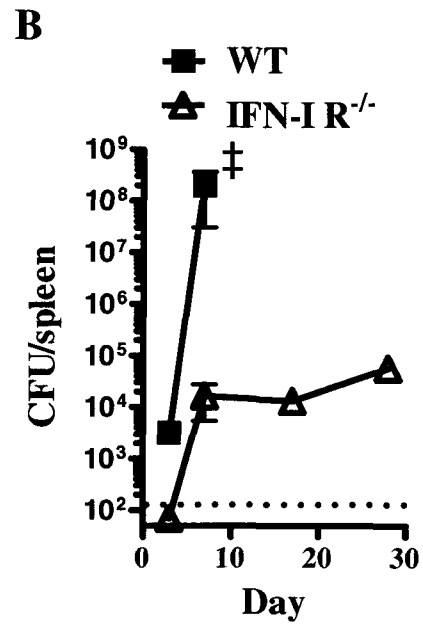
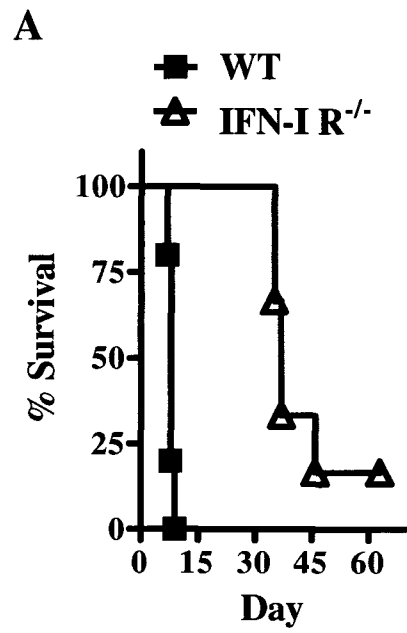
correlated with significantly higher ST burden in the spleen as compared to PBS treated mice throughout the course of infection (Fig. 5). At each time point evaluated, CFUs in CpG treated mice were 5-10 fold higher than PBS treated controls (Fig. 5). It is important to note, however, that the bacterial burdens in the CpG treated group did not reach lethal ST levels ($>10^6$ /spleen), yet B6.129F1 mice became susceptible. It is therefore likely that CpG treatment in ST infected mice results in hyperactivation of the innate immune response leading to host susceptibility.

4.3 Effects of IFN-I signalling on host survival

Since the nature of the inflammatory response following CpG treatment resulted in enhanced host susceptibility to ST infection and it is known that CpG-TLR-9 engagement results in strong production of type-I interferons (IFN-I), I investigated the role of IFN-I on host survival during lethal ST infection. To this end, wild-type C57BL/6 (WT) mice and IFN-I receptor-deficient mice (IFN-I R^{-/-}) (on the C57BL/6 background) were infected with 10^2 ST i.v. and the weight of individual mice was monitored daily. Mice were euthanized after displaying a drop in body weights of greater than 15%. Interestingly, IFN-I R^{-/-} mice displayed enhanced survival as compared to WT mice (Fig. 6, A) indicating that IFN-I-receptor engagement negatively influences host resistance to ST infection. Whereas WT mice succumbed to ST infection by day 7 p.i., susceptibility in IFN-I R^{-/-} mice was delayed up to day 35 p.i. (Fig. 6, A).

To evaluate the role of IFN-I receptor engagement on ST pathogenesis, ST burden in the spleens of infected WT and IFN-I R^{-/-} mice was evaluated throughout infection.

Fig. 6. Effects of IFN-I on host survival and bacterial burden during ST infection. Type I IFN receptor deficient mice (IFN-I R^{-/-}) and WT mice were infected with 10² ST i.v. Survival was assessed by monitoring the weights of individual mice daily. Where a >15% drop in body weight was observed, mice were euthanized; the end point indicated. Data are representative of three independent experiments, n=5 (A). ST burden in the spleen was assessed at day 3, 7, 15 and 30 p.i. At each time point spleens were aseptically removed and homogenized to a single cell suspension. To enumerate bacterial burden, 10-fold serial dilutions in 0.9% saline were plated on BHI agar plates and incubated at 37°C overnight. Data represent mean CFU values ± SEM and are representative of three independent experiments; n=3 (B). IFN-I R^{-/-} and WT mice were infected with 10² ST given intraperitoneally. ST burden in the spleen was assessed at day 3, 7 and 15 p.i. At each time point spleens were aseptically removed and homogenized to a single cell suspension. To enumerate bacterial burden, 10-fold serial dilutions in 0.9% saline were plated on BHI agar and incubated at 37°C overnight. Data represent mean CFU values ± SEM and are representative of two independent experiments; n=3 (C). WT and IFN-I R^{-/-} survival curves are significantly different according to the Gehan-Breslow-Wilcoxon test, p=0.0018 (A). ‡Indicates the survival limit for WT mice.



On days 3, 7, 15 and 30 p.i. spleens were aseptically removed and homogenized to a single cell suspension. Serial dilutions of spleen homogenates were plated on BHI agar and CFUs were enumerated after 24hr at 37°C. At all time points, IFN-I R^{-/-} mice harboured fewer ST than their WT counterparts (Fig. 6, B). By day 7 p.i., ST burden in the spleens of WT mice rose to lethal levels (10⁹), however burden in the spleens of IFN-I R^{-/-} mice peaked at 10⁴ ST per spleen and was maintained at this level for the remainder of infection (Fig. 6, B). These data suggest that IFN-I-receptor engagement plays a role in regulating ST clearance.

Furthermore, similar survival trends occurred when mice were infected with ST i.p.; IFN-I R^{-/-} mice displayed enhanced survival (data not shown) and lower ST burden in the spleen (Fig. 6, C) as compared to WT mice. These data indicate that IFN-I regulates ST clearance in a manner independent of the route of infection.

Interestingly, despite prolonged survival, IFN-I R^{-/-} mice eventually succumb to infection (Fig. 6, A), where host death is associated with high ST burden in the spleen (data not shown). These data suggest that the influence of IFN-I on ST clearance is limited to the early/ intermediate stages of ST infection.

4.4 IFN-I signalling regulates the host inflammatory response to ST infection

Since IFN-I R^{-/-} mice display prolonged survival during ST infection, I investigated the effects of IFN-I on the host inflammatory response by evaluating chemokine and cytokine expression following ST infection. To this end, WT and IFN-I R^{-/-} mice were infected with 10² ST i.v.; on days 3 and 7 p.i. chemokine and cytokine expression was assessed in the serum (Fig. 7) and spleens (Fig. 8) of infected mice. Day 3 and 7 p.i. blood samples were collected by cardiac puncture from WT and IFN-I R^{-/-} mice. Blood samples

were allowed to clot at 4°C and were centrifuged for serum collection. On days 3 and 7 p.i., spleens were aseptically removed and homogenized to a single cell suspension in the presence of the protease inhibitor, PMSF (phenylmethanesulphonylfluoride). The expression of chemokines and cytokines in serum (Fig. 7) and spleen homogenates (Fig. 8) was assessed using the R&D Systems Proteome Profiler Array which enabled the measurement of 40 different chemokines and cytokines from a single sample. Based on the ELISA principle, duplicate primary antibody spots were spotted on a membrane. After sample incubation, a secondary antibody cocktail was incubated on the membrane. Proteomic quantification was determined using chemiluminescence and spot densitometry. Protein expression was corrected to an internal positive control and subsequently represented as the fold change over the chemokine and cytokine expression of naive WT and IFN-I R^{-/-} mice respectively, for both serum (Fig. 7) and spleen (Fig. 8).

On day 7 p.i., all 40 chemokines and cytokines evaluated exhibited elevated expression in WT mice as compared to IFN-I R^{-/-} mice. This was true for both serum (Fig. 7) and spleen samples (Fig. 8). Interestingly, however, proteins that were most significantly upregulated were predominantly chemokines. Chemoattractants for M ϕ s (I-309, IP-10, MCP-1, MCP-5), DCs (IP-10), B cells (BLC), T cells (IP-10, MIG) and growth factors (G-CSF, GM-CSF) were upregulated following ST infection in WT serum. Despite chemokine upregulation relative to naive serum samples, chemokine expression in IFN-I R^{-/-} serum was muted relative to WT serum (Fig. 7). Surprisingly, few cytokines were upregulated in the serum following ST infection, and of those that were: IL-5, IL-6, IL-10, IL-12 and TNF α were only upregulated in the serum of WT mice (Fig. 7).

Fig. 7. Effects of IFN-I on serum chemokine/cytokine profile following ST infection. IFN-I R^{-/-} and WT mice were infected with 10² ST i.v. Day 7 p.i. blood samples were collected by cardiac puncture peri-mortem. Blood samples were clotted at 4°C for 1-2hrs. Samples were centrifuged, serum collected and frozen at -80°C until use. 200 µl of serum was screened for chemokine/ cytokine expression using the R&D Systems Protein Array Kit. Chemokine/cytokine expression in WT and IFN-I R^{-/-} serum samples were quantified using chemiluminescence detected using a Fluorochem 8900 imager (Alpha Innotech) (A). Densitometric expression values were enumerated using AlphaEase software and were corrected to the internal positive control (B). Data are expressed as mean fold change ± SEM over uninfected serum samples (day 0) from WT and IFN-I R^{-/-} mice, respectively (A, B). Data is representative of two independent experiments; n=3.

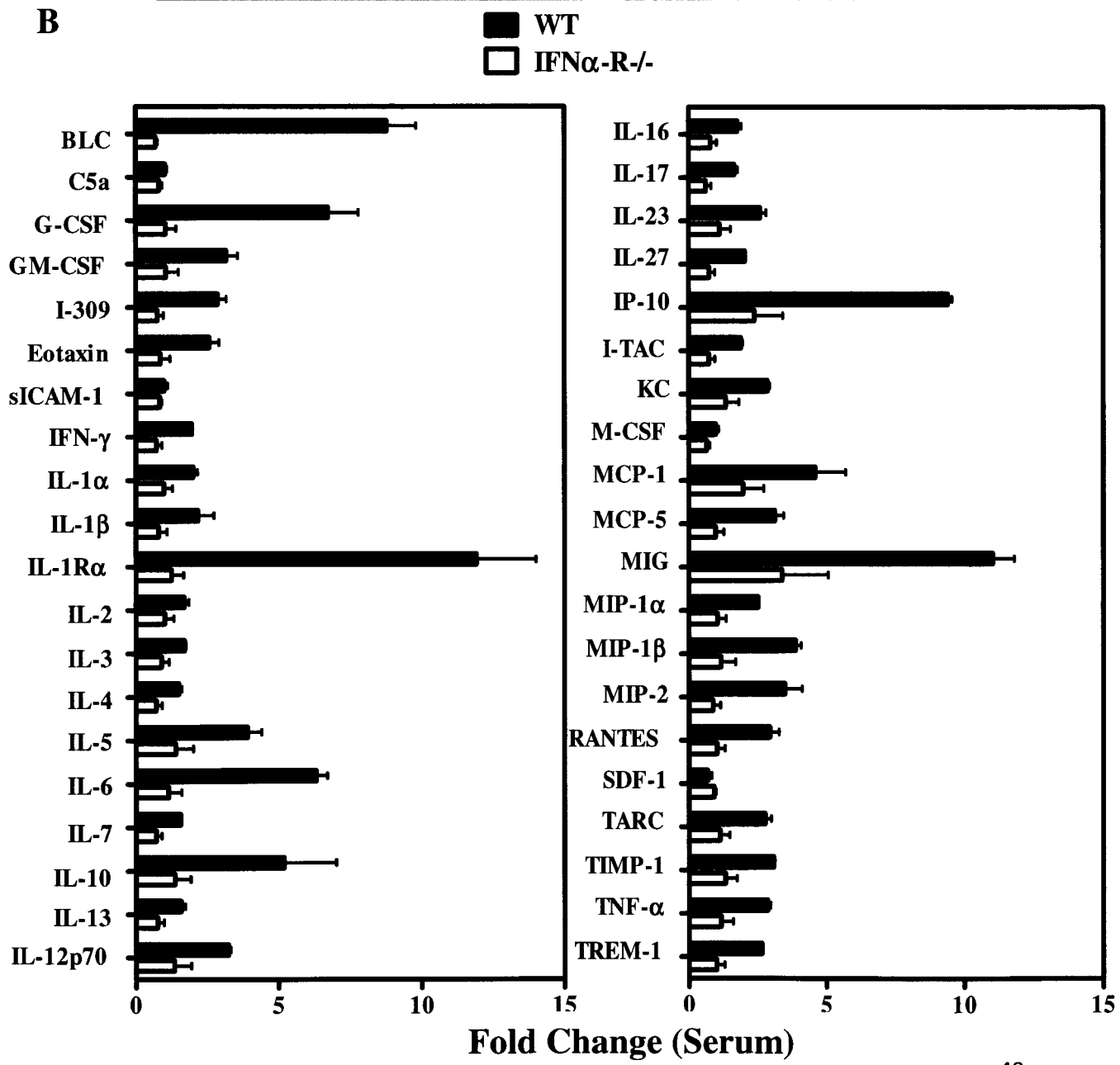
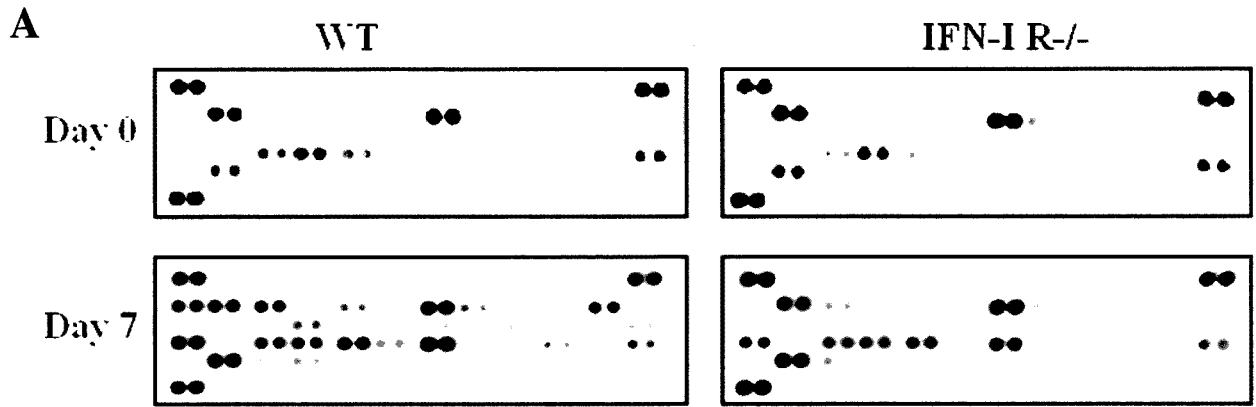
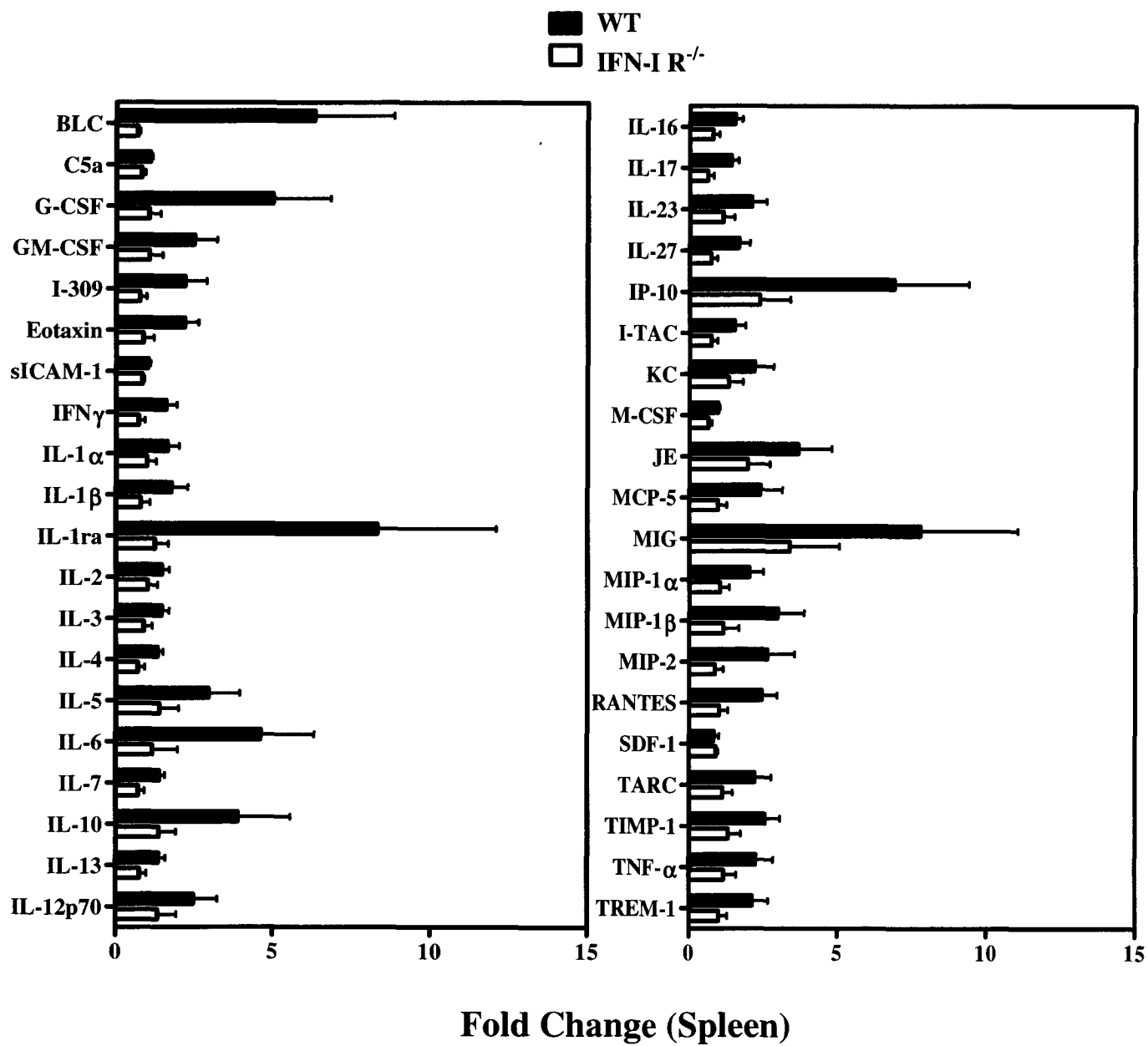


Fig. 8. Effects of IFN-I on chemokine/cytokine expression in the spleen following ST infection. IFN-I R^{-/-} and WT mice were infected with 10² ST i.v. Day 7 p.i. mice were euthanized and spleens were aseptically removed. Spleen samples were homogenized to single cell suspensions, treated with 10µg/ PMFA and frozen at -80°C until use. Protein concentration in spleen samples was enumerated using the Bradford assay. Subsequently, 1µg of protein from spleen sample homogenates was screened for chemokine/ cytokine expression using the R&D Systems Protein Array Kit. Chemokine/cytokine expression in WT and IFN-I R^{-/-} serum samples was quantified using chemiluminescence detected using a Fluorochem 8900 imager (Alpha Innotech). Densitometric expression values were enumerated using AlphaEase software and were corrected to the internal positive control. Data are expressed as mean fold change ± SEM over uninfected spleen samples from WT and IFN-I R^{-/-} mice, respectively. Data is representative of two independent experiments; n=3.



Chemokine and cytokine expression in the spleen exhibited a similar trend whereby the chemokines I-309, MCP-1, MCP-5, BLC, IP-10, MIG, G-CSF, GM-CSF were predominantly upregulated following ST infection (Fig. 8). As was observed for serum samples, chemokines and cytokines were more highly expressed in WT spleens than in the spleens of IFN-I R^{-/-} mice (Fig. 8). Furthermore, the cytokines IL-5, IL-6, IL-10, IL-12 and TNF α were significantly upregulated in WT spleen samples only, and not in IFN-I R^{-/-} spleens (Fig. 8). Qualitatively, chemokine and cytokine expression in WT and IFN-I R^{-/-} samples on day 3 p.i. in both serum and spleen displayed a similar expression pattern, albeit muted relative to protein expression on day 7 p.i. (data not shown). Quantitative RT-PCR analysis of some key cytokines (IFN γ , IL-6, IL-10, IL-12) was in agreement with the proteomic data showing upregulation in WT mice only (data not shown). Taken together these data indicate a muted inflammatory response in IFN-I R^{-/-} mice correlates with increased host survival.

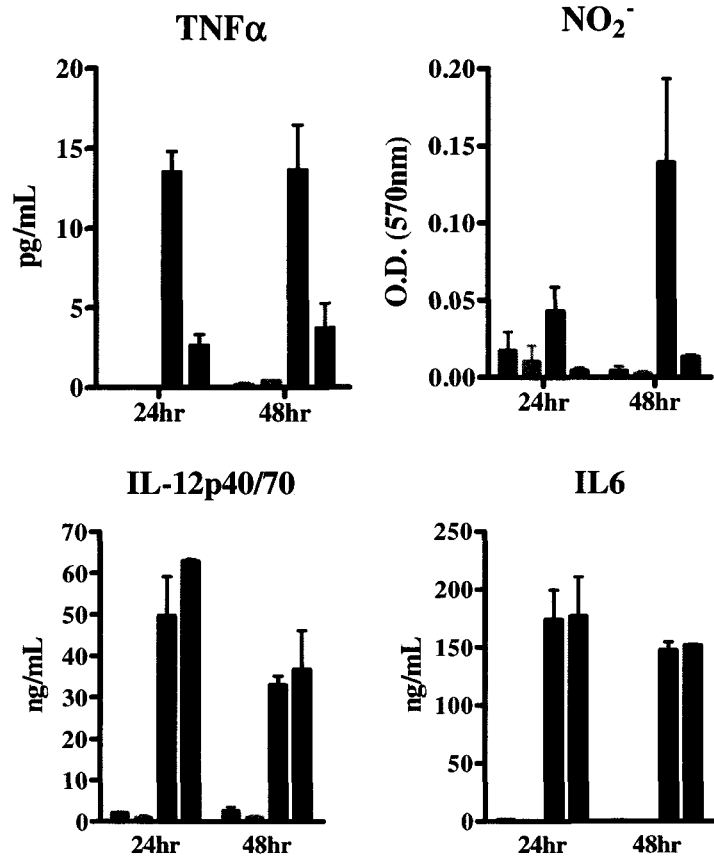
Macrophages (M Φ s) are key cellular mediators for controlling ST replication, however, chemokines and cytokines can be expressed by numerous cell types in the spleen. Measurement of chemokines and cytokines globally based on proteomic analysis in serum and spleen does not discriminate between protein expression by different cell types. To avoid this problem, the expression of inflammatory mediators was measured quantitatively in macrophages that were stimulated with ST. I examined the expression of the key innate immune mediators TNF α , NO $_2^-$, IL-12 and IL-6 in bone marrow-derived macrophages (BMMs) following ST infection *in vitro*. To this end, bone marrow was aseptically collected from naive WT and IFN-I R^{-/-} mice, and cultured with 10ng/ml M-CSF in RPMI+8%FCS (R8). Non-adherent cells were washed away on days 2 and 4 of the BMM

culture; on day 8 adherent cells (>95% CD11b⁺F4/80⁺) were collected, washed and seeded at 10⁵ BMMs/ well in 96 well microplates. BMMs were infected with 10 MOI ST for 30min. Subsequently, BMMs were treated with 50µg/ml gentamycin in R8 medium to eliminate extracellular ST. After 2hr at 37°C, BMMs were washed and supplemented with R8 containing 10µg/ml gentamycin. At 24hr and 48 hr p.i., culture supernatants were removed and stored at -80°C until use. TNFα expression in culture supernatants was enumerated using the WEHI-164 TNFα bioassay (Fig. 9, A). NO₂⁻ expression in culture supernatants was determined using the Greiss Reagent System (Fig. 9, A). IL-12p40/70 and IL-6 in culture supernatants were quantified relative to a standard curve using cytokine specific ELISAs (Fig. 9, B). ST infection induced the production of TNFα, NO₂⁻, IL-12p40/70 and IL-6 by both WT and IFN-I R^{-/-} BMMs (Fig. 9). Expression of IL-12p40/70 and IL-6 was similar at 24hr and 48hr p.i. in both WT and IFN-I R^{-/-} BMMs (Fig. 9, B), however, WT BMMs produced significantly more TNFα and NO₂⁻ than IFN-I R^{-/-} BMMs ; this was true at both 24hr and 48hr p.i. (Fig. 9, A). Together, these data suggest that IFN-I receptor engagement regulates the expression of TNFα and NO₂⁻, but not IL-12p40/70 and IL-6 in BMMs following ST infection.

To examine whether IFN-I regulates cytokine expression in other cell types, I examined the expression of TNFα, NO₂⁻, IL-12p40/70 and IL-6 in bone marrow derived dendritic cell (BMDC) cultures. To this end, bone marrow was aseptically collected from naive WT and IFN-I R^{-/-} mice, and cultured with 5ng/ml GM-CSF in R8. Non-adherent cells were washed away on days 2 and 4 of the BMDC culture; on day 6 non-adherent cells were collected (>95% CD11c⁺), washed and seeded at 10⁵ BMDCs/ well in 96 well

Fig. 9. Cytokine expression by M ϕ s during ST infection *in vitro*. Bone marrow (BM) cells from IFN-I R^{-/-} and WT mice were cultured with 10ng/ml M-CSF in R8. Non-adherent cells were removed on days 2 and 4 and the adherent cells remained in culture in M-CSF containing R8. From day 8 onwards, bone marrow M ϕ s (BMMs) generated using this protocol were used. BMMs were plated at 10⁵ cells/well in a 96-well plate and infected with 10 MOI ST. After 30 min, BMMs were treated with 50 μ g/ml gentamycin in R8 for 2 hrs to eliminate extracellular ST. BMMs were washed and incubated with 10 μ g/ml gentamycin in R8. At 24hr and 48hr p.i. culture supernatants were removed and stored at -80°C until use for cytokine quantitation. IL-12p40/70 and Il-6 expression in cell culture supernatants were quantified by cytokine ELISA. The TNF α -sensitive WEHI-164 cell bioassay was used to quantify TNF α expression in cell culture supernatants. NO₂⁻ expression was detected in cell culture supernatants using the Greiss reagent method. Data represents mean expression (or absorbance values) \pm SEM and are representative of three independent experiments; n=3.

- WT
- IFN-I R^{-/-}
- WT + 10MOI
- IFN-I R^{-/-} + 10MOI



microplates. BMDCs were infected with 10 MOI ST for 30min. Subsequently, BMDCs were treated with 50µg/ml gentamycin in R8 medium to eliminate extracellular ST. After 2hr at 37°C, BMDCs were washed and supplemented with R8 containing 10µg/ml gentamycin. At 24hr p.i., culture supernatants were removed and stored at -80°C until use. Cytokine expression was evaluated as described previously for BMMs. Similar to what was observed for cytokine expression by BMMs, expression of IL-12p40/70 and IL-6 expression was similar by BMDCs from WT and IFN-I R^{-/-} mice (Fig. 10, B). Again, significantly more TNFα and NO₂⁻ were produced by WT BMDCs than by IFN-I R^{-/-} BMDC cultures (Fig. 10, A). Taken together, these results suggest that IFN-I influences TNFα and NO₂⁻ expression by various cell types following ST infection.

4.5 IFN-I signalling influences innate cell population kinetics in the spleen

Since the expression of chemokines and cytokines was attenuated in IFN-I R^{-/-} mice following ST infection, I examined the influence of IFN-I deficiency on splenocyte populations during ST infection. To this end, WT and IFN-I R^{-/-} mice were infected with 10² ST i.v.. The spleens of infected animals were removed on day 0, 3 and 6 p.i. and homogenized to a single cell suspension. Cells were stained with fluorescently labelled antibodies and analyzed by flow cytometry. Specific cell populations were tracked in the spleens of infected animals based on their expression of the following cell markers: Natural Killer cells (NKs: CD3⁻DX5⁺ cells), Natural Killer T cells (NKTs: CD3⁺DX5⁺ cells), neutrophils (CD11b⁺Gr-1⁺ cells) dendritic cells (DCs: IA/IE⁺CD11c⁺ cells) and Macrophages (M_φs: CD3⁻CD11b⁺F4/80⁺). There was no significant difference in the NK, NKT, neutrophil and DC populations among WT and IFN-I R^{-/-} mice (Fig.11).

Fig. 10. Cytokine expression by DCs during ST infection *in vitro*. BM cells from IFN-I R^{-/-} and WT mice were cultured with 5ng/ml GM-CSF in R8. On day 2 and 4 non-adherent cells were disposed of after gentle washing. Adherent cells were cultured in R8 containing GM-CSF. From day 6 onwards, BMDCs developed which were non-adherent. BMDCs were plated at 10⁵cells/well in a 96-well plate and infected with 10 MOI ST. After 30 min, BMDCs were treated with 50µg/ml gentamycin in R8 for 2 hrs to eliminate extracellular ST. BMDCs were washed and incubated with 10µg/ml gentamycin in R8. At 24hr post-infection culture supernatants were removed and stored at -80°C until use for cytokine quantitation. IL-12p40/70 and IL-6 expression in cell culture supernatants were quantified by ELISA. The TNFα-sensitive WeHi-164 cell bioassay was used to quantify TNFα expression in cell culture supernatants. NO₂⁻ expression was detected in cell culture supernatants using the Greiss reagent method. Data represents mean expression (or absorbance values) ± SEM and are representative of three independent experiments; n=3.

- WT
- IFN-1R^{-/-}
- WT + 10MOI
- IFN-1R^{-/-} + 10MOI ST

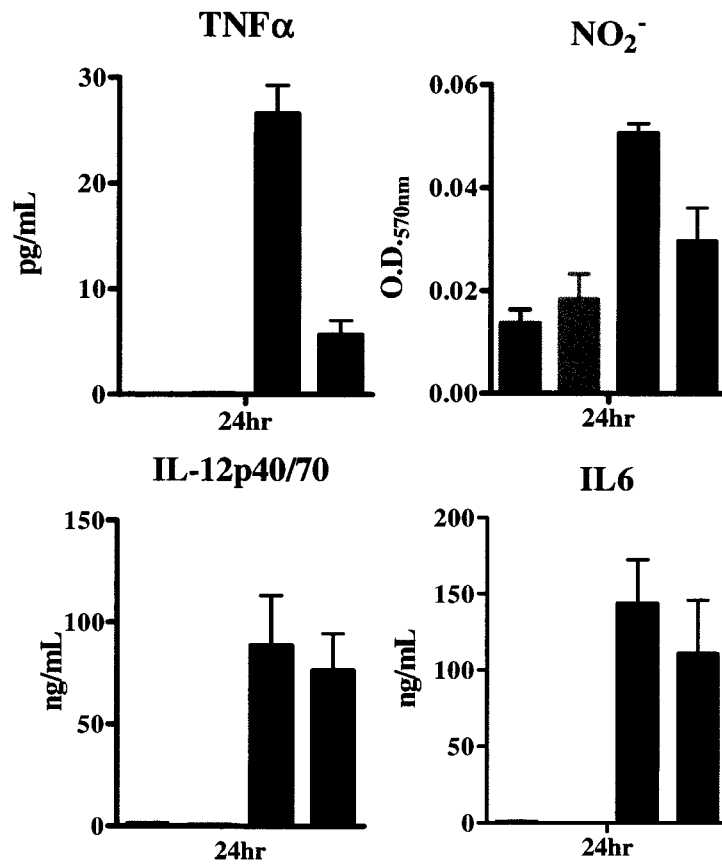
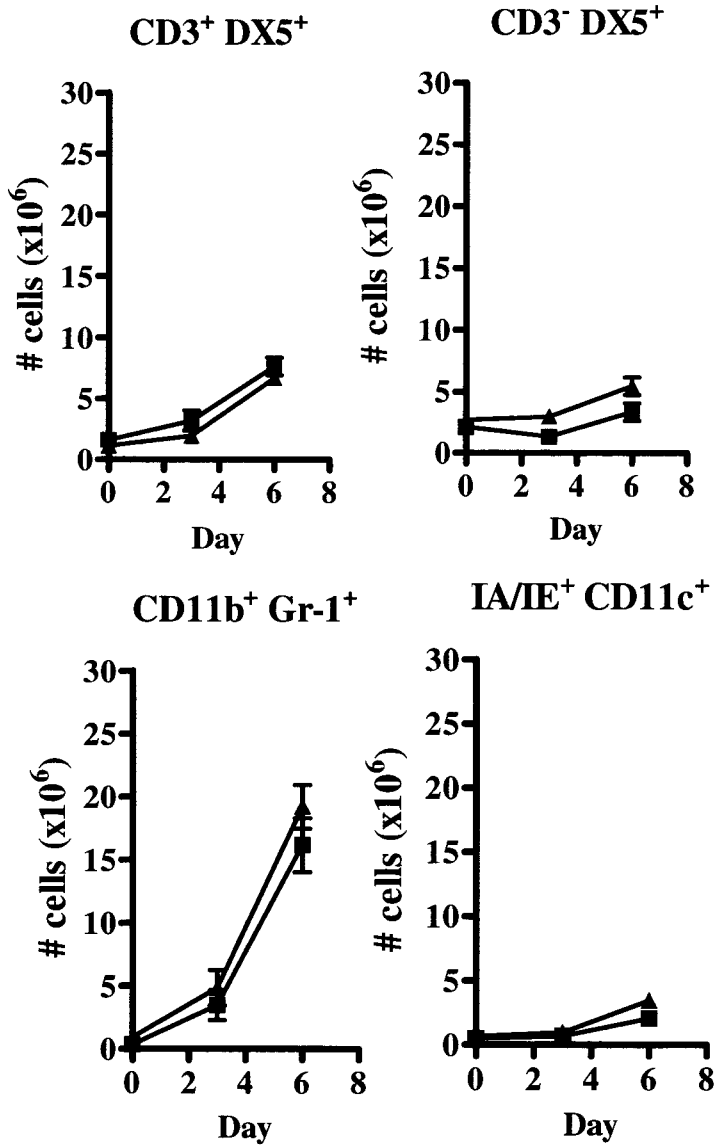


Fig. 11. Kinetic changes in NK-T, NK, Neutrophil and DC populations following ST infection. IFN-I R^{-/-} and WT mice were infected with 10² ST i.v. Spleens were aseptically removed on day 0, 3, and 6 p.i. Spleen samples were homogenized to single cell suspensions and stained with fluorescently labelled antibodies. Changes in the numbers of NK cell (CD3⁻, DX5⁺), NK-T cell (CD3⁺, DX5⁺), Neutrophil (CD11b⁺, Gr-1⁺) and DC (IA/IE⁺, CD11c⁺) populations in the spleen were assessed by flow cytometry (BDFacsCanto™, FacsDiVa™ software). Data represents mean cell counts ± SEM and are representative of two independent experiments; n=3.

■ WT
▲ IFN-I R-/-



However, there were significantly more M_{ϕ} s in IFN-I $R^{-/-}$ spleens than in WT spleens by day 6 p.i. (Fig. 12). Greater than two-fold more M_{ϕ} s were observed in IFN-I $R^{-/-}$ spleens on day 6 p.i. as compared to WT spleens (Fig. 12). These data indicate that IFN-I-receptor engagement negatively influences M_{ϕ} population size in the spleen following ST infection, but plays no role in regulating DC, neutrophil, NK or NKT population kinetics.

4.6 IFN-I receptor-deficiency enhances intracellular ST burden control

Since IFN-I was shown to influence M_{ϕ} populations in the spleen I examined the influence of IFN-I on ST pathogenesis at the cellular level. WT and IFN-I $R^{-/-}$ were infected with 10^2 ST i.v. On day 5 p.i. spleens were aseptically removed and homogenized to a single cell suspension. An aliquot of whole spleen cell suspensions was saved for CFU analysis after M_{ϕ} isolation. M_{ϕ} s ($F4/80^+$ or $CD11b^+$ cells) were isolated from spleen homogenates using the PE Selection kit from STEMCELL Technologies. In brief, whole spleen cell suspensions were labelled with anti-F4/80-PE or anti-CD11b-PE antibodies. Subsequently, cells were incubated with an anti-PE selection cocktail followed by incubation with magnetic particles. Sample tubes were placed in a magnet and supernatants were washed off eliminating the unlabelled cells. This procedure typically resulted in the isolation of M_{ϕ} s from the spleen that were >90% pure (data not shown). Following isolation, 10-fold serial dilutions of whole spleen cell suspensions and purified M_{ϕ} s, $F4/80^+$ (Fig. 13, A) or $CD11b^+$ (Fig. 13, B), were plated on BHI agar plates. After 24hr at 37°C, CFUs were enumerated. M_{ϕ} s isolated from IFN-I $R^{-/-}$ spleens harboured significantly fewer ST than did M_{ϕ} s from WT spleens. This was true for both $F4/80^+$ (Fig. 13, A) and $CD11b^+$ (Fig. 13, B) populations which displayed one log fewer ST in M_{ϕ} s isolated from

IFN-I R^{-/-} mice. Data represents the ST burden in the total number of isolated F4/80⁺ (Fig. 13, A) and CD11b⁺ (Fig. 13, B) M ϕ s.

Furthermore, IFN-I R^{-/-} M ϕ s display reduced ST burden irrespective of the route of infection. WT and IFN-I R^{-/-} mice were infected with 10² ST i.p. At day 5 p.i., F4/80⁺ cells were isolated from peritoneal exudates using the protocol described above for the PE Selection Kit. WT peritoneal M ϕ s harboured one log more ST than did IFN-I R^{-/-} M ϕ s (Fig. 14, B). On days 3, 6 and 15 p.i., peritoneal cell number was enumerated. As was observed for IFN-I R^{-/-} M ϕ s in the spleen during i.v. infection (Fig. 12), IFN-I R^{-/-} mice contained more cells in their peritoneal exudates than did WT mice (Fig. 14, A). Together, these results indicate that IFN-I-receptor engagement impairs bacterial elimination by M ϕ s.

To examine whether the influence of IFN-I on burden control was specific to M ϕ s, I evaluated ST burden in DCs isolated from infected spleens. CD11c⁺ cells were isolated from infected WT and IFN-I R^{-/-} spleens on day 5 p.i. using STEMCELL Technologies CD11c⁺ isolation kit, similarly to the methods described previously. DCs isolated from spleens by this method resulted in cell populations that were >90% CD11c⁺ (data not shown). Whole spleen and CD11c⁺ samples were diluted in a series of 10-fold dilutions and plated on BHI agar plates. After 24hr at 37°C, CFUs were enumerated. IFN-I R^{-/-} DCs harboured one log fewer ST than WT DCs in the spleen (Fig. 15) indicating that IFN-I signalling influences intracellular ST burden in multiple cell types, including DCs and M ϕ s.

Fig. 12. Kinetic changes in M ϕ populations in the spleen following ST infection. IFN-I R^{-/-} and WT mice were infected with 10² ST i.v. Spleens were aseptically removed on day 0, 3, and 6 p.i. Spleen samples were homogenized to single cell suspensions and stained with fluorescently labelled anti-CD3, anti-CD11b and anti-F4/80 antibodies. The numbers of macrophages (CD3⁻CD11b⁺F4/80⁺) were evaluated in the spleen by flow cytometry (BDFacsCanto™, FacsDiVa™ software). Data represent mean cell counts at each time point \pm SEM and are representative of two independent experiments; n=3.

■ WT
▲ IFN-1R^{-/-}

CD3⁻, CD11b⁺, F4/80⁺

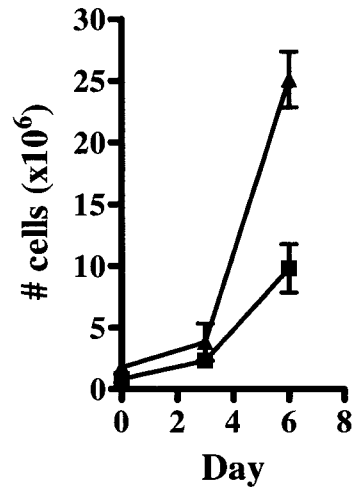


Fig. 13. Reduced ST burden in the M ϕ s of IFN-I R-deficient mice. IFN-I R^{-/-} and WT mice were infected with 10² ST i.v. At day 5 following infection spleens were aseptically removed and placed in dissociation medium in preparation for magnetic cell separation using StemCell PE isolation kit. In brief, single cell suspensions were prepared and labelled with anti-F4/80-PE (A) or anti-CD11b-PE (B) antibodies. Anti-PE magnetic beads separated PE-labelled cell populations. To enumerate bacterial burden 10-fold serial dilutions of single cell suspensions (whole spleen) and purified F4/80⁺ (A) or CD11b⁺ (B) cells were plated on BHI agar and incubated at 37°C overnight. CFUs were corrected for the total number of isolated F4/80⁺ (A) and CD11b⁺ (B) cells. Data represent mean CFU values \pm SEM and are representative of two independent experiments; n=2. [†]F4/80⁺ cells were pooled for burden assessment.

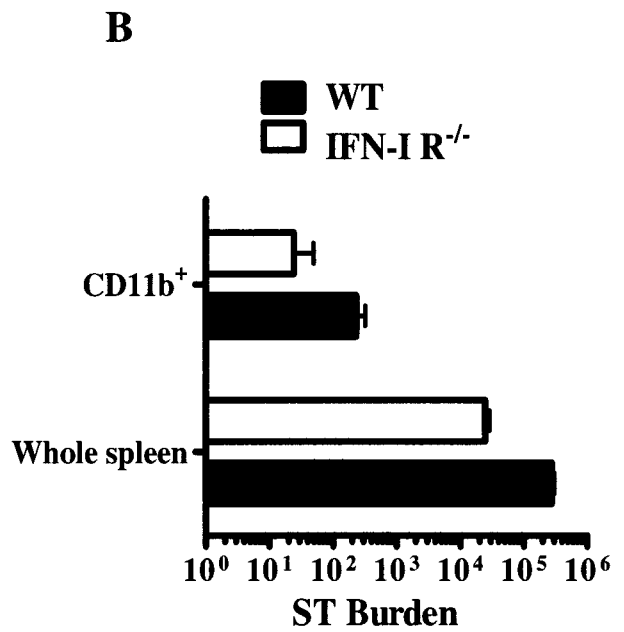
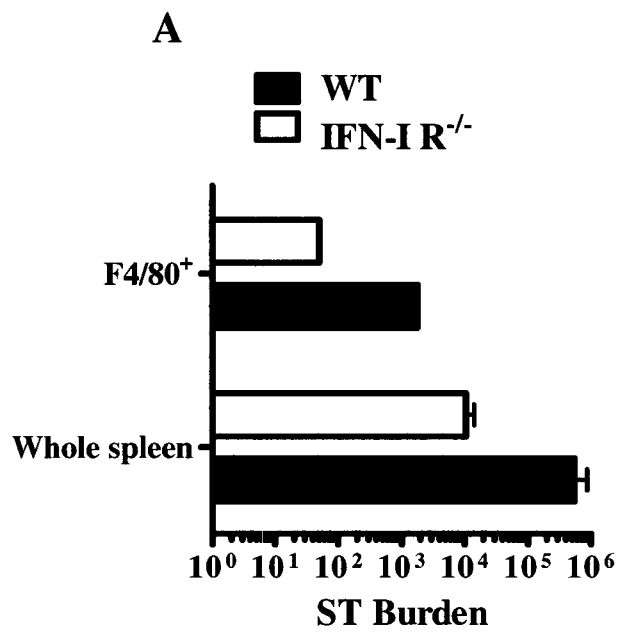


Fig. 14. Reduced ST burden in M ϕ s of IFN-I R-deficient mice in the peritoneum. WT and IFN-I R^{-/-} mice were infected with 10² ST given intraperitoneally. On day 3, 7 and 16 p.i. mice were euthanized and peritoneal cells were harvested through peritoneal lavage. Total peritoneal cell counts were recorded. Data represents mean cell counts \pm SEM and are representative of two independent experiments; n=3 (A). Peritoneal cells were obtained on day 5 following infection and were incubated with anti-F4/80-PE antibodies for magnetic separation using the StemCell PE isolation kit. To enumerate bacterial burden 10-fold serial dilutions of whole peritoneal lavage samples (whole peritoneum) and purified F4/80⁺ cells were plated on BHI agar plates and incubated at 37°C overnight. CFUs were corrected for the total number of isolated F4/80⁺ cells. Data represents mean CFU values \pm SEM, n=2 (B). ‡Indicates the survival limit for WT mice.

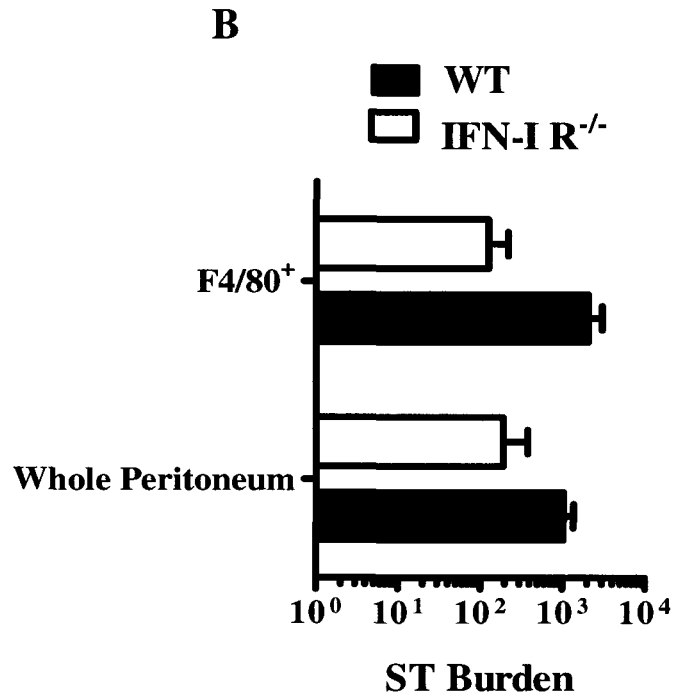
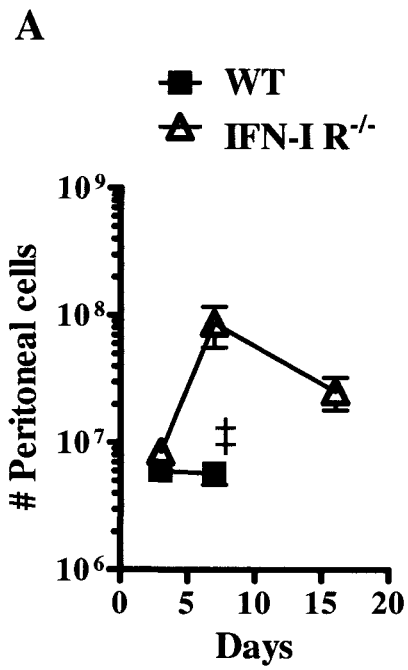
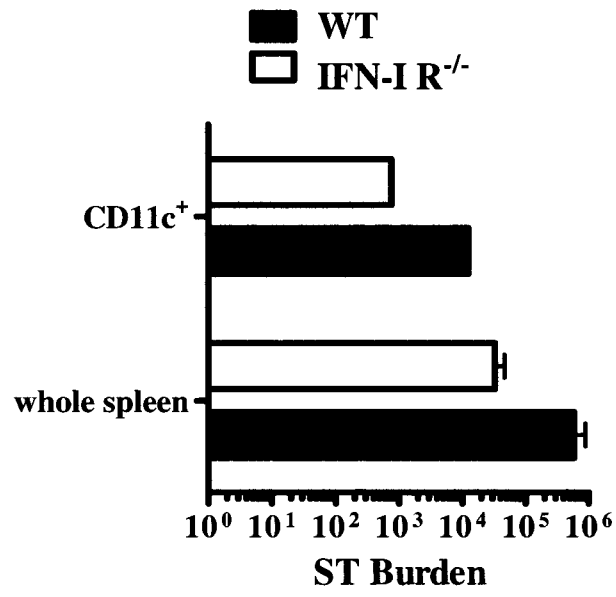


Fig. 15. Reduced ST burden in DCs of IFN-I R-deficient mice. IFN-I R^{-/-} and WT mice were infected with 10² ST i.v. Spleens were aseptically removed on day 5 post-infection and placed in dissociation medium in preparation for magnetic cell separation using StemCell CD11c isolation kit. In brief, single cell suspensions were prepared and labelled with anti-CD11c-PE. Anti-PE magnetic beads separated the PE-labelled CD11c⁺ cell populations. To enumerate bacterial burden 10-fold serial dilutions of single cell suspensions (whole spleen) and purified CD11c⁺ cells were plated on BHI agar plates and incubated at 37°C overnight. CFUs were corrected for the total number of isolated CD11c⁺ cells. Data represents mean CFU values ± SEM, n=3.



4.7 ST-induced IFN-I compromises the survival of BMMs but not BMDCs

Intracellular ST burden control could be regulated at several levels; IFN-I could influence intracellular ST burden control directly by influencing either initial ST invasion or other intracellular killing mechanisms. Conversely, IFN-I could regulate cell viability, thus indirectly control intracellular ST burden. Since IFN-I was demonstrated to influence not only M_{Φ} cytokine expression, but also the size of the M_{Φ} population in the spleen, I evaluated the influence of IFN-I on M_{Φ} viability. Bone marrow was aseptically collected from naive WT and IFN-I $R^{-/-}$ mice, and cultured with 10ng/ml M-CSF in R8. Non-adherent cells were washed away on days 2 and 4 of the BMM culture; on day 8 adherent cells (predominantly M_{Φ} s) were collected, washed and seeded at 10^5 BMMs/ well in 96 well microplates. BMMs were infected with 10 MOI ST for 30min. Subsequently, BMMs were treated with 50 μ g/ml gentamycin in R8 medium to eliminate extracellular ST. After 2hr at 37°C, BMMs were washed and supplemented with R8 containing 10 μ g/ml gentamycin. At 24hr and 48 hr post-infection, cell viability was assessed using the Neutral Red cytotoxicity assay kit. In this assay, Neutral Red dye is taken up by viable cells and incorporated into host cell lysosomes. Upon cell lysis Neutral Red dye is released into culture wells and absorbance is measured at 570nm, enabling relative quantification of cell viability. Interestingly, BMMs derived from IFN-I $R^{-/-}$ hosts displayed enhanced survival when compared with WT BMMs. This was true for BMM cultures at both 24hr and 48hr p.i. (data not shown, Fig. 16). Furthermore, visualization of BMMs by inverted microscopy revealed that IFN-I $R^{-/-}$ BMMs resist ST-induced cell death, displaying healthy M_{Φ} morphology and confluency, up to 72hr whereas infected WT BMMs began to die at 24hr post-infection (Fig. 17).

To examine whether IFN-I influences the survival of other cell types BMDC cultures were assessed for cell viability at 24hr p.i. For this purpose, bone marrow was aseptically collected from naive WT and IFN-I $R^{-/-}$ mice, and cultured with 5ng/ml GM-CSF in R8. Non-adherent cells were washed away on days 2 and 4 of the BMDC culture; day 6 non-adherent cells (predominantly DCs) were collected, washed and seeded at 10^5 BMDCs/ well in 96 well microplates. BMDCs were infected with 10 MOI ST for 30min. Subsequently, BMDCs were treated with 50 μ g/ml gentamycin in R8 to eliminate extracellular ST. After 2hr at 37°C, BMDCs were washed and supplemented with R8 containing 10 μ g/ml gentamycin. At 24 hr p.i. cell viability was assessed using the Neutral Red cytotoxicity assay kit. Briefly, BMDC culture supernatants were removed and replaced with media containing Neutral Red labelling solution. After 4 hr at 37°C, the labelling solution was removed and was replaced with fixing solution for 1 min. Subsequently cells were lysed and Neutral Red dye was solubilized using solubilisation solution. It is shown here that BMDC viability following ST infection was independent of IFN-I signalling; both WT and IFN-I $R^{-/-}$ BMDCs were susceptible to ST-induced cell death by 24hr p.i. (Fig. 18). This is consistent with the previous observation that IFN-I had no effect on the DC population size in the spleen (Fig. 11). Taken together, these results suggest that during ST infection IFN-I signalling mediates the direct or indirect elimination of $M\phi$ s, key cellular mediators of ST clearance. Suggesting a possible mechanism by which IFN-I signalling may enhance host susceptibility to ST infection.

Fig. 16. IFN-I receptor engagement enhances M ϕ susceptibility to ST-induced cell death. BM cells from WT and IFN-I R^{-/-} were cultured with 10ng/ml MCSF in R8 to derive BMMs. BMMs were plated at 10⁵ cells/well in a 96-well plate and infected with 10 MOI ST. Infected cells were treated with 50 μ g/ml gentamycin in R8 for 2 hrs to eliminate extracellular ST. BMMs were washed and incubated with 10 μ g/ml gentamycin in R8 up to 72 hrs. At 48hr p.i. culture supernatants were removed and replaced with R8 media containing neutral red dye. After 4 hrs at 37°C, cell cultures were washed with R8, fixed and lysed using the Neutral Red cytotoxicity assay kit. Absorbance at 570nm reflects cell viability. Data represents mean absorbance at 570nm \pm SEM and are representative of three independent experiments; n=3.

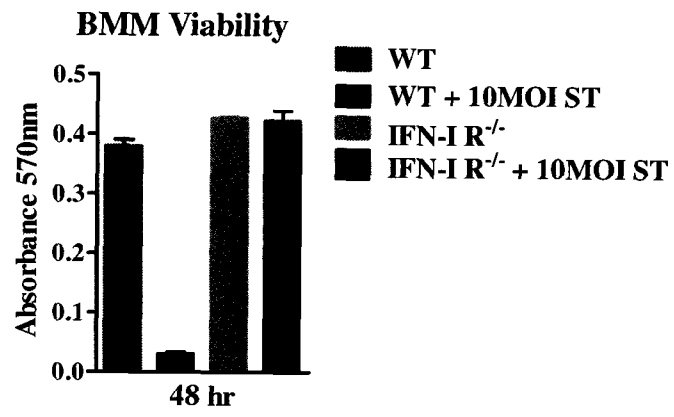
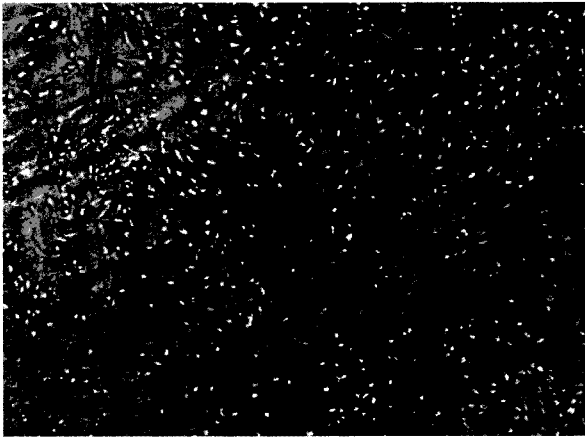


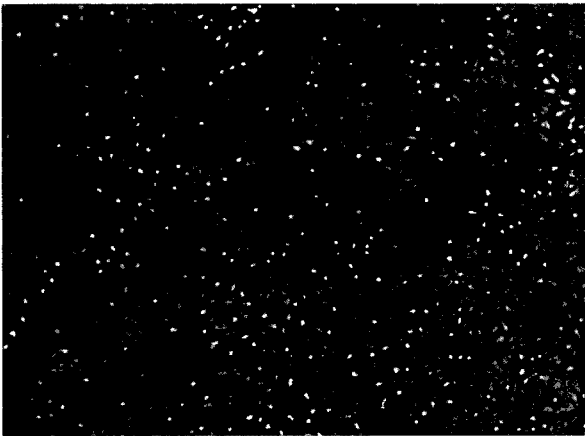
Fig. 17. IFN-I R-deficiency enhances M ϕ survival following ST infection. BM cells from WT and IFN-I R^{-/-} were cultured with 10ng/ml MCSF in R8 to derive BMMs. BMMs were plated at 10⁵ cells/well in a 96-well plate and infected with 10 MOI ST. Infected cells were treated with 50 μ g/ml gentamycin in R8 for 2 hrs to eliminate extracellular ST. BMMs were washed and incubated with 10 μ g/ml gentamycin in R8 for 72 hrs. BMMs were imaged on the Olympus IX81 inverted fluorescence microscope (40X) at 72 hr p.i. Data is representative of four independent experiments; n=3.



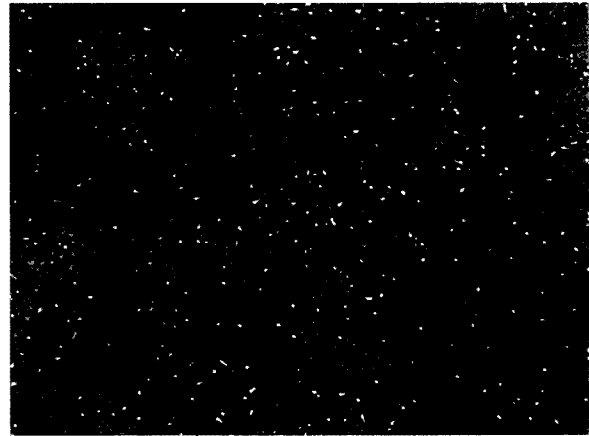
WT



WT + ST

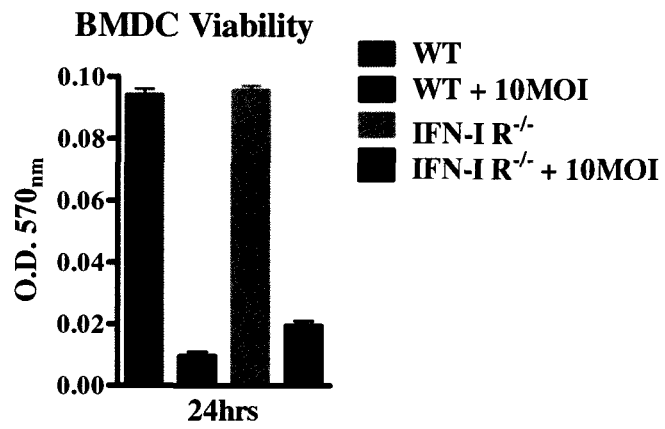


IFN-I R^{-/-}



IFN-I R^{-/-} + ST

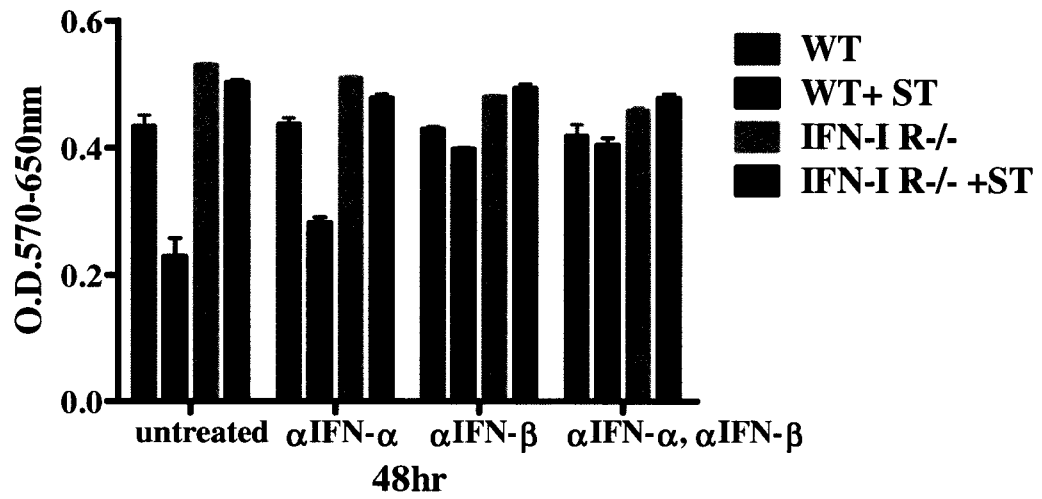
Fig. 18. DCs from WT and IFN-I R-deficient mice are equally susceptible to ST-induced cell death. BM cells from IFN-I R^{-/-} and WT mice were cultured with 5ng/ml GM-CSF in R8 to derive BMDCs. BMDCs were plated at 10⁵ cells/well in a 96-well plate and infected with 10 MOI ST. Infected cells were treated with 50µg/ml gentamycin in R8 for 2 hrs to eliminate extracellular ST. BMDCs were washed and incubated with 10µg/ml gentamycin in R8. 24 hrs p.i. cell supernatants were removed and replaced with R8 media containing neutral red dye. After 4 hrs at 37°C, cell cultures were washed with R8, fixed and lysed using the Neutral Red cytotoxicity assay kit. Absorbance at 570nm reflects cell viability. Data represents mean absorbance values at 570 nm ± SEM and are representative of three independent experiments; n=3.



4.8 IFN- β and not IFN- α enhances ST-induced M ϕ death

All members of the IFN-I family, IFN- α subtypes and IFN- β , signal through the same receptor; the IFN-I R. To determine which members of the IFN-I family are responsible for mediating ST-induced M ϕ death, BMM viability was assessed after incubation with different combinations of anti-IFN-I antibodies. Bone marrow was aseptically collected from naive WT and IFN-I R^{-/-} mice, and cultured with 10ng/ml MCSF in R8. Non-adherent cells were washed away on days 2 and 4 of the BMM culture; on day 8 adherent cells (M ϕ s) were collected, washed and seeded at 10⁵ BMMs/ well in 96 well microplates. BMMs were infected with 10 MOI ST for 30min. Subsequently, BMMs were treated with 50 μ g/ml gentamycin in R8 to eliminate extracellular ST. After 2hr at 37°C, BMMs were washed and supplemented with R8 containing 10 μ g/ml gentamycin and one of the following anti-IFN antibody treatments: untreated, 10 μ g/ml anti-IFN- α , 10 μ g/ml anti-IFN- β or 10 μ g/ml anti-IFN- α + 10 μ g/ml anti-IFN- β in combination. At 48hr p.i., cell culture supernatants were removed and replaced with media containing Neutral Red labelling solution. After 4 hr at 37°C, the labelling solution was removed and replaced with fixing solution for 1 min. Subsequently cells were lysed and Neutral Red dye was solubilized using solubilisation solution. Interestingly, BMM cell viability was restored when BMMs were treated with anti-IFN- β alone, or in combination with anti-IFN- α antibody (Fig. 19). Further, BMM were susceptible to ST infection when treated with anti-IFN- α alone (Fig. 19). These results indicate that it is ST-induced IFN- β production, and not IFN- α , that mediates susceptibility of BMMs to ST infection.

Fig. 19. IFN- β contributes to macrophage death during infection with ST. BM cells from WT and IFN-I R^{-/-} were cultured with 10ng/ml MCSF in R8 to derive BMMs. BMMs were plated at 10⁵cells/well in a 96-well plate and infected with 10 MOI ST. Infected cells were treated with 50 μ g/ml gentamycin in R8 for 2 hrs to eliminate extracellular ST. BMMs were washed and incubated with 10 μ g/ml gentamycin in R8 in combination with anti-IFN- α , anti-IFN- β , anti-IFN- α + anti-IFN- β or remained untreated. The concentration of each antibody was 10 μ g/ml. After 48hrs p.i., cell supernatants were removed and replaced with R8 media containing neutral red dye. After 4 hrs at 37°C, cell cultures were washed with R8, fixed and lysed using the Neutral Red cytotoxicity assay kit. Absorbance at 570nm reflects cell viability. Data represents mean absorbance values at 570 nm \pm SEM and are representative of a single experiment; n=3.



5. Discussion

5.1 Prelude

Salmonella spp. are infectious food-borne pathogens that have global health implications. Especially endemic in developing regions where sanitation remains problematic, *Salmonella* infections are increasingly common in developed nations where mass food production has led to an increase in food automation and distribution. *Salmonella* infections affect over 16 million people world-wide annually (4). A number of factors contribute to the severity of infection, including the dose of exposure, pathogen virulence and host immune status.

Infection of mice with *Salmonella typhimurium* (ST) mimics human typhoid fever, since dissemination of ST from the gut into the blood results in massive systemic inflammatory response, thus serving as a good model to study typhoid-like pathogenesis. Interestingly, in mice, susceptibility to (ST) is regulated by the expression of a phagosomal membrane-bound divalent cation exchanger natural resistance-associated macrophage protein (NRAMP) (17, 18). NRAMP, expressed on macrophage (M_{Φ}) phagosomal membranes actively pumps divalent cations out of the phagosome and into the cytoplasm thus limiting the availability of critical cofactors of bacterial enzymes to ST contained within the phagosome (6, 8, 9, 15, 22, 23). M_{Φ} function is therefore crucial for mediating host survival. Intracellular residence has also been demonstrated in other cell types such as dendritic cells (DCs) however, M_{Φ} s are predominantly the cellular reservoir of ST infection. It is clear that innate host responses, particularly those mediated by M_{Φ} s are crucial for mediating host protection from ST in mice (36, 38). Examination of the

mechanisms of host susceptibility and survival may lead to the identification of novel therapeutic targets for combating ST infection.

While adaptive responses have shown to be very important in mediating long-term protection from ST in resistant mouse strains, it is also evident that early inflammation following ST infection is crucial in determining host survival. Previous work in our lab has shown that resistant as well as susceptible hosts exhibit delayed T cell activation following ST infection when compared to other pathogens such as *Listeria monocytogenes* (LM) (Fig. 1). Whereas LM induces significant T cell activation within the first few days of infection, T cell activation following ST infection is enormously delayed (Fig. 1, B). Since susceptible (C57BL/6) mice succumb to ST infection prior to the onset of both CD4⁺ and CD8⁺ T cell activation (Fig. 2), this indicates a critical role for early innate mediators in regulating host protection during the early stages of ST infection.

5.2 Early inflammation is crucial for host survival to ST infection

Inflammation is a crucial part of the host response to bacterial infections. It is required for mediating initial pathogen clearance, while also initiating and influencing later adaptive responses. In the case of ST, where the adaptive response is significantly delayed (Fig. 1), early inflammatory responses are particularly important in mediating host survival. M_Φs have been well defined as important cellular effectors in controlling ST replication (35) early in the host response to ST since defects in M_Φ function result in enhanced host susceptibility to infection (35, 37). Since M_Φ function is thought to be a fundamental difference between genetically susceptible and resistant mouse strains, we evaluated the

contribution of specific innate mediators that are known to influence M_{ϕ} activation or function on host survival during ST infection in genetically susceptible C57BL/6 mice.

Host survival was assessed in mice deficient in key genes involved in M_{ϕ} function (IFN- γ or TNF- α/β receptor). Ablation of IFN- γ and TNF- α/β signalling rendered mice more susceptible to ST infection, with deficient mice succumbing to infection two days prior to wild-type (WT) mice (Fig. 3, A). Furthermore, mice deficient in the inducible nitric oxide synthase gene (iNOS2), an important mediator of the bactericidal effects of macrophages, were also more susceptible to infection than WT mice (Fig. 3, B). These results suggest that early inflammation participates in delaying fatality in susceptible hosts, and further indicate that the expression of these key mediators by M_{ϕ} s facilitates the control of ST.

To further investigate the influence of inflammation on host survival, we evaluated the influence of the pro- and anti-inflammatory cytokines IL-6 and IL-10, respectively, on host survival during ST infection in susceptible C57BL/6 mice. Despite previous reports of strong IL-6 production following ST infection in both susceptible and resistant mouse strains (9, 31, 32), IL-6 deficiency had no influence on host survival (Fig. 3, C). Interestingly, however deficiency of the anti-inflammatory cytokine IL-10 resulted in significantly enhanced host survival (Fig. 3, D). IL-10, a potent anti-inflammatory cytokine, serves to dampen the early inflammatory response by controlling the expression of several innate inflammatory mediators including TNF α , IFN- γ and type I interferons (IFN-I) (75), limiting host tissue damage. Since host survival is prolonged in the absence of IL-10 signalling, these results suggest that the early inflammatory response facilitates ST control. It has previously been demonstrated that neutralization of endogenous IL-10

during *Salmonella choleraesuis* infection resulted in enhanced inflammation and host resistance to infection (76) and that enhanced host susceptibility to ST infection was correlated with significantly higher levels of IL-10 mRNA expression (77) further supporting the important role played by inflammatory cytokines in facilitating the control of ST.

Since our results suggested that some key inflammatory cytokines (such as IFN γ and TNF α) facilitate the control of ST infection early on (Fig. 3), we examined the influence of amplifying the innate response further using the immune-stimulant CpG. Statistical relevance was difficult to ascertain in our previous survival studies given that C57BL/6 succumb to ST infection within 7 days. To avoid this problem, we evaluated survival in the resistant progeny of a cross between susceptible (C57BL/6) and resistant (129SvJ) mice. Contrary to our hypothesis, amplification of the innate response by CpG exacerbated infection and rendered resistant mice susceptible to ST (Fig. 5).

Interestingly, while enhancement of inflammation, achieved through IL-10-deficiency, resulted in improved host survival, deliberate amplification of inflammation by CpG administration was catastrophic. These results suggested to us that although the magnitude of the inflammatory response is important for mediating host protection against ST, equally important is the **nature** of the host inflammatory response. ST infection is multi-faceted where many pathogen-associated molecular patterns (PAMPs) are recognized by different host pathogen recognition receptors (PRRs) ultimately stimulating the production of a multitude of inflammatory mediators. Conversely, CpG is limited to signalling via TLR-9 and thus stimulates the production of a more limited array of inflammatory mediators such as type I interferons (IFN-I).

5.3 IFN-I signalling exacerbates ST infection

Synthetic CpG used in this study, mimics CpG motifs that are predominantly found in microbial DNA. As such, CpG-TLR-9 engagement stimulates the strong production of the type-I interferons (IFN-I) (25, 29). The expression of type-I interferons are crucial in the innate response to viral pathogens (60, 61), however their role in non-viral contexts remains largely unclear.

Few reports have examined the influence of IFN-I production during bacterial infections and previous works examining the issue present conflicting results on a limited number of bacterial pathogens. Some studies suggest IFN-I is crucial for mediating host protection from a number of extracellular pathogens including Group B Streptococcus and *E. coli* (63). Conversely, in the case of the intracellular pathogens such as *Listeria monocytogenes* (LM) and *Mycobacterium tuberculosis* (MTb) IFN-I signalling exacerbated infection resulting in enhanced host susceptibility (64-67). Given the sparse literature on the subject, making any conclusions would be premature, however the evidence accumulated thus far suggests that IFN-I may regulate host survival depending on unique host-pathogen interactions.

Given that CpG treatment rendered normally resistant hosts susceptible to ST and that IFN-I has been shown to influence host survival during bacterial infections, we evaluated the influence of IFN-I receptor-deficiency (IFN-I R^{-/-}) on host survival during ST infection in susceptible mice.

Interestingly, mice deficient in IFN-I signalling displayed significantly delayed susceptibility to ST infection when compared with WT (C57BL/6) mice. Whereas WT

mice succumbed to ST infection by day 7 p.i., survival was prolonged in IFN-I R^{-/-} mice up to day 35 p.i. (Fig.6, A). Enhanced survival was correlated with significantly reduced ST burdens in the spleens of IFN-I R^{-/-} mice throughout the course of infection. In contrast, uncontrolled ST replication in WT spleens resulted in increased ST burden that reached lethal levels (10⁸) immediately preceding host fatality (Fig. 6, B). Similarly, when mice were infected with ST intraperitoneally, IFN-I R^{-/-} mice had reduced ST burdens in the spleen and were significantly less susceptible to ST infection (Fig. 6, C; data not shown).

It should be noted, however that IFN-I R-deficiency did not render mice resistant to ST infection, it simply delayed the timing of susceptibility. This was evidenced by the fact that ST infection was fatal in IFN-I R^{-/-} mice near day 35 p.i. (Fig. 6, A) when burden in the spleen reached lethal levels (data not shown). An intriguing question that arose was that despite the considerable delay in host susceptibility to ST infection in IFN-I R-deficient hosts, they still succumb to infection. Why then, would the acquired immune response not be able to control the pathogen in these mice? This may be explained by several reports which suggest that IFN-I mediate a crucial link between innate and adaptive immunity (78). IFN-I were demonstrated to be crucial for cross-priming of CD8⁺ T cells in LCMV infection (79). Furthermore, IFN-I mediated cross-priming of CD4⁺ and CD8⁺ T cells was demonstrated to be essential for stimulating adaptive responses in CpG-based vaccines (80, 81). This critical role for IFN-I in linking innate and adaptive immunity could explain why the absence of IFN-I signalling results in host death despite observing enhanced survival early during ST infection. This is supported by our observation that neither CD4⁺ nor CD8⁺ T cells were activated in IFN-I R-deficient mice at any time during ST infection (data not shown) despite their survival beyond day 15 p.i., when T cell activation occurs in resistant

mice (Fig. 6; Fig. 2). It is not clear whether IFN-I is also crucial in mediating cross-presentation against bacterial pathogens. However, if it is, then this would mean that cross-priming is the only route through which antigen-presentation occurs during ST infection. Any abrogation of this pathway (such as a deficiency in IFN-I signalling) would compromise the development of the adaptive immune response and leave the host totally dependent on innate immune responses for protection against ST.

Given that previous reports imply a link between pathogen residence and the influence of IFN-I on host survival (63-67, 82), we evaluated intracellular ST burden throughout infection in innate cell populations previously demonstrated to harbour most of the intracellular ST, namely $M\phi$ s and DCs (38, 47). Not surprisingly, the disparity between ST burdens in whole spleen samples from WT and IFN-I $R^{-/-}$ mice was reflected in both $M\phi$ and DC populations isolated from the spleens of infected mice; WT cells harbouring significantly more ST than IFN-I $R^{-/-}$ cells (Fig. 13; Fig. 15). It is important to note, however that the ST burden in isolated $M\phi$ and DC populations cannot account for the entire splenic burden (Fig. 13; Fig. 15), thus it is likely that ST also resides extracellularly and/or within other cell types, which is consistent with previous reports (50). Furthermore, when mice were infected intraperitoneally, peritoneal $M\phi$ s isolated from WT peritoneal cavities contained significantly more ST than did $M\phi$ s isolated from the peritoneal cavities of IFN-I $R^{-/-}$ mice (Fig. 14).

Since ST can reside both intra- and extracellularly, it was of particular interest that IFN-I signalling during ST infection exacerbated ST infection and enhanced host susceptibility (Fig. 6). These results are in line with those observed during infection with

the intracellular pathogen LM which demonstrated enhanced host survival and significantly reduced LM levels in hosts deficient in IFN-I signalling (64-66).

5.4 Ablation of IFN-I signalling reduces ST-induced inflammation

Prolonged host survival to ST infection in IFN-I-deficient hosts suggested that IFN-I plays a role in regulating early inflammation during ST infection. To investigate this, we evaluated the expression of inflammatory mediators following ST infection in WT and IFN-I R^{-/-} mice.

Protein sampling from spleen and serum revealed an interesting trend that expression levels of all cytokines and chemokines tested were upregulated in WT mice relative to IFN-I R^{-/-} mice. This trend was observed throughout the duration of ST infection in WT mice. Surprisingly, very few cytokines were significantly upregulated following ST infection in either the spleens or serum of WT and IFN-I R^{-/-} mice (Fig. 7, 8). Similarly, qRT-PCR analysis confirmed that IL-5, IL-6, IL-10, IL-12 and TNF- α mRNA levels were only slightly upregulated in the spleens of WT mice just prior to death (data not shown).

While cytokines were only minimally upregulated, chemokine expression was markedly enhanced in the spleen and serum of WT mice. Notably, IP-10, I-309, MIG, MCP-1, MCP-5, BLC displayed significant upregulation in both the spleen (Fig. 7) and serum (Fig. 8) of WT mice relative to IFN-I R^{-/-} mice. The majority of chemokines upregulated following ST infection are known to be involved in M ϕ chemotaxis. Furthermore, the growth factors G-CSF and GM-CSF, involved in M ϕ and DC maturation were also upregulated in WT mice (Fig. 7, 8) which supports an important role for M ϕ s during ST infection.

Since chemokines were predominantly upregulated following ST infection we addressed the possibility that IFN-I signalling may be important in regulating innate cell trafficking. The composition of splenocyte population was thus evaluated by flow cytometry in WT and IFN-I R^{-/-} mice. Interestingly, NKT (CD3⁺ DX5⁺), NK (CD3⁻ DX5⁺), neutrophil (CD11b⁺ Gr-1⁺) and DC (IA/IE⁺ CD11c⁺) populations were similar in the spleens of WT and IFN-I R^{-/-} mice throughout the course of ST infection (Fig. 11). However, M_Φs in the spleens of IFN-I R^{-/-} mice were significantly more numerous than in the spleens of WT mice (Fig.12). Furthermore, significantly higher M_Φ yields were consistently obtained from the spleens or peritoneum of IFN-I R^{-/-} mice (data not shown, Fig. 14). The disparity between M_Φ population size between WT and IFN-I R^{-/-} hosts suggests that IFN-I signalling during ST infection regulates M_Φ populations either by mediating their elimination, preventing their expansion or influencing their trafficking within the host. Regardless, these results indicate that the disparity in M_Φ numbers in WT versus IFN-I R-deficient hosts may be tied to host survival.

5.5 IFN-I signalling regulates macrophage survival

Since there was a positive correlation between macrophage number and host survival, we were interested in evaluating the influence of ST-induced IFN-I signalling on M_Φs more closely. To this end, cytokine production by bone marrow derived M_Φs (BMMs) from WT and IFN-I R^{-/-} mice, infected with ST *ex vivo*, was evaluated. Interestingly, while IL-12p40/70 and IL-6 expression was unchanged in the absence of IFN-I signalling, WT BMMs produced significantly more TNF α and NO₂⁻ than did IFN-I R^{-/-} BMMs following ST infection (Fig. 9). Similarly, TNF α and NO₂⁻ expression, but not IL-12p40/70 or IL-6

was regulated by IFN-I signalling following ST infection in DCs (Fig. 10). IFN-I-dependent NO_2^- expression has been demonstrated previously in macrophages (83) and although $\text{TNF}\alpha$ and NO_2^- are important for M_Φ activation and intracellular killing, respectively, they have also been shown to contribute to cell death by apoptosis (66) and necrosis (69).

The observation that WT mice display reduced M_Φ numbers following ST infection, coupled with increased $\text{TNF}\alpha$ and NO_2^- expression suggested that IFN-I signalling regulates M_Φ viability during ST infection. This suggestion is in line with previous reports that demonstrated IFN-I-mediated M_Φ and T cell death following LM infection (66, 69, 70, 84). Furthermore, M_Φ s treated *in vitro* with IFN- α were far more susceptible to LPS-induced apoptosis (85) suggesting the possibility that IFN-I could regulate M_Φ viability following ST infection. In fact, BMMs derived from WT mice were significantly more susceptible to ST-mediated cell death than BMMs derived from IFN-I R^- mice (Fig. 16 - 17). Interestingly, despite similar trends in cytokine production by BMDCs and BMMs derived from WT and IFN-I R^- mice following ST infection, BMDC viability was independent of IFN-I signalling at 24hr post-infection. This could indicate that cell survival is differentially regulated by IFN-I signalling, however, since DC populations are known to be transient, we cannot exclude the possibility that IFN-I signalling could regulate DC survival at earlier time points than those evaluated in this study.

Together these results suggest that IFN-I-mediated elimination of M_Φ s, key players in ST clearance, could account for the disparity in host response observed in this study.

5.6 IFN- β regulates macrophage death during ST infection

Since it was clear that IFN-I signalling correlated with reduced M_{Φ} death and host susceptibility we were interested in determining which members of the IFN-I family were responsible for the detrimental host outcome observed following ST infection. We evaluated M_{Φ} viability after ST infection in BMMs derived from WT and IFN-I $R^{-/-}$ hosts with or without anti-IFN- α or anti-IFN- β antibody treatments, alone or in combination. As previously discussed ST induced cell death in WT, but not IFN-I $R^{-/-}$ BMMs. Interestingly, treatment with antibodies against both IFN- α and IFN- β restored WT BMM viability. Furthermore BMM viability was restored following treatment with anti-IFN- β alone. Treatment of cells with anti-IFN- α antibodies alone however, was incapable of restoring BMM viability (Fig. 19). These results therefore indicate that M_{Φ} death is mediated by ST-induced IFN- β signalling through the IFN-I R. Here it is indirectly demonstrated that IFN- β is not only produced following ST infection, but that it regulates M_{Φ} survival and ultimately contributes to host susceptibility.

To examine the mechanism of IFN- β signalling, we evaluated host survival to ST infection in mice that were deficient in the MyD88-independent signalling adapter molecule TRIF. Mice deficient in this signalling pathway displayed enhanced survival, suggesting that IFN- β is produced, at least in part, following the MyD88-independent pathway. IFN-I production following viral infection was demonstrated to be exclusively mediated through MyD88-independent signalling (28). However, since the absence of TRIF did not enhance survival significantly, it cannot account entirely for the effects of ST-induced IFN- β production; whether MyD88-dependent signalling is able to compensate

for the lack of TRIF, or whether both pathways are required for ST-induced IFN- β production will require further investigation into signalling mechanisms of the host response following ST infection.

6. Conclusion

Type I interferons (IFN-I) are important cytokines in anti-viral host defence mechanisms, deriving their name from their function that led to their discovery; their capability to interfere with the process of viral replication. Since then, many other functions have been elucidated for this diverse family of cytokines. As important early mediators of the host response to viral infections, IFN-I prevent viral replication and spread by inhibiting viral replication within infected host cells and mediating the elimination of infected cells (60, 86). As a group of potent inflammatory cytokines, type-I interferons stimulate massive cytokine production resulting in the induction of an 'anti-viral' state in neighbouring cells further preventing viral spread (60, 61). Furthermore, despite lacking a clear understanding of the mechanisms of action, IFN-I continue to be effective treatments for both autoimmune disorders and various types of cancer. This is thought to be largely due to the anti-inflammatory effects of IFN-I, dampening the production of IL-12, TNF α and IFN γ (87, 88). IFN- β treatment of patients with Multiple Sclerosis results in slowed disease progression and fewer clinical relapses (89, 90). IFN-I has also demonstrated anti-proliferative and anti-angiogenic functions that make it an important cytokine for defence against numerous types of cancer (91-93). Recently, novel exploration of the innate

functions of IFN-I outside of its otherwise familiar viral context has shed light on an important role for IFN-I during bacterial infections (63-67). Here we report that IFN-I significantly influence the host response to infection with *Salmonella typhimurium* (ST).

The results of this study highlight the importance of innate host responses previously unexamined with respect to ST infection. Part of the innate host response to ST infection is the production of IFN-I, likely produced following signalling through both the MyD88-independent and λ -dependent pathways. ST-induced IFN-I production significantly enhances pathogenesis leading to an uncontrolled systemic infection that results in host lethality within 7 days of infection. Enhanced susceptibility to ST infection was correlated with a reduction in the number of macrophages in the spleens of infected animals. Furthermore, IFN- β signalling induced macrophage death in bone marrow-derived macrophages, and was associated with significantly higher levels of TNF- α and NO $_2^-$. Whether elevated levels of these mediators cause the observed death of macrophages remains unclear. Regardless of the mechanism, we propose that in its elimination of macrophages, IFN-I limit the ability of the host to clear ST thus rendering hosts more susceptible to infection (Fig. 20).

Importantly, susceptibility to ST infection has been associated with massive inflammation, generally thought to be due to LPS-mediated endotoxic shock (9). Our results indicate that IFN-I signalling significantly contributes to early inflammation following ST infection since both protein and mRNA expression of multiple cytokines and chemokines were significantly reduced in IFN-I R-deficient mice. The balance of pro- and anti-inflammatory mediators is crucial for eliminating pathogens, while still maintaining host integrity. As such, it is conceivable that the host inflammatory response was

significantly amplified in WT hosts as a direct result of the limited number of macrophages. Since ST replicates uncontrollably in WT mice, chemokine over-expression could be an indication of a potent attempt by the host to attract, or activate the few macrophages that remain viable to clear ST infection at the cost of potential tissue damage. This suggestion is supported by the fact that there was a negative correlation between increased inflammation and the size of macrophage populations in the spleen, however this remains to be addressed directly.

Here it is demonstrated that IFN- β is produced endogenously following ST infection, and that it serves a crucial role in regulating host survival. While elimination of infected cells during viral infections is important for pathogen elimination, in the context of the facultatively intracellular pathogen, ST, IFN- β -induced M ϕ death reduces the host's ability to clear ST enabling uncontrolled systemic replication. High ST levels in combination with massive inflammation results in enhanced host susceptibility to infection (Fig. 20). Further studies into the role of IFN-I signalling in macrophage regulation are required to elucidate whether its effects are limited to regulating cell viability or whether IFN-I control other aspects of macrophage function. Finally, although it remains to be addressed directly, crucial links between innate and adaptive immunity have been shown to be dependent on IFN-I signalling during viral infections. This could explain why despite displaying prolonged survival beyond the point when T cells normally become activated in resistant mice, IFN-I R^{-/-} mice still succumb to ST infection. IFN-I have been demonstrated to be important for the priming of both CD4⁺ and CD8⁺ T cells as well as initiating antibody-mediated responses all of which are important mediators of the host adaptive response to ST (79-81, 94). Thus, paradoxically, IFN-I signalling is detrimental for the

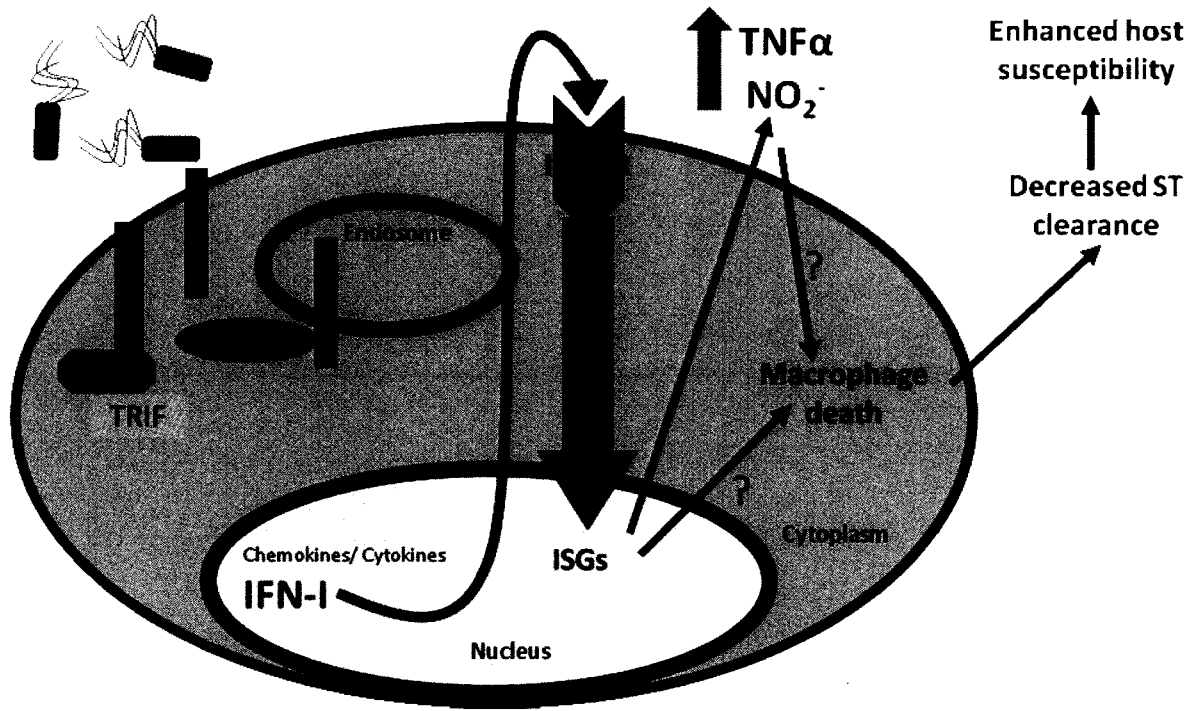
innate response to ST infection, but may be crucial for initiating later adaptive responses important for ST clearance. Investigation into the role of IFN-I at different time points during ST infection in innately susceptible and resistant mice may provide further insight into the importance of this cytokine in regulating the host response to infection.

Importantly, this study provides further evidence that IFN-I is an important mediator of the host response to non-viral pathogens and is the first report of its pathological role during ST infection. Our results support the findings of several studies on IFN-I mediated susceptibility to the intracellular pathogens LM and MTb (64-67) and contrasts other studies addressing the role of IFN-I during infections with extracellular pathogens (63). Since the role of IFN-I during ST infection exhibits similarities with those of LM and MTb, this would suggest that the ability of ST to reside intracellularly may be significant in determining the influence of IFN-I on host survival although this remains to be addressed directly. Future investigations into the role of IFN-I during other non-viral pathogen infections will provide insight into this hypothesis.

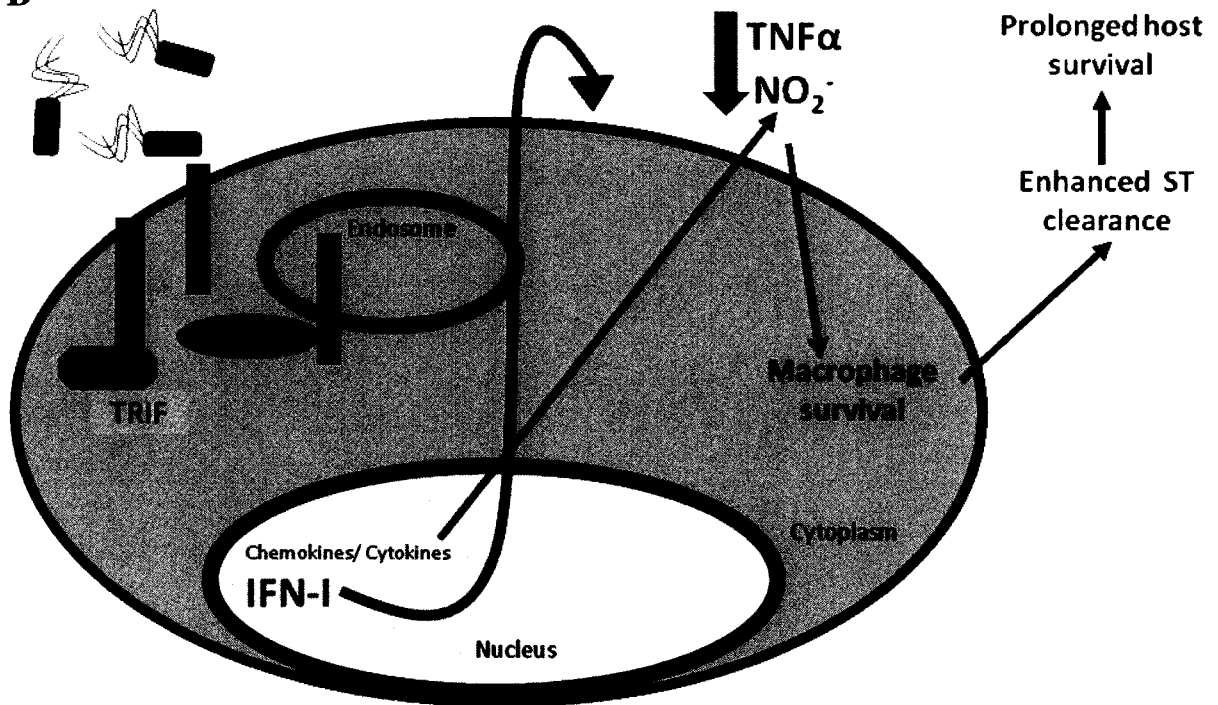
Fig. 20. Model depicting cellular events in the presence or absence of IFN-I signalling that ultimately lead to disparity in host survival

The recognition of ST by host cells resulted in the production of IFN-I along with a number of other cytokines through both the MyD88-dependent and -independent pathways. IFN- β produced following ST infection signalled through the IFN-I receptor (IFN-I R) and the JAK-STAT signalling cascade leading to the production of interferon stimulated genes (ISGs). Importantly, upregulation of TNF α and NO $_2^-$ expression was dependent on IFN-I signalling and was correlated with reduced M ϕ viability in WT mice. ST infection was exacerbated as a result of reduced ST clearance due to IFN-I-mediated M ϕ death, leading ultimately to enhanced host susceptibility (A). In the absence of the IFN-I R, M ϕ s remained viable throughout ST infection, thus IFN-I R $^{-/-}$ mice displayed enhanced ST clearance and prolonged survival (B).

A



B



7. References

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EDUCATION

Masters of Science Candidate

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Microbiology & Immunology; University of Ottawa, Ottawa ON

- Research-based thesis program under the supervision of Dr. Subash Sad, National Research Council Canada, Institute for Biological Sciences (NRC-IBS)
- Study of innate responses to lethal infection with *Salmonella typhimurium* with particular interest on the role of type-I interferons in modulating host responses

Bachelor of Science (Honours)

Biology (Biomedical Science); Laurentian University, Sudbury ON

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- Course and laboratory experience focused largely on Anatomy, Genetics, Biochemistry, Immunology and Microbiology/ Infectious Disease
- Honours thesis project entitled 'Lungworm Infections in Ontario Fisher (*Martes pennanti*); Prevalence, Intensity and Geographical Distribution'; under the supervision of Dr. G.H. Parker
- Laurentian University President's Honour Roll

SPECIAL SKILLS

Languages

- English
- French (conversational)

Technical Skills

- Operated under level-1 and level-2 laboratory standard operating procedures
- Accredited with Canadian Council on Animal Care (live animal procedures; cardiac puncture, organ harvesting and processing)
- Performed quantitative real-time PCR, bacterial culture and microbiological assays (Level-2 pathogens), cell purification, flow cytometry, proteomic analysis (cytokine ELISAs, protein arrays), primary cell culture

Computer Skills

- Microsoft Word, Excel and PowerPoint; Graph Pad Prism, FACSDiva, ABI Prism and AlphaEase Imaging software; SPSS; KROLL Pharmacy software, McKesson on-line ordering

CAREER-RELATED EXPERIENCE

Lecturer, Immunology

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Department of Biology, Nipissing University, North Bay ON

- Developed course materials including lectures, assignments and evaluations
- Evaluated students based on understanding and practical application of theory discussed in lectures.

Laboratory Instructor, Biology, Microbiology and Genetics

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Department of Biology, Nipissing University, North Bay ON

- Provided laboratory skills instruction for Cellular and Molecular Biology, Microbiology and Genetics.
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- Teaching Assistant, Biochemistry** **2009**
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- Taught principles of carbohydrate properties, demonstrated and instructed common laboratory techniques
 - Evaluated lab reports in a timely fashion with constructive feedback
- Tutor, Biology** **2006-7**
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- Presenter**
Department of Biochemistry, Microbiology and Immunology, University of Ottawa, Ottawa ON **2009**
- Presented seminar entitled 'Disruption of Type-I Interferon (IFN-I) Signalling Reveals a Detrimental Role for IFN-I in the Pathogenesis of *Salmonella typhimurium*'
- NRC-IBS, Ottawa ON; Canadian Society for Immunology Conference, Mont-Tremblant PQ; Department of Biochemistry, Microbiology and Immunology, University of Ottawa, Ottawa ON** **2008-9**
- Presented poster on novel thesis research entitled 'Interferon-alpha receptor-deficient mice display prolonged survival during infection with *Salmonella typhimurim* compared to wild-type C57BL/6 mice'
- Ontario Biology Day, McMaster University, Hamilton ON** **2007**
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- Thesis Defense, Department of Biology, Laurentian University, Sudbury ON** **2007**
- Defended undergraduate thesis
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National Research Council of Canada, Institute for Biological Sciences, Ottawa ON **2007**
- Conducted live animal research
 - Studied the pathogenesis and host response to level-2 infectious pathogens
 - Standardized laboratory protocols
 - Accumulated and analyzed data
- Department of Biology, Laurentian University, Sudbury ON** **2006**
- Acted as a summer student researcher including data collection and analysis
 - Studied wildlife parasitology, necropsied a variety of vertebrate specimens for infection analysis
 - Applied statistical analyses of gathered data
- Pharmacy Assistant** **2002-5**
- Assisted filling prescriptions, placed orders on-line, maintained over-the-counter and prescription stocks.
 - Entrusted with opening, closing and additional business operations
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Let's Talk Science, Ottawa ON **2008**
- Queen Elizabeth Elementary School, Ottawa ON
 - Science Fair Judge and Class Visitor
- Volunteer Science Fair Judge**
Canada-Wide Science Fair, Ottawa ON **2008**
- Junior/Intermediate Life Sciences Judge

AWARDS

- University of Ottawa Graduate Entrance Scholarship, 2007-9
- NRC-IBS Group Achievement and Teamwork Award, 2007-8
- Laurentian University In-Course Scholarship, 2006-7
- Laurentian University Presidents Honour Roll, 2005-7
- Laurentian University Entrance Scholarship, 2003-4
- Ontario Scholar's Award, 2003
- BMLSS All-Round Graduate Award, 2003
- BMLSS Biology Award, 2003
- University of Toronto National Biology Competition, 2003

REFEREES

Available upon request