Evaluation of the role of type-1 interferon signaling in the pathogenesis of *Salmonella* Typhimurium

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PREFACE

CONTRIBUTION OF COLLABORATORS

The *Irf9*- deficient mice were provided by Dr. Karen Mossman (McMaster University, Hamilton, ON, Canada). The Seahorse assay was performed using the Seahorse Bioscience XF24 Extracellular Flux Analyzer from Dr. Mary-Ellen Harper laboratory (University of Ottawa). They also provided all the key reagents used for the Seahorse assay.

APPROVALS

The experimental protocols used were approved by the University of Ottawa Animal Care Committee and include the protocols BMI1638 and BMI1639. A Biohazardous Materials Use Certificate was obtained from the University of Ottawa Office of Risk Management, Environmental Health and Safety.

ABSTRACT

Innate immunity operates independently of prior exposure to pathogens. There are several signal transduction pathways that play a key role in inflammatory and immune responses. Critical signaling cascade in the interest of my research is type-1 interferon (IFN) signaling pathway in response to infection with *Salmonella* Typhimurium (ST). The role of type-I interferons is well established in the context of a viral infection; however, their role in bacterial infections is not clear. In my thesis I aimed to understand the role of type-1 IFNs in bacterial pathogenesis, and scrutinize the mechanism adopted by various components of type-1 IFN signaling, especially ISGF3 complex in response to *Salmonella* Typhimurium. My results indicate that type-I IFN signaling is detrimental to host survival. I further investigated the mechanism through which type-1 IFN signaling results in host susceptibility against *Salmonella*. My results indicated that the three transcription factors downstream of IFNAR1 have different impacts in mounting an innate immune response against ST. IRF9 and STAT2 promote susceptibility against ST whereas STAT1 through IFNAR1-signaling, promotes enhanced expression of pro-inflammatory cytokines and protection against ST. I also observed that the monocytes/macrophages lineage in *Ifnar1*$^{-/-}$ mice is responsible for conferring the enhanced resistance against ST. Furthermore, my work determined that expression of type-I IFN signaling compromises the fitness of macrophages by reducing mitochondrial respiration, glycolysis and myelopoiesis.

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LIST OF ABBREVIATIONS

2-DG	2-Deoxy-D-Glucose
AIM-2	Absent in Melanoma
ALR	Aim-2 Like Receptors
Apaf	Apoptotic Protease Activating Factor-1
APC	Antigen Presenting Cell
ATP	Adenosine Triphosphate
ALDOLA C	Aldolase C
BCG	Bacillus Calmette-Guérin
BCR	B Cell Receptor
BMM	Bone Marrow Derived Macrophages
BSA	Bovine Serum Albumin
CD	Cluster of Differentiation
CFU	Colony Forming Unit
CCAC	Canadian council on Animal Care
CLP	Common Lymphoid Progenitor
CMP	Common Myeloid Progenitor
CO_2	Carbon diaoxide
CTL	Cytotoxic T- lymphocytes
DAMP	Danger Associated Molecular Pattern
DC	Dendritic Cell
DNA	Deoxyribose Nucleic Acid
ELISA	Enzyme-Linked Immunosorbent Assay
ER	Endoplasmic Reticulum
ETC	Electron transport chain
ECAR	Extracellular acidification rate
Fasl	Fas ligand
FBS	Fetal Bovine Serum
GAF	Gamma-Activated Factor
GAS	Gamma-Activated Sequence
GMP	Granulocyte-Monocyte Progenitor
GPI	Glucose-6 phosphate isomerase
HIV	Human Immunodeficiency Virus
HRP	Horse Radish Peroxidase
HSC	Hematopoietic Stem Cell
IFN	Interferon
IFN-α/β	Interferon Alpha/Beta
IFN-γ	Interferon Gamma
IFNAR	Interferon Alpha/Beta Receptor
IFNGR	Interferon Gamma Receptor
IL	Interleukin
iNTS	invasive non-typhoidal strains
i.p.	Intraperitoneal
i.v.	Intravenous
IRF	Interferon Regulatory Factor

ISG	Interferon Stimulated Genes
ISGF	Interferon Stimulated Gene Factor
ISRE	Interferon Stimulated Response Elements
JAK	Janus Kinase
LB	Luria Broth
LCMV	Lymphocytic Choriomeningitis Virus
LPS	Lipopolysaccharide
LT-HSC	Long-Term Hematopoietic Stem Cell
MAPK	Mitogen Activated Protein Kinase
M cells	Microfold cells
MCSF	Macrophage Colony Stimulating Factor
MEP	Megakaryocyte-Erythrocyte Progenitor
MHC	Major Histocompatibility Complex
MK-2	MAPK activated protein kinase 2
MLKL	Mixed Lineage Kinase Domain Like Kinase
MOI	Multiplicity of Infection
MP	Myeloid progenitors
MPPs	Multipotent progenitors
Mtb	*Mycobacterium tuberculosis*
MyD88	Myeloid Differentiation Primary Response Gene-88
NF-κB	Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells
NK cell	Natural Killer Cell
NLR	NOD-Like Receptors
NR	Neutral Red
NRAMP	Natural Resistance Associated Macrophage Protein
NTS	Non-Typhoidal *Salmonella*
OCR	Oxygen Consumption Rate
OD	Optical Density
OXPHOS	Mitochondrial Oxidative phosphorylation
PAMP	Pathogen Associated Molecular Pattern
PBS	Phosphate Buffer Saline
PBS-T	Phosphate Buffer Saline with Tween
PIP	Phosphatidylinositol Phosphates
poly(I:C)	Polyinosinic:polycytidylic acid
PRR	Pattern Recognition Receptor
RIPK	Receptor-Interacting Protein Kinase
RLR	Retinoic Acid-Inducible Gene-I-Like Receptors
RNA	Ribonucleic Acid
RNA seq	RNA Sequencing
RPM	Revolutions Per Minute
RPMI	Roswell Park Memorial Institute Medium
SCV	*Salmonella* Containing Vacuole
SIF	*Salmonella* Induced Filaments
SIP	*Salmonella invasion protein*

SPI	*Salmonella* Pathogenicity Islands
ST	*Salmonella enterica* serovar Typhimurium
STAT	Signal Transducer and Activator of Transcription
ST-HSC	Short-Term Hematopoietic Stem Cell
T3SS	Type Three Secretion System
TCR	T-Cell Receptor
TGF	Transforming Growth Factor
T_h	T- helper cell
TLR	Toll-Like Receptor
TMB	Tetramethylbenzidine
TNF-α	Tumour Necrosis Factor Alpha
TNFR	TNF-alpha Receptor
TRIF	TIR-Domain-Containing Adapter-Inducing Interferon-beta
TYK	Tyrosine Kinase
WHO	World Health Organization
WT	Wild Type

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1. INTRODUCTION

1.1 The immune system

Immune system is comprised of various cell types that provide protection against numerous pathogens. Innate (natural) immunity operates independently of the prior exposure to pathogens and mediates protection in a nonspecific manner. It operates swiftly and has no memory of the encounters, unlike the adaptive immunity (Delves and Roitt, 2000). On the other hand, adaptive immunity is pathogen specific and generates immunological memory which provides long-term protection against the pathogen (Parkin and Cohen, 2001). Both innate and adaptive immune responses cooperate to provide comprehensive protection against pathogens (Parkin and Cohen, 2001).

1.1.1 Innate immune system

Innate immunity serves as the first line of defense, although it is less specific in nature. There are various white blood cells involved in innate immunity, such as monocytes, neutrophils, eosinophils, basophils, and natural killer cells. Almost all the cell types are of myeloid origin except NK cells, which are of lymphoid origin. Each cell type is responsible for a specific function (Chaplin, 2010). The key function of NK cells is to target cells possessing low levels of surface proteins known as class-I major histocompatibility complex molecules (MHC-I) when compared to healthy cells. (Delves and Roitt, 2000).

An important cell type of innate immunity is the macrophage, which differentiates from monocytes after migrating into tissues, when a pathogen breaches the first line of defense and causes damage or infection. However, this process also involves various signaling proteins (cytokines) produced by diverse immune cells that play a significant role in inflammatory response (Medzhitov, R. & Janeway, 2000). After their activation macrophages secrete various inflammatory cytokines which promote pathogen clearance.

All cytokines except the IL-1 cytokine family possess a secretion signal which promotes their export to the extracellular milieu. Cytokines of the IL-1 family do not have the secretory signal and require additional processing (cleavage) in order to be exported to the extracellular milieu, and this processing is mediated by inflammasomes **(**Coondoo A, 2011). Among all the cell types of innate immunity, the first cell type to migrate to the site of infection are the neutrophils (Parkin and Cohen, 2001). They perform phagocytosis via pseudopodia, engulfing pathogens in a vesicle known as phagosome, which then fuses with the lysosome to form a phagolysosome via the endocytic pathway. During this process various enzymes and microbicidal agents get released such as reactive oxygen species (ROS), which help in the digestion of ingested pathogens (Kindt et al., 2012). Similarly, activated neutrophils and IFN-γ play a key role in attracting and stimulating activation of phagocytes such as macrophages. Activated neutrophils also attract dendritic cells to the site of infection. Pathogen control is enhanced with the help of cytokines which are produced during this process (Parkin and Cohen, 2001).

Dendritic cells are also phagocytic in nature and are directly involved in pathogen control. In addition, they have the ability to migrate to lymph nodes and present antigens to naïve T cells of the adaptive immune system leading to T cell activation. Thus, dendritic cells are known as the professional antigen presenting cells (APCs) which act as a bridge between the innate and adaptive immune system (Janeway, 2001b).

1.1.2. Adaptive immune system

The adaptive immunity is critical when innate immunity is ineffective against infectious pathogens. The adaptive immune system involves T and B lymphocytes. Each of these cells bear unique antigen-specific receptors from a diverse repertoire of random receptor gene rearrangements, generated through VDJ recombination (Kindt et al., 2012).

Antigen specific T and B cells are derived from hematopoietic stem cells, differentiation of B cells into plasma cells produce antigen-specific antibodies. Activated T cells proliferate into effector cells through the action of APCs (Marshall, J. S.,et al 2018). The receptors of these cells that are involved in antigen recognition are the T cell receptor (TCR) for T cells and the B cell receptor (BCR) for B cells which are basically cell-surface bound antibodies belonging to the class of immunoglobulins (Parkin and Cohen, 2001).

Each single T cell has its unique TCR, which has the capacity to proliferate and differentiate on receiving specific signal. They are activated when they recognize antigenic peptides processed and presented through Major Histocompatibility complex (MHC) by antigen-presenting cells (APCs) such as dendritic cells, macrophages, B cells and fibroblasts (Huang, M. and Dong, J. 2021; Delves et al., 2000). MHC-I, known as human leucocyte antigen, is present on all nucleated cells, whereas MHC-II is found on certain cells of immune system such as macrophages, dendritic cells and B cells. The MHC protein presents fragments of antigens (peptides) after pathogen is phagocytosed by DCs. (Marshall, J. S.,et al 2018). T cells are activated upon encountering an APC that displays unique antigenic peptide bound to its MHC molecule (Janeway,2001b; Medzhitov and Janeway,2000; Marshall, J. S.,et al 2018). This MHC-antigen complex then activates the TCR, and the activated T cell secretes the cytokines which further amplifies the T cell response, through a process that is known as co-stimulation (Delves et al., 2004). MHC-I molecules present endogenous peptides, which are processed in the cytosol, to CD8 T cells.

On the other hand, the exogenous peptides which are processed in the phagolysosomes are presented to CD4 T cells. This antigen presentation process stimulates the T cells to proliferate and differentiate into effector T cells (Parkin and Cohen, 2001).

Cytokines present during the activation of CD4 T cells can influence their differentiation to various types of effector T cells (Delves et al., 2000). Th1 response activates bactericidal activities of macrophages along with production of IFN-γ, (Marshall, J. S.,et al 2018). Th1 derived cytokines also contribute to differentiation of B cells that secrete specific isotypes that are able to control intracellular infections better. Th2 response is characterized by production of cytokines such as IL-4 and IL-5, which promote the development of IgE producing B cells. Th 17 response is characterized by the secretion of IL17 cytokine, which is associated with inflammatory responses (Marshall, J.S et al., 2018). Another important subset of CD4+ T cells is the regulatory T cell (T reg). These cells can suppress and limit immune responses and hence play a key role in the development of immune tolerance (Kindt et al.,2012; Parkin and Cohen,2001).

In contrast to CD4 T cells, activation of CD8 T cells results in their differentiation towards cytotoxic cells that rapidly kill infected cells. They are activated when the TCR recognizes a specific antigen bound to MHC class I molecules (Medzhitov and Janeway,2000). Effector cells generated as a result of clonal expansion of cytotoxic T cells releases granzymes into the cytoplasm of infected target cells which results in rapid cell death of infected cells and consequent control of infection. Once infection subsides, majority of effector cells die and are cleared by apoptosis, whereas some are retained as long-lived memory cells (Marshall, J. S.,et al 2018). On the other hand, CD4+ T cells, also known as T- helper cells (Th) cells play an important role in maximizing the immune response (Marshall, J. S.,et al 2018). They recognize antigen presented by MHC class II molecules

and once activated they express numerous pro-inflammatory and anti-inflammatory cytokines (Janeway,2001b; Medzhitov and Janeway,2000).

While CD4 T cells and CD8 T cells recognize exogenous versus endogenous respectively, alternative pathway known as "Cross presentation pathway" promotes recognition of exogenous antigens by CD8 T cells. Even though, various APCs can cross-present, only dendritic cells are the most common and efficient cell type that mediates cross-presentation. Cross-presentation has been studied in the context of bacterial infections such as *Listeria monocytogenes* and *Mycobacterium tuberculosis* and in the context of antigens released from dying cells. Dendritic cells (DC) phagocytose infected apoptotic cells and mediate cross-presentation of antigens to CD8 T cells, thus allowing for effective cytotoxic T lymphocytes (CTL) responses against the pathogens (Jung S et al, 2002).

Unlike T cells, B lymphocytes can recognize the antigens in their environment with the help of their B cell receptor (BCR) (Delves et al., 2000; Marshall, J. S.,et al 2018). After antigen recognition at the cell surface the antigen is internalized, processed and antigenic peptides presented to CD4 T cells through MHC class II which results in the secretion of cytokines that aid in the class switching of B cell response towards various isotypes (Parkin and Cohen,2001). Differentiation of B cells results in the generation of a plasma cells which secrete high levels of antibodies that control infections directly. A subset of B cells differentiates to memory B cells which continue to express antigen-binding receptors and can then quickly recognize their specific antigen upon secondary exposure, re-differentiate into plasma cells and secrete antibodies at a faster rate (Parkin and Cohen, 2001). Whereas plasma cells are short lived and often undergo apoptosis, memory B cells promote lifelong immunity against the pathogen (Kindt et al.,2012).

1.1.3. Generation of hematopoietic cells

Hematopoietic stem cells (HSCs) are generated in the bone marrow by a process known as hematopoiesis. This process can also occur in minor hematopoietic sites such as spleen and liver (Zhu and Emerson, 2002). HSCs possess long term self-renewal capacity and play a crucial role in maintenance of the immune system. These cells have an ability to proliferate and re-establish homeostasis upon injury (Rosenbauer and Tenen, 2007). HSCs are common precursor cells from which other blood cells and immune cells are generated (Petvises and O'Neill, 2012). LT- HSCs known as long- term HSC give rise to short term HSCs (ST- HSC) (Rosenbauer and Tenen, 2007). ST-HSC then acts as a precursor for common myeloid precursors (CMPs) and common lymphoid precursors (CLPs). CLPs give rise to all lymphoid cells including B, T and NK cells. Moreover, CLPs also gives rise to the lymphoid subset of dendritic cells (Zhu and Emerson, 2002). After maturation naïve T cells migrate to the thymus where the self-reactive T cells are eliminated. After their "education" in the thymus, naïve T cells migrate to the secondary lymphoid organs such as spleen and lymphoid tissues. On the other hand, CMPs serve as a precursor to all myeloid progenitors (CMPs) and erythroid cells (Kondo et al.,1997).

Granulocyte-monocyte progenitors (GMPs) and megakaryocyte-erythrocyte progenitors (MEPs) are two subclasses of CMPs. CMPs express CD16 /32 and CD34, the two markers that can be used to discriminate these progenitors as follows: CMPs: CD34 (+ve) / CD16/32 (- ve), GMPs: CD34 (+ve) / CD16/32 (+ ve) and MEPs: CD34 (-ve) / CD16/32 (- ve) (Rosenbauer and Tenen, 2007).

CMPs also lead to generation of the myeloid subset of dendritic cells, granulocytes, and monocytes. These cells migrate to the tissue via bloodstream. Various cytokines such as M-CSF and GM-CSF play key roles in macrophage -monocyte lineage cells. M-CSF acts in homeostasis and is known to signal through its receptor (CSF-1R) and IL-34, that acts

as a second ligand of CSF-1R and regulate macrophage and monocyte cell numbers, without having any impact on their activation status. GM-CSF is activated during inflammatory conditions, and it promotes the differentiation of monocytes into macrophages or to other immune cells such as dendritic cells (Ushach and Zlotnik, 2016).

1.2. *Salmonella* infection

Salmonella is a gram-negative bacterium that belongs to the family Enterobacteriaceae (Eng et al., 2015). *Salmonella enterica* and *Salmonella bongori* are the two main species of this genus (Reeves et al.,1989). *Salmonella enterica* is divided into 6 subspecies, which are further classified into serovars Out of 2500 serovars identified, majority of serovars belong to *Salmonella enterica* subspecies *enterica* (Reeves et al.,1989).

Broadly there are two serovars, non-typhoidal serovar such as *Salmonella* Typhimurium (*S*. Typhimurium) and Enteritidis and typhoidal serovars such as *Salmonella enterica* Typhi and Paratyphi (Hume et al., 2017). *S.* Typhimurium infects humans and invades intestinal epithelial barrier and stimulates inflammation along with fluid secretion (Hume et al., 2017). Moreover, it is the causative agent of non-typhoidal *Salmonella* infection (iNTS), often associated with immune suppression, and development of septicemia in immunocompromised patients (Feasey et al., 2012 ;Hume et al., 2017).

My work is based on the non-typhoidal strain of *Salmonella* known as *S*. Typhimurium (ST). It is widely used in murine models because it can cause disease in susceptible animals. One such susceptible strain is C57BL/6J; it was observed that ST can mimic human typhoid fever in this strain of mice. One of the strengths of this mouse model is the discovery of effectors governing ST virulence, known as *Salmonella* pathogenicity islands (SPIs) (Santos et al., 2001). Partial resistance of *Salmonella* is associated with a gene known as natural resistance associated macrophage protein 1, also known as *Nramp1* gene (Vidal et

al., 1995). The NRAMP-1 protein actively pumps divalent cations into the cytoplasm, this makes ST inaccessible to metabolic cofactors (Gruenheid et al., 1999). Strains that have a mutation in the *Nramp1* gene, cannot actively pump cations into the cytoplasm, which leads to accumulation of these ions in the phagosome that promotes intracellular pathogen replication (Vidal et al., 1995). These strains can relent to infection rapidly even when infected with a minimal dosage of bacteria as low as 10^2 (Broz et al., 2012a).

1.2.1 Mechanism of infection

Salmonella infection occurs through the oral route and is mainly acquired through ingestion of contaminated food or water (Eng et al., 2015). *Salmonella* can survive the extreme conditions of the gastrointestinal tract and can invade the intestinal mucosa (Haraga et al., 2008). *Salmonella* breaks the gut epithelial barrier and can be taken up by specialized epithelial cells called microfold cells (M cells). Thus, the bacteria get internalized, into APCs such as macrophages and dendritic cells and this favors rapid dissemination *Salmonella* through the lymphatics and blood stream (Monack et al., 2004; Hume et al.,2017; Kaur and Jain,2012).

1.2.2 *Salmonella* and its virulence

Salmonella can induce its own uptake into the host cells such as macrophages by a process known as macropinocytosis (Garcia-del Portillo and Finlay, 1994). In this process, the phagosome that contains the bacteria, fuses with a lysosome to form a vacuole known as a *Salmonella* containing vacuole (SCV) (Haraga et al., 2008). Various virulence factors are responsible for intracellular survival of *Salmonella* (Alpuche-Aranda et al.,1992).

Salmonella injects virulence factors (effector proteins) into the host cell cytoplasm using the molecular machinery encoded by the Type 3-Secretion System (T3SS). These effector proteins are segregated mainly within 12 Salmonella pathogenicity islands (SPIs). These are mainly gene clusters located in the DNA of *Salmonella.* Out of 12 SPIs, SPI-1 and SPI-II code for T3SS (Sırıken, 2013)

The SPI-1 T3SS effector protein SopB is responsible for bacterial entry into epithelial cells through ruffle formation and membrane extension (Lou et al., 2019), this also restricts secretion of digestive enzymes in SCV which promotes bacterial survival. Moreover, SopB protein also leads to recruitment of neutrophils at the site of infection (Wallis and Galyov, 2000). Other SPI-1 effector proteins such as *Salmonella invasion protein* (SipA and SipC), play a key role in pathogen uptake (Hume et al., 2017). Another SPI-1 encoded gene known as *invA* gene is responsible for bacterial invasion into epithelial cells (Galán and Curtiss,1991). Infection with *Salmonella* Typhimurium that have an inactive *invA* gene, have a dysfunctional SPI-I T3SS, which results in a failure to infect hosts following an oral challenge (Galán and Curtiss, 1991).

In contrast to SPI-I, SPI-II genes promote bacterial survival and replication within infected cells. SifA and SifB are SPI-II T3SS effector proteins, that stimulate the formation of *Salmonella* induced filaments (SIFs) from the SCV, that play a key role in intracellular bacterial replication (Abrahams and Hensel, 2006). They also allow systemic spread of infection by limiting antigen presentation to DCs and consequently suppressing immune response (Waterman and Holden, 2003). Various effector proteins encoded by SPI-II have different impact on the ability of *Salmonella* to cause a virulent infection. *ssaR* is one of the key regulatory proteins in the SPI-II T3SS and disabling of this effector protein results in a highly attenuated, non-lethal infection (Knodler and Steele-Mortimer,2003).

1.3 The inflammatory response

Inflammation is the body's response to defend itself against harmful stimuli such as pathogens. Usually, acute inflammation is observed in response to injury or infection; however, inability to control a pathogen can lead to chronic inflammation (Chen, L., et al 2016). Inflammatory response is initiated as soon as the cell signaling begins, which leads to increased blood flow towards the pathogen or other stimuli. This results in the generation of heat and causes redness of the infected area. Additionally, accumulation of fluids causes swelling to occur in the infected area (Fullerton and Gilroy, 2016).

Inflammatory mediators such as cytokines, antimicrobial peptides and reactive oxygen species get released as a response to phagocytosis of invading pathogens, these modulators help in amplifying inflammatory response (Kindt et al., 2012; Medzhitov,2008).

The first step in the initiation of immune response is the recognition of pathogens by the cell surface receptors present on immune cells. These receptors are very selective in nature and are known as pattern-recognition receptors (PRRs), which detect structural motifs known as pathogen-associated molecular patterns (PAMPs), conserved within each

microbial species (LaRock et al., 2015). Interaction of these PRRs with PAMPs followed by downstream signaling leads to activation of innate immune response (Medzhitov, 2008; Medzhitov and Janeway, 1997).

1.3.1 TLR Signaling

Toll- like receptors (TLRs) are the first identified pattern recognition receptors present in mammals that play vital roles in immune response (Akira et al., 2006). These receptors are mainly present on the cell surface of immune cells (macrophages and dendritic cells) as well as non-immune cell types (fibroblasts and epithelial cells) (Kawasaki and Kawai, 2014). Some of these receptors are also present in the endosomal compartment of cells (Medzhitov,2001).

Each TLR binds to a distinct PAMP, which enables recognition of a broad variety of viruses, bacteria and fungi by the immune system. There are various types of PAMPs expressed by *Salmonella* such as Lipopolysaccharide (LPS), which is a component of outer membrane of gram-negative bacteria which binds to TLR4, and flagellin which binds to TLR5, and lipoproteins that are detected by TLR 1/2/6 (Gilchrist et al., 2015).

LPS binding to TLR4 activates two distinct signal transduction pathways. The myeloid differentiation response gene-88 dependent pathway (Myd88) leads to NF-κB (Nuclear Factor kappa -light-chain-enhancer of activated B cells) activation and induces secretion

of pro-inflammatory cytokines, such as IL12 and TNFα. (Gilchrist et al., 2015; Medzhitov, 2001).

LPS-TLR4 signaling also induces a TRIF- dependent pathway (Kawasaki and Kawai, 2014) which leads to expression of type-1 interferons through activation and phosphorylation of transcription factors of the IRF family such as IRF-3 (Interferon regulatory factor) (Akira and Takeda, 2004; Baccala et al., 2007; Häcker et al., 2011).

1.3.2 Interferon Signaling

Interferons (IFNs) play a pivotal role in heightening the immune response, as the host cells release interferons in response to infection with pathogens. Interferons are classified into three families, type-I, type-II and type-III. The type-I interferons (IFN- α/β) are most widely expressed (Akira and Takeda,2004, McNab et al.,2015). Production of type-I IFNs (IFN-α/β) as mentioned earlier can be stimulated by various bacterial and viral PAMPS which activate the transcription factor NF-kB and MAPK (Mitogen activated protein kinase) pathways resulting in the transcription and finally translation of IFN-α/β. Following secretion, IFN-α/β signaling is achieved by binding of IFN-α/β to a common heterodimeric cell surface receptor termed IFNAR, (IFNAR1 and IFNAR2) which results in the recruitment of tyrosine kinases, Janus kinase 1 (JAK1) and tyrosine kinase (TYK2) respectively. This triggers the Janus kinase (JAK)-mediated phosphorylation of tyrosine, providing the docking sites for the recruitment of signal transducer and activator of transcription proteins (STAT1 and STAT2) (Darnell J.E et al.,1994, Stark G.R et al.,1998). This results in the phosphorylation of STAT1 and STAT2 which interact with interferon regulatory factor 9 (IRF9) to form a heterotrimeric transcription factor complex termed the interferon-stimulated gene factor 3 (ISGF3). After the assembly of ISGF3, this transcriptional complex translocates to the nucleus and binds to the interferon-stimulated response elements (ISRE) which are located within the promoters of interferon-regulated genes (IRGs). Thereafter, this modulates the transcription of several interferon stimulated genes (ISGs) **(Figure 1)** (Ivashkiv and Donlin, 2014; McComb et al., 2014). As part of ISGF3 it has been shown that STAT1 and IRF9 are required for sequence-specific recognition and stable binding with DNA, whereas STAT2 provides transcriptional modulation but is unable to interact with DNA directly (Bluyssen H. A et al., 1997).

Type-I interferon signaling is considered as the primary controller of viral infection as it

interferes with the viral replication cycle through multiple innate immune mechanisms which results in rapid control of infection (Au-Yeung et al., 2013; McNab et al., 2015).

Other important classes of IFN are type-II (IFN-γ) and type-III (IFN-λ) (Megan L et al.,2019). Type-II IFN, which consists of IFN-γ, binds to its receptor IFNGR which employs JAK1 and JAK2. Phosphorylation of STAT1 by JAK1/2, leads to the dimerization of STAT1, also referred to as the gamma activated factor (GAF). This homodimer then translocates to the nucleus and initiates expression of several ISGs by binding to γ-interferon activated sequences (GAS) **(Figure 1) (**Begitt et al., 2014). In contrast to other interferons, type-III IFNs engage with a heterodimeric receptor to initiate signal transduction, which consist of the IFNLR1 chain (IL-28Rα) and the IL-10R2 chain. Importantly, the IL-10R2 chain is also a part of the heterodimeric receptor of all the IL-10 family (IL-10, IL-22 and IL-26) (Megan L et al.,2019).

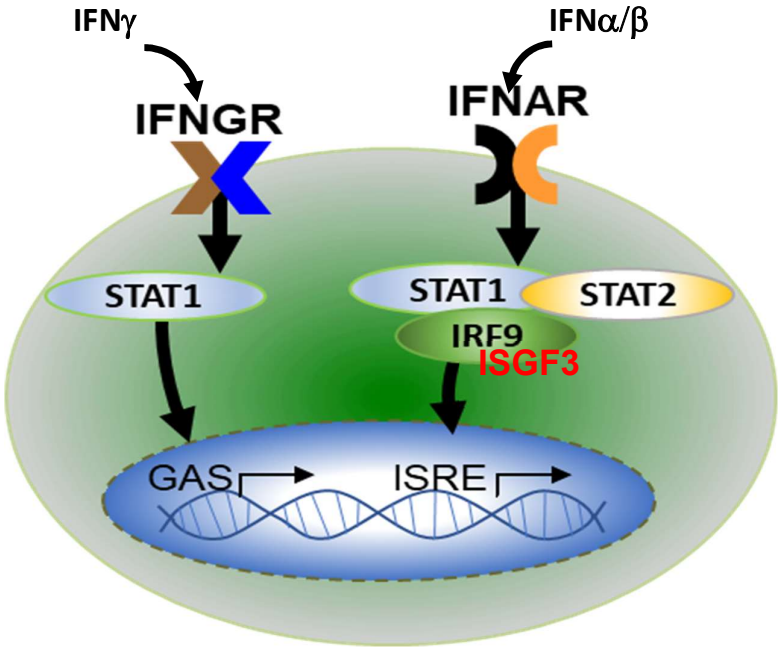

IFNγ
IFNα/β
IFNGR
IFNAR
STAT1
STAT1
STAT2
IRF9
ISGF3
GAS
ISRE

Figure 1. Pictorial overview of type-I (IFN-α/β) and type-II (IFN-γ) signaling pathways.

Binding of type-I IFN (IFN-α/β) to either subunit of common heterodimeric cell surface receptor termed IFNAR, results in the assembly of a trimeric transcriptional complex called as the interferon stimulated gene factor-3 (ISGF3), which is composed of IRF9, STAT1, and STAT2. This complex then translocates to the nucleus and binds to the interferon-stimulated response elements (ISRE), that modulates gene transcription.

On the other hand, binding of type-II IFN (IFN- γ), binds to its receptor (IFNGR), which lead to formation of STAT1 homodimers that translocate to the nucleus and binds γ-interferon interferon activated sequences (GAS), which initiates transcription.

1.4 Cell Death

Cell death is an important mechanism that is responsible for survival of living organisms, by maintaining a homeostatic state in the body (Pasparakis and Vandenabeele, 2015). Pathogens like *Salmonella* use a mechanism through which they manipulate the host's cell development and death machinery to ultimately benefit the pathogen itself (Knodler and Brett Finlay, 2001).

Programmed cell death also known as apoptosis emerged during late 1989 and has proven to be very important for normal development and removal of self-reactive immune cells (Cookson and Brennan, 2001). In this ordered process, the cell brings about its own lysis, which is observed in various phenomena such as cell renewal and lymphocyte selection. In this form of cell death, plasma membrane remains intact, and no intracellular contents ooze out, therefore it is also known as the silent form of cell death (Blander, 2014; Green et al., 2009).

On the contrary, accidental cell death in response to trauma or extreme physicochemical conditions include forms of cell death such as necrosis, necroptosis and pyroptosis (Blander, 2014; Pasparakis and Vandenabeele, 2015).

1.4.1 Apoptosis

Apoptosis is a well characterized form of cell death that exhibits morphological changes such as plasma membrane blebbing, cell shrinkage and cellular fragmentation into small apoptotic bodies. It has been described as a natural phenomenon to remove unwanted components without affecting or harming nearby areas (Kerr et al., 1972). These apoptotic bodies possess intact plasma membranes which help restrict release of

components to the outside environment thus preventing inflammation (Nagata and Tanaka,2017; Ravichandran and Lorenz,2007; Vandivier et al.,2006).

Apoptotic cascades can be distinguished into intrinsic and extrinsic pathways (Eum and Lee, 2011). Intrinsic pathway is activated by various internal factors such as stress, hormones, radiations, toxins, hypoxia, or metabolic perturbations sensed by host cells (Elmore, 2007). During this process, the pro-apoptotic mediator known as cytochrome c is released from mitochondria which binds to apoptotic protease activating factor 1 (Apaf-1), in the presence of ATP; this binding leads to oligomerization of Apaf-1 molecules to form apoptosome (Bao and Shi, 2007). Apaf-1 interacts with pro caspase -9, which is the initiator caspase and triggers its cleavage and activation to caspase-9 (Bao and Shi, 2007). Activated caspase-9 further interacts with executioner caspases such as pro-caspase 3, 7 and 6, which further leads to formation of apoptotic bodies and brings about other morphological and biochemical changes (Ghobrial et al., 2005).

The extrinsic apoptotic pathway is induced in response to extracellular factors such as death receptor (Fas). Binding of Fas to its ligand, Fas ligand (Fas-L) brings about conformation changes, that leads to the cleavage of pro-caspase-8 to active caspase-8, which further leads to activation of caspase-3, eventually resulting in apoptotic cell death (Fadeel and Orrenius,2005).

1.4.2 Necrosis and necroptosis

Necrosis is known as a toxic form of cell death and primarily refers to the changes associated with cell death post injury (Nagata and Tanaka, 2017; Yang et al., 2015b). In contrast to apoptosis, necrosis involves the disruption of plasma membranes of injured

cells and subsequent release of intracellular contents to the outside environment which leads to successive inflammatory reactions (Kono and Rock, 2008; Wallach et al., 2016). Necroptosis is a type of programmed cell death (Pasparakis and Vandenabeele, 2015) and is highly regulated. It involves death receptors such as tumor necrosis factor (TNFR1 and TNFR2) and Fas (Schilling et al., 2014). When apoptosis is inhibited, this process involves the formation of a necrosome which contains the receptor-interacting protein kinase 1 (RIPK1) and RIPK3. RIPK3 auto phosphorylates and leads to the recruitment of mixed lineage kinase domain like kinase (MLKL) (Wallach et al., 2016; Yang et al., 2015b). Phosphorylated MLKL further binds to phosphatidylinositol phosphates (PIPs), which leads to build up of osmotic pressure thus causing the plasma membrane to rupture and trigger inflammation (Dondelinger et al., 2014).

1.4.3 Pyroptosis

In contrast to cell death by apoptosis, which is anti-inflammatory, cell death by pyroptosis results in rapid rupture of cell membrane and release of the intracellular contents to the exterior milieu. Some of the released contents are danger-associated molecular patterns (DAMPs) which can induce activation of innate immune cells. Thus, cell death by pyroptosis results in excessive inflammation. Pyroptosis occurs following the recognition of the trigger and assembly of inflammasomes in the host cell cytoplasm. The final step in the assembly of inflammasomes is the recruitment and activation of caspases which induce cell death. Caspase-1 and caspase-11 are the two main caspases that constitute the final step in inflammasome signaling (Bergsbaken et al., 2009; Jorgensen and Miao,2015). Pyroptosis; wherein “Pyro” means fire, whereas “ptosis”, represents falling, which is similar to the terminology used for other forms of programmed cell death (Cookson and Brennan, 2001). Pyroptosis is also associated with

the rupture of plasma membrane as seen in necrosis. It has been known that *Salmonella invasion protein B (*SipB), a SPI-I T3SS encoded protein, activates pyroptosis and inflammasome signaling (Hersh et al.,1999).

In addition to this, intracellular LPS or hexa-acyl Lipid A component of gram-negative bacteria activates a distinct pyroptotic pathway which is mediated by caspase-11 (Hagar et al., 2013). Activation of caspase-11 can take up to 24 hours to be detected as compared to caspase-1 (Broz et al., 2012b). It has been shown that caspase-11 plays a very crucial role in disease pathogenesis, as its absence can make mice extremely susceptible to infection by bacteria that escape the phagosome and reside in cytosol (Aachoui et al., 2013).

RIG-I-like receptor (RLR), the AIM2-like receptor (ALR), and the nucleotide binding domain and leucine-rich repeat-containing (NLR) proteins are the other host immune cell receptors that can recognize PAMPs present in the cytosol (Takeuchi and Akira, 2010). Among them NLRs and ALRs play a key role in activating pro-inflammatory caspases-1 and 11 respectively through the formation of canonical and non-canonical inflammasomes (Kayagaki et al., 2011).

Activation of inflammatory caspases then cleaves the pro- IL-1β and pro- IL-18, which leads to the release of pro-inflammatory cytokines like IL-1β and IL-18 and stimulates the lysis of infected cells (Broz et al.,2012b). IL-1β acts as an endogenous pyrogen that stimulates chemokine production and fever (Delaleu and Bickel, 2004). IL-18 helps in T cell maturation and macrophage activation by inducing IFN-γ production (Nakanishi et al., 2001).

When pyroptosis of infected cells occurs, it abolishes the biological niche of bacteria, which makes them more susceptible for killing by other phagocytes such as neutrophils; thus, pyroptosis serves as a protective mechanism against various pathogens (Lara-Tejero et al., 2006; Mariathasan et al., 2005; Molofsky et al., 2006).

1.5 RATIONALE

It has been known that chronic viral infection predisposes the host to bacterial invasion, but the mechanism is not clear. Type-I IFN is one of the key inflammatory cytokines produced at high levels during viral infections which is indispensable for viral control. However, persistent expression of inflammatory cytokines can lead to increased cell death of immune cells which in turn may lead to immune suppression (Almand et al., 2017). Type-1 IFN signaling is detrimental to host survival during infection with various intracellular bacteria such as *Listeria monocytogenes*, *Salmonella* Typhimurium and *Mycobacterium tuberculosis* (M*tb*) (Auerbuch et al., 2004; Robinson et al., 2012). Conversely type-I IFN can lead to protection against some bacteria such as (*E. coli*) (Mancuso et al., 2007). Previous research in our lab has shown that *Ifnar1*$^{-/-}$ mice display increased resistance against *S.* Typhimurium which correlates with reduced bacterial burden in comparison to WT mice. Engagement of type-I interferon receptor results in the assembly of the trimeric transcription factor complex ISGF3 that is composed of STAT1, STAT2 and IRF9. Therefore, understanding the role of these transcription factors in the pathogenesis of bacterial infection is of utmost importance.

1.6 HYPOTHESIS

STAT1 through IFNAR1 signaling, promotes enhanced expression of pro-inflammatory cytokines and provides protection against ST.

1.7 OBJECTIVES

1: Evaluate the role of ISGF3 complex in promoting susceptibility to bacterial infection.

2: Evaluate the cell type in Ifnar1-deficient mice that is responsible for the enhanced resistance against ST.

3: Evaluate the impact of type-I interferon signaling on macrophage function.

2. MATERIALS AND METHODS

2.1 Mice

Experiments were performed in accordance with the Canadian Council on Animal Care (CCAC) guidelines and the Ethics Board and the Animal Care Committee at University of Ottawa. All animals used in my thesis work were housed and maintained at the University of Ottawa Animal facility. Breeding and experimental procedures were approved and timely reviewed by the University of Ottawa Animal Care Committee and Ethics Board. Mice used for each experiment were age and sex matched, female mice were used for all the experiments in my thesis and the preferred age of mice for experiments was 5-6 weeks old. The following mice (C57BL/6J, *Stat1*$^{-/-}$, *Stat2*$^{-/-}$, *Ifn-γ*$^{-/-}$, *Ifnar1*$^{-/-}$) used in my experimental models were obtained from the Jackson Labs, Bar Harbor, USA. *Irf9*$^{-/-}$ mice were provided by Dr Karen Mossman, McMaster University, Hamilton, ON, Canada. B6. *Stat1*$^{-/-}$ *Irf9* $^{-/-}$ and B6. *Ifn-γ*$^{-/-}$ *Irf9*$^{-/-}$ double knockout mice were generated in house by crossing the corresponding single knock-out mice.

2.2 Bacterial strain

Salmonella enterica serovar Typhimurium (ST), SL1344 strain was used for infection. 1 ml frozen stock (ST WT) were stored in Lab at -80 °C.

2.3 Generation of bone marrow-derived macrophages

To obtain bone marrow derived macrophages (BMMs), mice were sacrificed by CO_2 inhalation, followed by cervical dislocation. Mouse hind legs were extracted and with the help of cotton gauge the fur and muscles were removed. Bone marrow was flushed out of the bones with the help of 26-gauge needle, cells were then passed through a 100 μm cell strainer to remove debris followed by centrifugation at 500 g for 5 min. The cell pellet was

resuspended in RPMI containing 8% fetal bovine serum (FBS), hereto referred as R8, containing 50 µg/ml gentamycin. Cell number was calculated using a hemocytometer. Plastic petri dishes of size fifty millimeter were coated with macrophage colony stimulating factor (M-CSF) using an L shaped stick at a concentration of 5 ng/ml. Fifteen million cells were added to each petri dish and R8 media was added to make up a final volume of 10 ml. All the petri dishes were incubated at 37°C, for 6 days, for macrophage differentiation.

2.4 In *vitro* ST infection

BMMs were harvested post macrophage differentiation from the petri dishes. Firstly, the media was aspirated, and the cells were washed with phosphate buffer saline (PBS), following which, the cells were scraped off from the petri dishes while in PBS, using a cell scrapper. The cells were centrifuged at 500 g for 5 min, pellet was resuspended in R8 media without gentamycin. After counting, the cells were seeded at a density of 3 X 10^5 cells/300 µl per well in a 24 well flat bottom plate. The following day, infection was performed on adherent cells. ST vials used for infection were stored in -80°C, glycerol was removed after spinning and pellet was resuspended in R8 media without gentamycin. BMMs were infected with 10 MOI (multiplicities of infection) of the ST bacteria for 15 min. Higher MOIs were used to measure the impact on cell death. Post infection with ST. cells were spun at 2500 RPM for 6 minutes, and the 24 well plate was incubated at 37°C for a duration of 15 minutes in a 5% CO_2 incubator. After 15 min gentamycin (50 µg/mL) was added to eliminate extracellular bacteria, this incubation was performed for 2 hours at 37°C, after which media was replaced with fresh R8 media containing lower concentration of gentamycin (10µg/mL). Supernatants were collected at 6 hour and 24-hour post-infection. Cell death assays were performed at 24-hour post-infection only.

2.5 *In vivo* infection and assessment of bacterial burden.

Animals were infected intravenously (i.v.) with 200 CFU of the ST WT through the lateral tail vein. Volumes was administered in 100 µL of PBS. Bacterial burden was measured in spleen or bone marrow, on day 4, 5 or 6 as per the requirement of experiments. Whole spleen was extracted from the mouse with the help of frosted glass slides it was mashed to obtain a homogenate of the spleen in 10 ml R8 without gentamycin. The spleen homogenate in R8 was further serially diluted and from each dilution 100 µl of cell suspension was plated onto LB agar plates with streptomycin; as this strain of *Salmonella* is resistant to streptomycin (Pezzella et al., 2004). These Plates were then incubated for a duration of 24 hours in a 5% CO_2 incubator. Next day, colony forming units (CFU) were counted visually.

2.6 Neutral Red assay

Neutral Red (NR) assay was performed to determine percentage of cell death after ST infection. Neutral Red solution (5%) was made in R8 media and was filtered through a 0.22 µm filter. At 24-hour post infection of macrophages, the filtered 5% NR solution was added to macrophages and incubated for 15-30 min at 37°C. After the incubation period extracellular dye was removed by aspiration and the metabolized dye was solubilized by solubilization agent that consists of ethanol and acetic acid in water. Viability of cells was determined by the absorbance of the dye measured at 570 nm. The absorbance measured is directly proportional to cell viability.

2.6.1 Zombie yellow assay

Cell death was also evaluated by staining cells with Zombie yellow. Cells were first washed with PBS, and then stained with Zombie yellow (1:100 dilution) in PBS. Cells

were then incubated in dark for 30 min at room temperature after which, they were washed and acquired on the flow cytometer.

2.7 Flow cytometry

Infected cells (spleen and bone marrow-derived immune cells) were extracted, resuspended in R8 media without gentamycin and placed on ice. Whole spleen was removed and homogenized using frosted glass slides and passed through a 70 μm cell strainer to obtain a single-cell suspension. To extract the bone marrow cells, hind limbs were extracted, and bone marrow was flushed out of the bones with the help of 26- gauge needle and filtered through a 100 μm cell strainer, in R8 media without gentamycin in case of infected sample, and in R8 media with gentamicin for uninfected samples.

Cells (3-6 × 10^6) were centrifuged and washed with staining buffer (1% bovine serum albumin in PBS). Meanwhile, antibody cocktail was prepared **(Table 1)**. Cells were first blocked with FcBlock (anti-CD16/32) at 4°C in dark. After 10-minute incubation, staining was performed with various cell surface markers as indicated in table below. Since the cell surface receptors added are light sensitive, cells were protected from light and incubated at 4°C for 30 minutes in the dark. Post- incubation, staining buffer was added to cells and cells were centrifuged for 10 min. Cell supernatant was aspirated and cells were fixed in the fixing buffer (PBS containing 1% paraformaldehyde and 0.02% sodium azide). Cells were then acquired on Fortessa flow cytometer.

Table 1. Flow cytometry staining antibodies

Antibody	Conjugated fluorophore	Source	Catalog number
CD11c	eFluor 450	eBioscience	48-0114-80
CD11b	PE/Cy7	eBioscience	2500112-81
Ly6G	FITC	eBioscience	11-5931-82
Ly6C	PE	eBioscience	12-5932-82
F4/80	APC-eFluor 780	eBioscience	47-4801-80
Lin*	FITC	eBioscience	22-7770-72
c-kit	PE/Cy7	eBioscience	25-1171-81
Sca-1	PerCP-Cy5.5	eBioscience	45-5981-80
CD34	eFluor 450	eBioscience	48-0341-80
IL7R	APC	eBioscience	17-1271-82
CD16/32	PE	eBioscience	12-0161-81
Zombie Yellow™	BV 605	BioLegend	423103
Annexin	APC Cy7	BioLegend	640919
Ly-6G/Ly-6C	PE	eBioscience	12-5931-82
CD4/CD8	FITC/PE	eBioscience	MA5-17009

*Lin cocktail

2.8 Cytokine analysis

Supernatants collected from each well at 6 hours post infection were analyzed for TNF-α production and supernatants collected at 24-hour post infection were analyzed for IL12, IL-6, IL-10 and IL1β production. Enzyme-linked immunosorbent assays (ELISAs) was the technique used to measure cytokine production. The commercial kits used to perform ELISAs are listed in table below **(Table 2)**. High binding 96-well plates were coated with 50 µl of the antigen specific capture antibody and were left overnight at 4°C. Plates were sealed with a special plate sealer. Dilution of the antibody used varied with the ELISA kit for each cytokine. The following day, plate washer was used to wash the plates with PBS-T wash buffer (1X PBS and 0.5% Tween 20 in H_2O). 150 µl of (10% FBS in PBS) was used as a blocking buffer for IL-10, IL-12, and TNFα and for IL-1β (1% BSA in PBS) was used. Plates were incubated for a minimum of 1 hour at room temperature, followed by washing with PBS-T using plate washer. Meanwhile, standards were prepared in assay diluent as per the user manual, 50 µl of standards (various dilutions) were added to the corresponding wells of the washed plate and 50 µl of samples were added in triplicates. Plate was then incubated for 2 hours at room temperature. Post-incubation, plates were washed and 50 µl of detection antibody with streptavidin-HRP mixture was added to all the wells and incubated in the dark for 1 hour at room temperature. The plates were washed again and finally 50 µl of tetramethylbenzidine (TMB) was added and the plates were left in dark for approximately 10-15 minutes or until the development of blue color. Lastly to stop the reaction, 25 µl of stop solution (2 N sulphuric acid in H_2O) was added. Absorbance was measured at 450 nm on FilterMaxTM F5 plate reader and the obtained data was analyzed using SoftMax Pro software.

Table-2 Commercial ELISA kits

No.	Cytokine	Source	Catalog Number
1.	Mouse IL-1β	R&D Systems	DY401
2.	Mouse IL-12	BD OptEIA™	555256
3.	Mouse TNF-α	BD OptEIA™	555268
4.	Mouse IL-6	BD OptEIA™	555240
5.	Mouse IL-10	BD OptEIA™	555252

2.9 Antibody depletion experiment

At Day 0, all mice were infected intravenously (i.v.) via the lateral vein with 200 CFU of S. Typhimurium (ST). For depletion of neutrophils: anti-Ly6G antibody was injected daily (200 μg/day i.p. at -3,-1,0,1,2,3,4 dpi). For depletion of both neutrophils and monocytes, anti- Ly6C/G antibody was injected daily (200μg/day i.p, at -3,-1,0,1,2,3,4 dpi). For depletion of Lymphoid subsets, anti-CD4/CD8 antibodies were injected daily (200 μg/day i.p, at 0,1,2,3,4 dpi). On the other hand, anti-IgG1 control antibody were injected daily to control animals. Bacterial burden was evaluated in the spleens of infected mice at 5 dpi by plating serial dilutions of the splenic homogenates on LB agar plates.

2.10 Mitochondrial bioenergetics assay

96-well Seahorse tissue culture plate (Seahorse Bioscience # 101085-004) was coated with cell adhesive Cell-Tak (Corning #354240) to ensure macrophage adherence. BMMs were seeded at a density of 1.5×10^5 cells/well in the Cell-Tak coated plate and the plate was incubated overnight at 37 °C. 24-hour post seeding, cells were infected with ST for 24 hours. Cells were then thoroughly washed with warm Seahorse assay media XF RPMI Medium pH 7.4 to remove any traces of R8 media. For both glycolysis and Mito stress tests, the media was made up of 1 mM sodium pyruvate and 2 mM Glutamine, and only for Mito stress test 10 mM Glucose was added additionally. Plate was kept for one hour in a non-CO2 incubator maintained at 37°C, to allow cells to attain a state of equilibrium. Oxygen consumption rates (OCR, in pmol/min) was the first parameter we calculated on a Seahorse Bioscience XF24 Extracellular Flux Analyzer, to assess mitochondrial respiration. First, basal OCR was measured at the resting stage before addition of inhibitors. Then various inhibitors such as Oligomycin (2 μM), FCCP (1 μM), and Rotenone/Antimycin A (1 μM) were added sequentially, and any OCR fluctuations were also recorded. Similarly, extracellular acidification rate (ECAR, in mph/min) was recorded on a Seahorse Bioscience XF96 Extracellular Flux Analyzer. Basal ECAR as well as the ECAR fluctuations in response to sequential addition of glycolysis stress test inhibitors: Glucose (10 mM), Oligomycin (2 μM) and 2-deoxyglucose (30 mM) were recorded and data was interpreted using Agilent Seahorse wave software.

2.11 Statistical analysis

The error bars depicted in my data show standard error of mean±s.e.m. One-way ANOVA, paired or unpaired t-test were used depending on number of variables involved and factors to be compared. Statistical analysis was performed using GraphPad Prism 8 software.

3. RESULTS

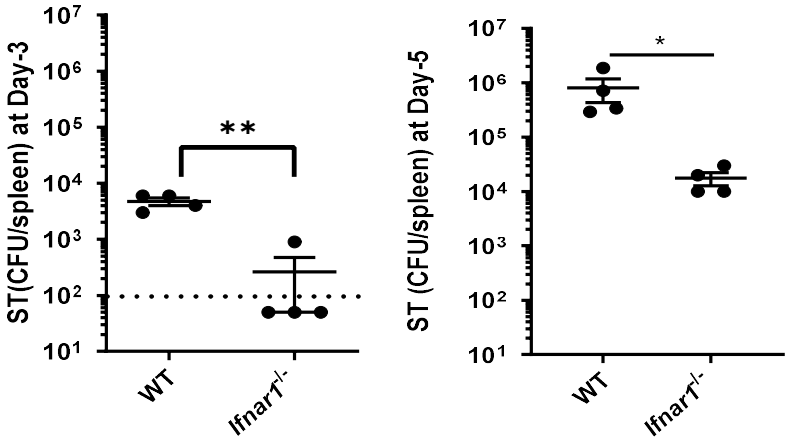
3.1 Evaluating the role of ISGF3 complex in promoting susceptibility to bacterial infection.

3.1.1 *Ifnar1*$^{-/-}$ mice display slow down in vivo growth of ST.

Role of type-1 interferon in regulating bacterial pathogenesis has not been fully established. Our lab has previously reported that type-I IFN signaling is detrimental to host survival when challenged with *Salmonella* Typhimurium (ST) (Robinson et al., 2012).

In vivo experiment was performed to evaluate whether type-I interferon signaling impairs the control of ST at early time point (day 3). It was observed that at day 3 post infection, ST was detectable in all the WT mice whereas in *Ifnar1*$^{-/-}$ mice ST was detectable in only 1 of the 4 mice (**Figure 2 A**). At day 5 post infection the bacterial burden increased in both WT and *Ifnar1*$^{-/-}$ mice (**Figure 2 B**) but the bacterial burden in *Ifnar1*$^{-/-}$ mice was still significantly lower than WT mice. Upon monitoring the survival of infected mice, all WT mice reached a humane endpoint and were sacrificed whereas the *Ifnar1*$^{-/-}$ mice displayed an enhanced duration of survival (**Figure 2 C**). We also evaluated the bacterial burden in the bone marrow of infected mice and observed that at day 3 post infection the bacterial burden was below the threshold of detection in both WT and *Ifnar1*$^{-/-}$ (**Figure 2 D**).

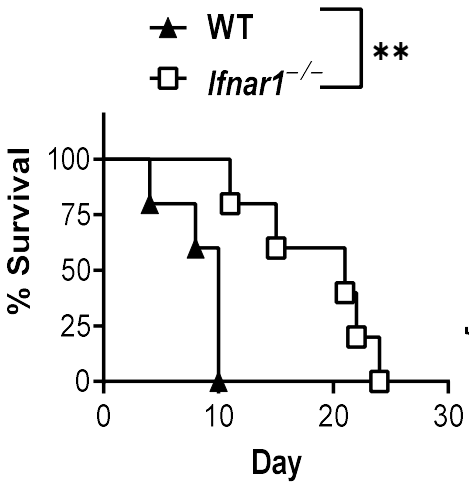
Overall, these results indicate that type-1 IFN signaling compromises the control of ST and this is detectable as early as day 3 of infection. Since the impact of type-I IFN is detectable at an early stage and considering that the development of acquired immune response is delayed against phagosomal pathogens such as ST (Luu et al., 2006; Srinivasan et al., 2004), it is likely that the increased resistance of *Ifnar1*$^{-/-}$ is due to a modulation of monocyte-macrophage mediated immune response.


ST(CFU/spleen) at Day-3
10^7
10^6
10^5
10^4
10^3
10^2
10^1
**
WT
Ifnar1-/-

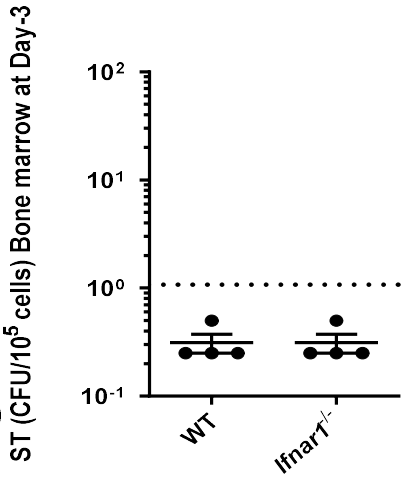
(A)

ST (CFU/spleen) at Day-5
10^7
10^6
10^5
10^4
10^3
10^2
10^1
*
WT
Ifnar1-/-

(B)


WT
Ifnar1−/−
**
% Survival
100
75
50
25
0
0
10
20
30
Day

(C)


ST (CFU/10^5 cells) Bone marrow at Day-3
10^2
10^1
10^0
10^{-1}
WT
Ifnar1-/-

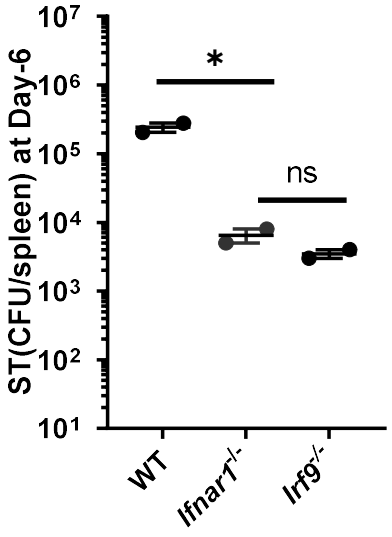
(D)

Figure 2. Impact of IFNAR1 signaling on host susceptibility to infection with *S.* Typhimurium.

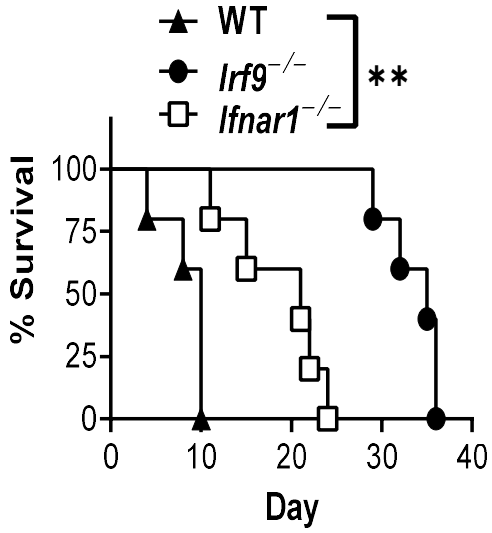
WT and *Ifnar1*$^{-/-}$ mice were infected intravenously (i.v.) with 200 CFU of *S.* Typhimurium (strain SL1344). At day 3 **(A)** and 5 **(B)** post infection, mice were euthanized, and spleens were isolated and serial dilutions prepared in PBS and 100 µl of each dilution was plated onto LB + streptomycin plates. These plates were incubated at 37^0C for 24h and the colony forming units (CFUs) were calculated. Survival of infected mice was evaluated by monitoring the endpoint such as piloerection, poor movement, weight loss **(C)** as described in the materials and methods section. Bone marrow was isolated from infected mice and the bacterial CFU was evaluated per 10^5 cells **(D)** after plating serial dilutions on LB agar plates as mentioned above. There were a minimum of 4 mice per group in each experiment. Statistical significance was calculated by unpaired student's t-test using GraphPad Prism 8 software; **$p<0.01$ and*$p<0.05$.

3.1.2 *Irf9*- deficient mice display enhanced survival upon infection with ST when compared to WT and *Ifnar1*-deficient mice.

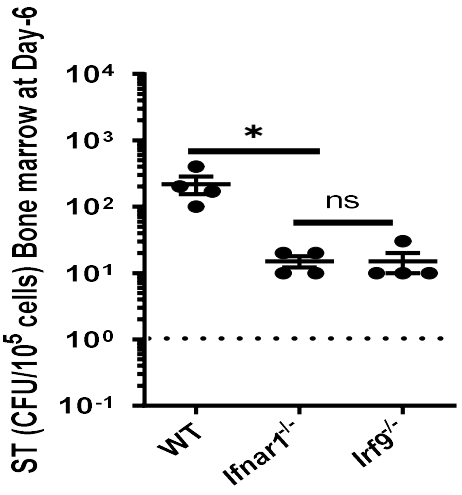
Phosphorylated STAT1 and STAT2 interact with interferon regulatory factor 9 (IRF9) to form a heterotrimeric transcription complex, termed as the interferon- stimulated gene factor 3 (ISGF3). It has been shown that in this complex STAT1 and IRF9 are required for sequence-specific recognition and stable binding with DNA, whereas STAT2 provides transcriptional modulation but is unable to interact with DNA directly (Bluyssen H. A.; Levy D. E et al 1997). Thus, I evaluated whether *Irf9*-deficient mice will also display increased resistance against ST. At day 6 post infection both *Ifnar1*$^{-/-}$ and *Irf9*$^{-/-}$ mice had reduced bacterial burden in the spleens in comparison to WT mice (**Figure 3A**). I performed a survival experiment to see whether the two knockout mice display similar resistance following infection with ST. It was observed that *Irf9*- deficient mice survived for longer period when compared to WT and *Ifnar1*-deficient mice following a challenge with ST (**Figure 3B)**. The bacterial burden was also evaluated in the bone marrow of infected mice at day 6. While there was detectable ST burden in WT bone marrow the burden in *Ifnar1*$^{-/-}$ and *Irf9*$^{-/-}$ mice was reduced as compared to WT (**Figure 3C**). The difference in the host survival of *Ifnar1*$^{-/-}$ and *Irf9*$^{-/-}$ mice indicates that there are significant signaling differences between these mice in spite of the fact that these two genes are expected to follow the same signaling pathway.


ST(CFU/spleen) at Day-6
10^7
10^6
10^5
10^4
10^3
10^2
10^1
*
ns
WT
Ifnar1-/-
Irf9-/-

(A)


WT
Irf9−/−
Ifnar1−/−
**
% Survival
100
75
50
25
0
0
10
20
30
40
Day

(B)


ST (CFU/10^5 cells) Bone marrow at Day-6
10^4
10^3
10^2
10^1
10^0
10^{-1}
*
ns
WT
Ifnar1-/-
Irf9-/-

(C)

Figure 3. Differential survival of WT, *Ifnar1*- and *Irf9*- deficient mice following infection with ST.

The indicated groups of mice were infected intravenously (i.v.) with 200 CFU of *S.* Typhimurium (strain SL1344). On day 6 **(A)** post infection, mice were euthanized, and spleens were collected, and serial dilutions prepared in PBS and 100 µl from each dilution was plated onto LB + streptomycin plates. These plates were incubated at 37^0C overnight after which the CFUs were calculated. Survival of infected mice was evaluated **(B)** as described in experimental methods. Bone marrow was isolated from infected mice and the bacterial CFU was evaluated per 10^5 cells **(C)** after plating serial dilutions on LB agar plates as mentioned above. Each dot represents a single mouse. Statistical significance was calculated by unpaired student's t-test using GraphPad Prism 8 software;*$p<0.05$.

3.1.3 *Stat2*$^{-/-}$ *and Irf9*$^{-/-}$ are highly resistant against ST

Having observed that *Ifnar1*$^{-/-}$ and *Irf9*$^{-/-}$ mice show differential survival in response to infection with ST. I tested the impact in *Stat2*$^{-/-}$ mice. WT and *Irf9*$^{-/-}$ were also infected for comparison. It was observed that both *Stat2*$^{-/-}$ and *Irf9*$^{-/-}$ mice show enhanced resistance to ST infection which was much greater than that observed in WT mice (**Figure 4**). Previous results in the lab have indicated that *Stat1*$^{-/-}$ mice do not display enhanced resistance against ST. Rather, *Stat1*$^{-/-}$ mice display enhanced fatality with all the mice dying between days 5-6. These results suggested that STAT1 and IRF9 have opposing roles in controlling ST infection; while STAT2 and IRF9 exacerbate bacterial infection, STAT1 promotes bacterial control, even though all three form the ISGF3 transcriptional complex.

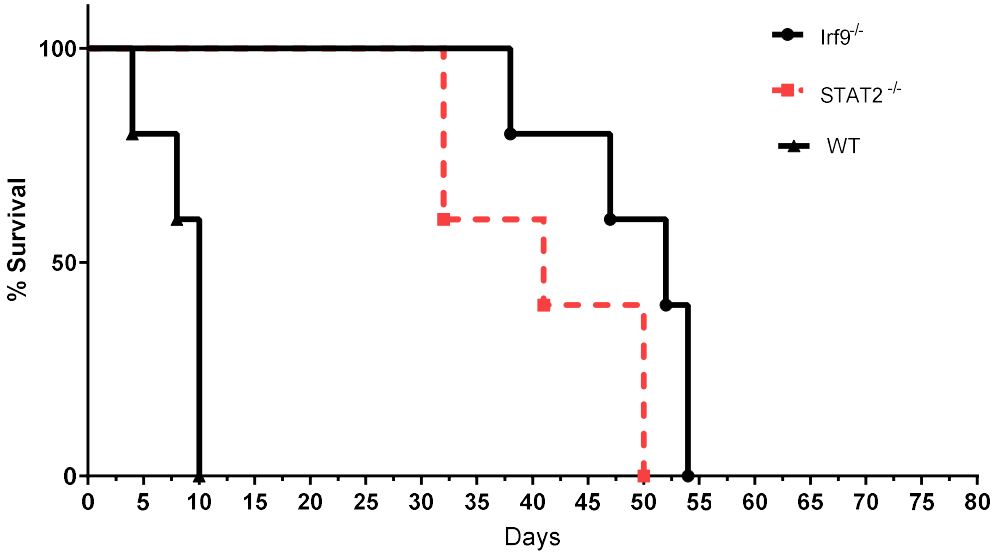

Irf9-/-
STAT2 -/-
WT
% Survival
100
50
0
0 5 10 15 20 25 30 35 40 45 50 55 60 65 70 75 80
Days

Figure 4. Differential survival of WT, *Stat2*- and *Irf9*- deficient mice.

WT, *Stat2*- and *Irf9*- deficient mice were infected intravenously (i.v.) with 200 CFU of *S.* Typhimurium (strain SL1344). Survival of infected mice was evaluated by monitoring the endpoint such as piloerection, poor movement, weight loss as described in the materials and methods section and survival curve was plotted.

3.1.4 STAT1 signaling through the type-I interferon pathway promotes protection against ST.

Since *Stat1*$^{-/-}$ mice were highly susceptible to ST infection (unpublished results of the lab), this means that STAT1 plays a protective role. While STAT2 and IRF9 mediate signaling only following IFNAR1 engagement, STAT1 can induce signaling after engagement of IFNAR1 (type-I IFN) as well as IFNGR (type-II IFN). Thus, it was important to determine whether the protective role of STAT1 was through type-I or type-II IFN pathway. Hence two double knockouts were generated (*Stat1*$^{-/-}$*Irf9*$^{-/-}$ and *Ifng*$^{-/-}$*Irf9*$^{-/-}$*).* In the *Ifng*$^{-/-}$*Irf9*$^{-/-}$ mice, STAT1 signaling through the type-II interferon (IFN-γ) will not occur because of the absence of IFN-γ but STAT1 and STAT2 can still signal through the IFNAR. This mouse strain will help us determine the role of STAT1-STAT2 (in the absence of IRF9) through the IFNAR. In the *Stat1*$^{-/-}$*Irf9*$^{-/-}$ mice, IFN-γ signaling will not occur because of the absence of STAT1, and IFNAR signaling will be blunted because of the absence of STAT1 and IRF9. The only transcription factor available for IFNAR signaling in this strain is STAT2. An in vivo experiment was performed wherein mice of various genotypes were infected parallelly with 200 CFU of ST and the bacterial burden was assessed day 4 post infection. The bacterial burden was increased in *Stat1*$^{-/-}$ and *Ifng*$^{-/-}$ mice in comparison to WT mice, implying that STAT1 signaling through the IFNGR pathway plays a protective role in controlling the infection (**Figure 5**). Bacterial burden in *Irf9*$^{-/-}$ was reduced in comparison to WT mice (**Figure 5**).

Among the two double knockouts significantly high bacterial burden was displayed in *Stat1*$^{-/-}$*Irf9*$^{-/-}$ mice than *Ifng*$^{-/-}$*Irf9*$^{-/-}$ **(Figure 5).** Furthermore, double deficiency of *Stat1* and *Irf9* resulted in a very high bacterial burden following a challenge with ST which was much higher than WT mice. Abrogation of IFN-γ in *Irf9*$^{-/-}$ mice resulted in the escalation

of the bacterial burden to WT levels. The CFU differential between the two double knockout mice (*Stat1*$^{-/-}$*Irf9*$^{-/-}$ versus *Ifng*$^{-/-}$*Irf9*$^{-/-}$) was 50-100-fold with *Stat1*$^{-/-}$*Irf9*$^{-/-}$ mice having a much higher bacterial burden in comparison to *Ifng*$^{-/-}$*Irf9*$^{-/-}$.

Based on the transcription factors available in these two double knockout strains of mice following IFNAR1 engagement one concludes that STAT1 signaling downstream of IFNAR1 mediates a protective response against ST.

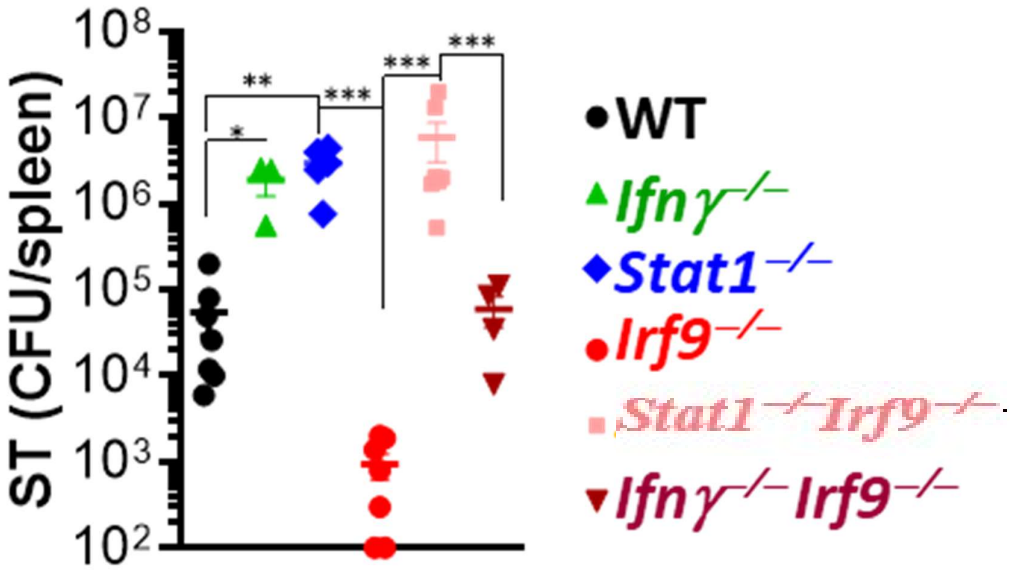

ST (CFU/spleen)
10^8
10^7
10^6
10^5
10^4
10^3
10^2
*
**
***
***
***
WT
Ifnγ−/−
Stat1−/−
Irf9−/−
Stat1−/−Irf9−/−
Ifnγ−/−Irf9−/−

Figure 5. Deficiency of STAT1 abrogates the resistance of *Irf9*$^{-/-}$ mice

C57BL/6J mice of various genotypes indicated in the figure above were infected intravenously (i.v.) with (200 ST). At day 4 post infection spleens were removed and homogenized with frosted end glass slides. Serial dilutions were prepared in PBS and 100 µl of each dilution was plated onto LB agar plates. After 24 hour incubation at 37^0C, bacterial colonies (CFU) were counted. Statistical analysis was done by unpaired student's t-test using GraphPad Prism 8 software; where *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

3.2 Evaluating the cell type in *Ifnar1*$^{-/-}$ mice that is responsible for providing enhanced resistance against ST infection

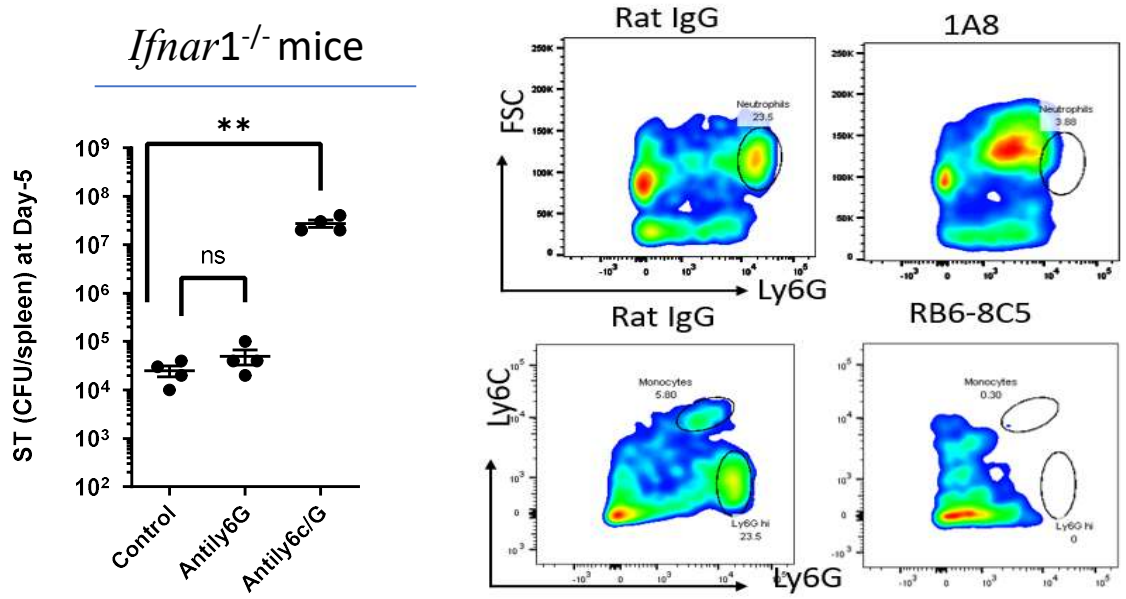
3.2.1 Depletion of monocyte/macrophage lineage results in abrogation of the resistance in *Ifnar1*$^{-/-}$ mice.

As described previously *Ifnar1*$^{-/-}$ exhibit enhanced resistance against ST. We were therefore interested in determining which cell type in these mice may be responsible for this protection. Amidst the myeloid cell populations, neutrophils are the first ones to reach the site of infection. Moreover, monocytes differentiate into highly phagocytic macrophages upon immigrating to various organs and responding to various stimulants (Janeway, 2001a). Our lab has previously reported that *Ifnar1*$^{-/-}$ mice have significantly higher numbers of splenic monocytes/macrophages in comparison to WT mice at day 5 post infection with ST. We thus depleted monocytes and neutrophils in infected *Ifnar1*$^{-/-}$ mice and evaluated the bacterial burden in the spleens of these mice at day 5 post infection. Clone 1A8 antibody is against the Ly6G which is selectively present on neutrophils. We depleted neutrophils using this antibody. There is no available antibody that specifically binds only monocytes. Instead, the clone RB6-8C5 antibody binds to both Ly6C (present on monocytes) and Ly6G (present on neutrophils). This antibody depletes both monocytes and neutrophils (Daley, J.M et al.,2008). We confirmed by flow cytometry that daily injection of groups of *Ifnar1*$^{-/-}$ mice with the 1A8 antibody resulted in depletion of neutrophils whereas depletion with the RB6-8C5 resulted in depletion of monocytes and neutrophils **(Figure 6A)**. It was observed that the bacterial burden in neutrophil depleted *Ifnar1*$^{-/-}$ mice was similar to that of the bacterial burden in *Ifnar1*$^{-/-}$ mice that received a control IgG (**Figure 6A**). This result suggests that the enhanced resistance in *Ifnar1*$^{-/-}$ mice

is not due to neutrophils. On the other hand, a substantial increase in bacterial burden was observed in *Ifnar1*$^{-/-}$ mice when both monocytes and neutrophils were depleted **(Figure 6A).** These results suggest that neutrophil depletion alone did not have an impact on the resistance of *Ifnar1*$^{-/-}$ mice whereas depletion of monocyte/macrophage lineage resulted in complete abrogation of the resistance **(Figure 6A).**

Previous work in our laboratory has demonstrated that depletion of NK cells did not result in an increased bacterial burden in *Ifnar1*$^{-/-}$ mice, indicating that the enhanced resistance of *Ifnar1*$^{-/-}$ mice is not related to the activity of NK cells. Although the impact of IFNAR1 deficiency on bacterial burden is detected at an early time point, we decided to evaluate whether the depletion of other cell subsets of the lymphoid lineage would influence the resistance of *Ifnar1*$^{-/-}$ mice**.** We used anti- CD4/CD8 antibody to deplete the T cells in *Ifnar1*$^{-/-}$ mice. It was observed that the depletion of the T cell population did not result in an increase in the bacterial burden of *Ifnar1*$^{-/-}$ mice (**Figure 6B**). Rather, there was a slight reduction in the bacterial burden of *Ifnar1*$^{-/-}$ mice. (**Figure 6B)**. This, along with the previous work in the laboratory, suggests that the lymphoid cell subsets do not confer enhanced resistance to *Ifnar1*$^{-/-}$ mice against ST infection **(Figure 6B).** Moreover, my results have also shown that the impact of type-I IFN is detectable at an early stage of the infection (Figure 2A) whereas the T cell activation is delayed during infection of mice with *S.* Typhimurium, therefore it is likely that the increased resistance of *Ifnar1*$^{-/-}$ is due to a modulation of the innate immune response itself (Luu et al., 2006; Srinivasan et al., 2004). Taken together, these results indicate that, it is indeed the monocytes that promote heightened resistance of *Ifnar1*$^{-/-}$ mice against a challenge with ST.

(A)


Ifnar1-/- mice
ST (CFU/spleen) at Day-5
**
ns
Control
Antily6G
Antily6c/G
Rat IgG
1A8
FSC
Ly6G
Neutrophils
23.5
Neutrophils
3.88
Rat IgG
RB6-8C5
Ly6C
Ly6G
Monocytes
5.80
Monocytes
0.30
Ly6G hi
23.5
Ly6G hi
0

(B)

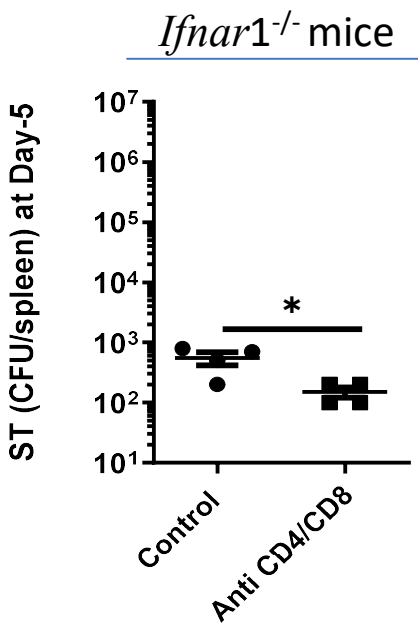

Ifnar1-/- mice
ST (CFU/spleen) at Day-5
*
Control
Anti CD4/CD8

Figure 6. Depletion of monocyte/macrophage lineage results in abrogation of the enhanced resistance of *Ifnar1*$^{-/-}$ mice.

6A For depletion of neutrophils, anti-Ly6G antibody was injected daily (200 µg/day i.p, at -3,-1,0,1,2,3,4 dpi) and for depletion of both neutrophils and monocytes, anti- Ly6C/G antibody was injected daily (200µg/day i.p, at -3,-1,0,1,2,3,4 dpi**).** Depletion of cell types was confirmed by flow cytometry. Control mice received IgG**.** At Day 0, all *Ifnar1*$^{-/-}$ mice were infected intravenously (i.v.) via the lateral vein with 200 CFU of *S.* Typhimurium (ST), and the bacterial burden was evaluated in the spleens of infected mice at 5 dpi by plating serial dilutions of the splenic homogenates on LB agar plates. Data confirming depletion of cell type by flow cytometry is also shown .Statistical significance was calculated by unpaired student's t-test using GraphPad Prism 8 software **p<0.01; *p<0.05; ns-nonsignificant.

6B For depletion of Lymphoid subsets, anti-CD4/CD8 antibodies were injected daily (200 µg/day i.p**,** at 0,1,2,3,4 dpi) in *Ifnar1*$^{-/-}$ mice which were infected intravenously (i.v.) via the lateral vein on day 0 with 200 CFU of *S.* Typhimurium (ST). Bacterial burden of infected mice was evaluated in the spleen at 5 dpi by plating serial dilutions of splenic homogenates on LB agar plates. Statistical significance was calculated by unpaired student's t-test using GraphPad Prism 8 software **p<0.01; *p<0.05.

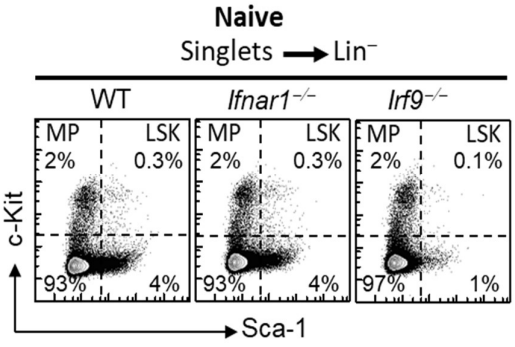
3.2.2 Type-I interferon signaling does not impact the myelopoiesis of naïve mice.

As described previously monocyte/macrophages promote protection in *Ifnar1*$^{-/-}$ mice, and our lab previously reported that macrophage numbers are increased in these mice. We were therefore interested in evaluating whether the deficiency of IFNAR1 results in a modulation of myeloid cell numbers in the bone marrow itself, which is the main site of generation of immune cells (Zhu and Emerson, 2002).

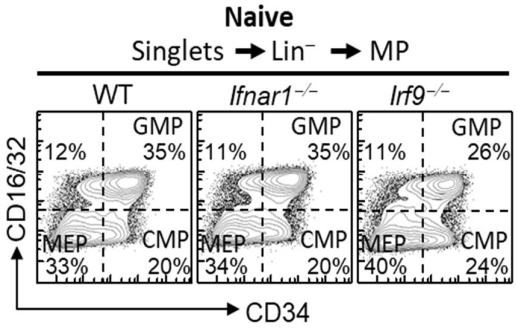
To address this possibility, myeloid and lymphoid progenitors in the bone marrow of naïve WT, *Ifnar1*$^{-/-}$ and *Irf9*$^{-/-}$ mice were compared by flow cytometry. HSCs differentiate into common myeloid progenitors (CMPs) and common lymphoid progenitors (CLPs). CMPs in turn differentiate into granulocyte monocyte-progenitors (GMPs) and megakaryocyte-erythrocyte progenitors (MEPs). GMPs give rise to granulocytes (neutrophils, eosinophils, basophils, mast cells) and monocytes. MEPs give rise to erythrocytes and to megakaryocytes (Zhu and Emerson, 2002). CLPs give rise to T cells, B cells and NK cells. Dendritic cells can be derived from myeloid or lymphoid progenitors.

Bone marrow cells were extracted from hind leg bones of naïve WT, *Ifnar1*$^{-/-}$ and *Irf9*$^{-/-}$ mice and cells were treated with FcBlock (anti CD16/32) to prevent non-specific binding of antibodies. The proportions and numbers of HSCs and various progenitor subsets were analyzed by flow cytometry based on the markers indicated in (**Table-1**). Prior to infection with ST, it was observed that the deficiency in IFNAR1 or IRF9 had no impact on LSKs (c-kit^{+} Lin^{-} Sca-1^{+}) and the myeloid progenitors (MP) **(Figure 7A**). Moreover, there were no significant differences in the proportions of CMPs, GMPs and MEPs, as their percentages were comparable among WT, *Ifnar1*$^{-/-}$ and *Irf9*$^{-/-}$ mice **(Figure 7B**). No modulation was observed in lymphoid progenitors as well (data not shown).

Taken together, these results suggest that IFNAR1 and IRF9 do not seem to play a major role in regulating the differentiation of HSCs and progenitors at steady state conditions. The next aim was to determine the impact of type-1 IFN signaling on hematopoiesis during ST infection, especially as *Ifnar1*$^{-/-}$ mice were less susceptible to ST.


Naive
Singlets → Lin⁻
WT
Ifnar1⁻/⁻
Irf9⁻/⁻
MP
LSK
2%
0.3%
2%
0.3%
2%
0.1%
93%
4%
93%
4%
97%
1%
c-Kit
Sca-1

(A)


Naive
Singlets → Lin⁻ → MP
WT
Ifnar1⁻/⁻
Irf9⁻/⁻
GMP
12%
35%
11%
35%
11%
26%
MEP
33%
CMP
20%
MEP
34%
CMP
20%
MEP
40%
CMP
24%
CD16/32
CD34

(B)

Figure 7. Modulation of myeloid or lymphoid progenitors was not observed in the bone marrow of naïve *Ifnar1*$^{-/-}$ mice.

Bone marrow cells were obtained from naïve WT, *Ifnar1*$^{-/-}$ and *Irf9*$^{-/-}$ mice as mentioned above. Cells were treated with FcBlock (anti CD16/32) to prevent non-specific binding of antibodies and stained with fluorophore-conjugated antibodies against HSC markers (Table-1) and acquired on a flow cytometer **(A-B)**. Representative FACS plots showing the gating strategy for hematopoietic progenitor subsets:-(A) LSK: Lin^{-} Sca-1^{+} c-Kit^{+}, MP: Lin^{-} Sca-1^{-} c-Kit^{+}; (B) based on MP gate: CMP: CD16/32^{lo} CD34^{+}, GMP: CD16/32^{+}CD34^{+}, MEP: CD16/32^{-}CD34^{-} were acquired. Data are pooled from at least 3 independent experiments.

3.2.3 Enhanced myelopoiesis in the bone marrow of *Ifnar1*$^{-/-}$ and *Irf9*$^{-/-}$ mice.

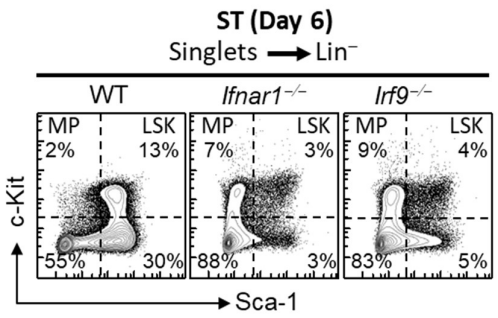
Our lab previously reported that there are significantly higher numbers of myeloid cells in the periphery of *Ifnar1*$^{-/-}$ mice in comparison to WT mice at day 5 post infection with ST. Myelopoiesis is the process in which innate immune cells such as neutrophils, dendritic cells and monocytes develop from myeloid progenitor cells. We were therefore interested in determining whether the increased number of myeloid cells in the periphery of *Ifnar1*$^{-/-}$ mice is related to modulation of myelopoiesis in the bone marrow of *Ifnar1*$^{-/-}$ mice. After observing that there was no modulation of myeloid progenitors in the bone marrow of naïve *Ifnar1*$^{-/-}$ and *Irf9*$^{-/-}$ mice**,** we decided to determine the impact of type-1 IFN signaling on hematopoiesis during ST infection, especially as *Ifnar1*$^{-/-}$ mice were less susceptible to ST. During infection, HSC pool depletes and adapts to the peripheral demand of immune cells via differentiation into lineage restricted progenitors in bone marrow (Essers et al., 2009; Hartner et al., 2009). Thus, WT, *Ifnar1*$^{-/-}$ and *Irf9*$^{-/-}$ mice were infected intravenously with 200 CFU of ST in 100 µl of PBS. At day 6 post infection, the hind leg bones of infected mice were flushed, and bone marrow cells were isolated and treated with FcBlock (anti CD16/32) to prevent non-specific binding of antibodies. This was followed by staining with fluorophore-conjugated antibodies against HSC markers (**Table-1**) with subsequent acquisition on a flow cytometer.

All HSCs are contained within the LSK (Lin^- $Sca\text{-}1^+$ $c\text{-}Kit^+$) population (Spangrude et al., 1988; Morrison and Weissman, 1994; Osawa et al., 1996). Firstly, these precursor populations were analyzed in the bone marrow of WT, *Ifnar1*$^{-/-}$ and *Irf9*$^{-/-}$ mice day 6 post infection with ST. It was observed that the LSK counts were reduced post infection in *Ifnar1*$^{-/-}$ and *Irf9*$^{-/-}$ mice as compared to WT **(Figure 8A).** In contrast to the previous observations with naïve mice, there was a ST-induced increase in MP proportions in *Ifnar1*$^{-/-}$ and *Irf9*$^{-/-}$ mice as compared to WT (**Figure 8A)**.

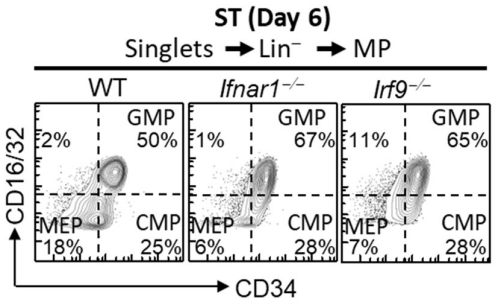
There was a very subtle increase in CMPs proportions in *Ifnar1*$^{-/-}$ and *Irf9*$^{-/-}$ mice as compared to WT. CMP differentiation towards GMPs occurs at the expense of MEPs and this differentiation was more biased in *Ifnar1*$^{-/-}$ and *Irf9*$^{-/-}$ mice as compared to WT mice. Infection of *Ifnar1*$^{-/-}$ and *Irf9*$^{-/-}$ *mice* with ST, resulted in an increase in GMP proportions and a reduction in MEPs as compared to infected WT mice (**Figure 8B).**

The absolute cell number of these gated populations of the progenitor cells were quantified. ST induced significant increase in MP numbers in *Ifnar1*$^{-/-}$ and *Irf9*$^{-/-}$ *mice as* compared to WT **(Figure 8C).** Furthermore, infection with ST led to a reduction in LSK numbers in *Ifnar1*$^{-/-}$ and *Irf9*$^{-/-}$ *mice* as compared to WT **(Figure 8D).** Subsequently, the ST-induced increase in CMP numbers was significantly more marked in *Ifnar1*$^{-/-}$ and *Irf9*$^{-/-}$ *mice* as compared to WT **(Figure 8E).** Accordingly, enhanced differentiation of CMPs to GMPs in the bone marrow of infected *Ifnar1*$^{-/-}$ and *Irf9*$^{-/-}$ *mice* resulted in a significant increase in their GMP counts as compared to WT **(Figure 8F).** This is indicative of increased myelopoiesis in the bone marrow of infected *Ifnar1*$^{-/-}$ and *Irf9*$^{-/-}$ *mice*, which is likely responsible for increased innate resistance against a challenge with ST.

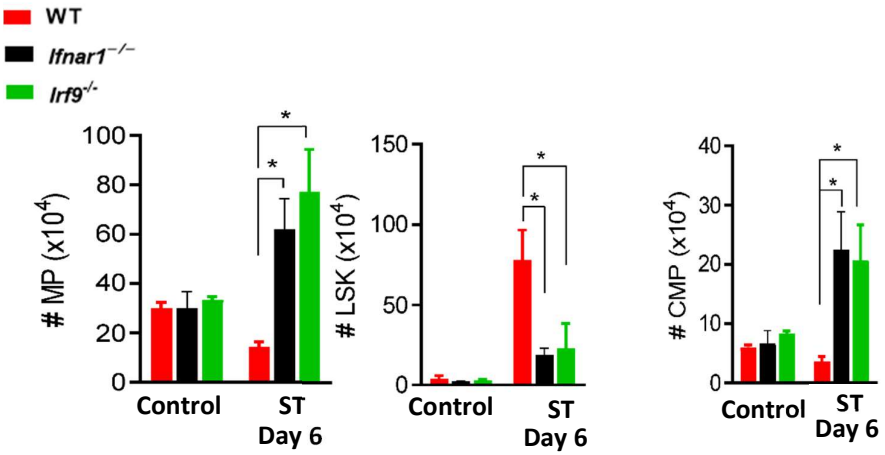
Collectively, these results suggests that type-I interferon signaling inhibits the myelopoiesis of myeloid progenitors, which in turn restricts the availability of monocytes and macrophages in the periphery. Thus, *Ifnar1*$^{-/-}$ *mice* harbor increased numbers of myeloid progenitors, which explains why there is an increase in the number of macrophages in these mice.


ST (Day 6)
Singlets → Lin⁻
WT
Ifnar1⁻/⁻
Irf9⁻/⁻
MP
LSK
2%
13%
7%
3%
9%
4%
55%
30%
88%
3%
83%
5%
c-Kit
Sca-1

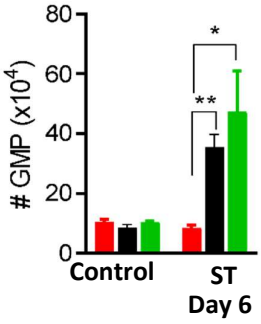
(A)


ST (Day 6)
Singlets → Lin⁻ → MP
WT
Ifnar1⁻/⁻
Irf9⁻/⁻
GMP
MEP
CMP
2%
50%
18%
25%
1%
67%
6%
28%
11%
65%
7%
28%
CD16/32
CD34

(B)


WT
Ifnar1⁻/⁻
Irf9⁻/⁻
MP (x10⁴)
LSK (x10⁴)
CMP (x10⁴)
Control
ST Day 6

(C) (D) (E)


GMP (x10⁴)
Control
ST Day 6

(F)

Figure 8. Type-I interferon signaling restricts myelopoiesis during infection of mice with ST.

WT, *Ifnar1*$^{-/-}$ and *Irf9*$^{-/-}$ mice were infected with 200 CFU of ST. At day 6 post infection bone marrow cells were extracted from infected WT, *Ifnar1*$^{-/-}$ and *Irf9*$^{-/-}$ mice as mentioned above. Cells were treated with FcBlock (anti CD16/32) to prevent non-specific binding of antibodies and stained with fluorophore-conjugated antibodies against HSC markers (Table-1) and acquired on a flow cytometer **(A-B).** Representative FACS plots showing the gating strategy for hematopoietic progenitor subsets:- (A) LSK: Lin^- $Sca\text{-}1^+$ $c\text{-}Kit^+$, MP: Lin^- $Sca\text{-}1^-$ $c\text{-}Kit^+$; (B) based on MP gate: CMP: $CD16/32^{lo}$ $CD34^+$, GMP: $CD16/32^+CD34^+$, MEP: $CD16/32^-CD34^-$ were acquired.

(C-F) Graphs display the absolute cell number of the gated population in the bone marrow. Cell types are indicated on y-axis. Data are pooled from at least 3 independent experiments with a total of 6 mice per group, presented as mean ± SEM. Statistical significance was calculated by unpaired two-tailed Student's t-test: *$p<0.05$

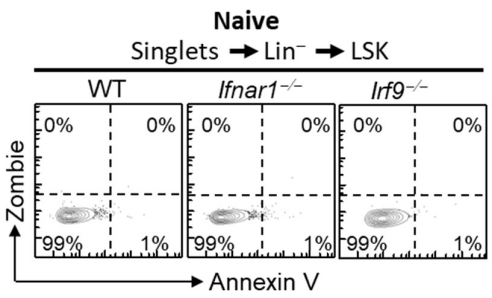
3.2.4 Reduced cell death of myeloid progenitors in infected *Ifnar1*$^{-/-}$ and *Irf9*$^{-/-}$ mice.

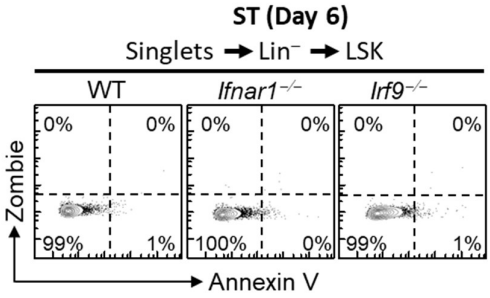
As described in the previous figures, type-1 IFN signaling inhibits the myelopoiesis of myeloid progenitors, which may explain the increase in the number of macrophages in *Ifnar1*$^{-/-}$ and *Irf9*$^{-/-}$ mice, which in turn may be responsible for the increased innate resistance against a challenge with ST. It has been previously reported that, *Ifnar1*$^{-/-}$ macrophages are less susceptible to *S.* Typhimurium–induced cell death than were wild-type macrophages (Robinson et al.,2012). Cell death programs are critical in HSC lineage decisions (Kanayama et al.,2017). Cell death can be one of the key mechanisms by which HSC and progenitor populations promote lineage biasing. Thus, we investigated whether the increase in the number of macrophages in *Ifnar1*$^{-/-}$ mice is because of reduced cell death in these mice.

Thus, WT, *Ifnar1*$^{-/-}$ and *Irf9*$^{-/-}$ mice were infected intravenously with 200 CFU of ST in 100 µl of PBS. At day 6 post infection, the hind leg bones of mice were flushed, and bone marrow cells were isolated and stained with Zombie yellow and Annexin V stains to measure the degree of necrotic and apoptotic cell death.

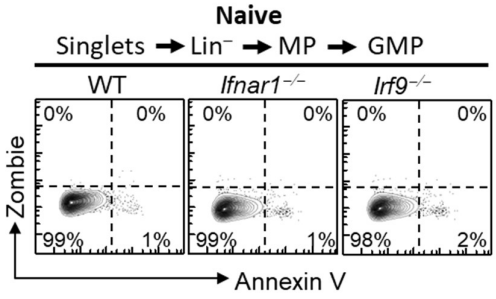
Salmonella can induce different types of cell death in macrophages such as apoptosis, necroptosis, pyroptosis, or autophagic cell death (Hu and Zhao, 2013). Upon evaluating the percentage of cell death, we observed that there was no evidence of necrotic or apoptotic cell death in the LSK progenitors of naïve or infected WT, *Ifnar1*$^{-/-}$ and *Irf9*$^{-/-}$ mice **(Figure 9A).** Similar to the LSK population GMPs did not show any potent augmentation of cell death upon infection with ST. In spite of that, the degree of early apoptotic death was reduced in the GMP population of infected *Ifnar1*$^{-/-}$ and *Irf9*$^{-/-}$ mice compared to WT, with minimal degree of necrotic and late apoptotic death detected in these subsets **(Figure 9B).** Compared to LSKs and GMPs, there was more degree of cell

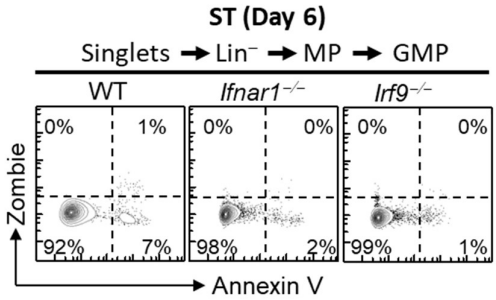
death overall in the CMP population. We observed that there was less degree of apoptotic and late apoptotic/necrotic cell death in the CMPs population of infected *Ifnar1*$^{-/-}$ and *Irf9*$^{-/-}$ compared to WT **(Figure 9C)**. Overall, reduced degree of cell death was observed in myeloid progenitors particularly in CMPs of infected *Ifnar1*$^{-/-}$ and *Irf9*$^{-/-}$.


Naive
Singlets → Lin⁻ → LSK
WT
Ifnar1⁻/⁻
Irf9⁻/⁻
0%
0%
0%
0%
0%
0%
99%
1%
99%
1%
99%
1%
Zombie
Annexin V

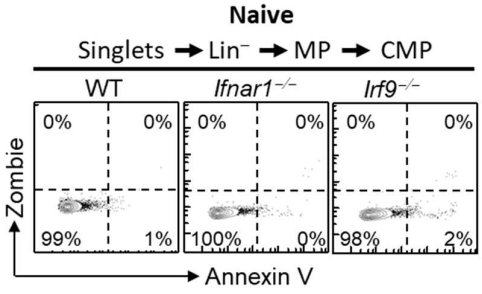

ST (Day 6)
Singlets → Lin⁻ → LSK
WT
Ifnar1⁻/⁻
Irf9⁻/⁻
0%
0%
0%
0%
0%
0%
99%
1%
100%
0%
99%
1%
Zombie
Annexin V

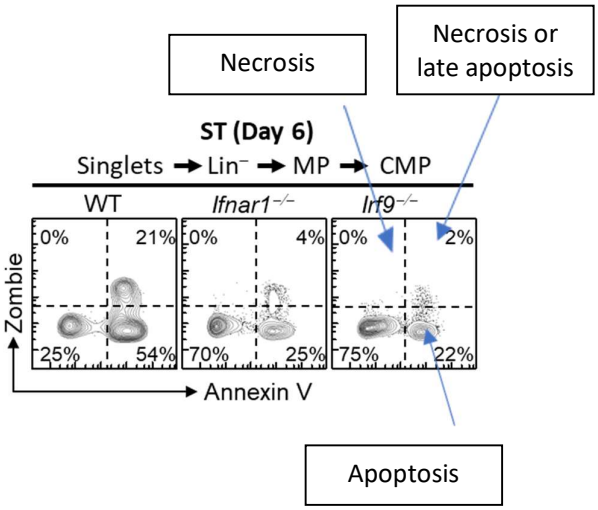
(A)


Naive
Singlets → Lin⁻ → MP → GMP
WT
Ifnar1⁻/⁻
Irf9⁻/⁻
0%
0%
0%
0%
0%
0%
99%
1%
99%
1%
98%
2%
Zombie
Annexin V


ST (Day 6)
Singlets → Lin⁻ → MP → GMP
WT
Ifnar1⁻/⁻
Irf9⁻/⁻
0%
1%
0%
0%
0%
0%
92%
7%
98%
2%
99%
1%
Zombie
Annexin V

(B)


Naive
Singlets → Lin⁻ → MP → CMP
WT
Ifnar1⁻/⁻
Irf9⁻/⁻
0%
0%
0%
0%
0%
0%
99%
1%
100%
0%
98%
2%
Zombie
Annexin V


Necrosis
Necrosis or late apoptosis
ST (Day 6)
Singlets → Lin⁻ → MP → CMP
WT
Ifnar1⁻/⁻
Irf9⁻/⁻
0%
21%
0%
4%
0%
2%
25%
54%
70%
25%
75%
22%
Zombie
Annexin V
Apoptosis

(C)

Figure 9. Reduced cell death of myeloid progenitors of infected *Ifnar1*$^{-/-}$ and *Irf9*$^{-/-}$ mice.

WT, *Ifnar1*$^{-/-}$ and *Irf9*$^{-/-}$ mice were infected with 200 CFU of ST. At day 6 post infection bone marrow cells were extracted from naïve and infected WT, *Ifnar1*$^{-/-}$ and *Irf9*$^{-/-}$ mice as mentioned above. Cells were stained with Zombie yellow and Annexin V stains and degree of necrotic and apoptotic cell death respectively was assessed by flow cytometry. Representative FACS plot showing the degree of necrotic and apoptotic death of various subsets **(A)** LSK **(B)** GMPs and **(C)** CMPs in naïve and infected WT, *Ifnar1*$^{-/-}$ and *Irf9*$^{-/-}$ mice.

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3.3 Evaluating the impact of type-I interferon signaling on macrophage function.

3.3.1 Impact of type-I interferon signaling on metabolism in macrophages.

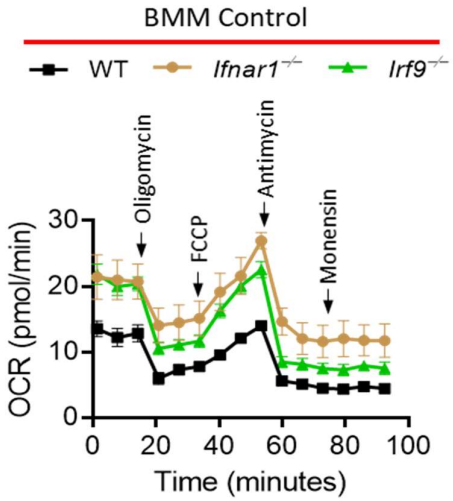
3.3.1.1 *Ifnar1*$^{-/-}$ and *Irf9*$^{-/-}$ macrophages display enhanced oxidative phosphorylation.

The increased myelopoiesis observed in the bone marrow of infected *Ifnar1*$^{-/-}$ and *Irf9*$^{-/-}$ *mice* may be responsible for increased innate resistance against a challenge with ST. Since metabolism plays a key role in survival and function of cells, we first evaluated the impact of type-1 IFN signaling on glycolysis and oxidative phosphorylation in bone marrow-derived macrophages (BMMs). Bone-marrow derived macrophages were generated from WT, *Ifnar1*$^{-/-}$ and *Irf9*$^{-/-}$ mice as described in experimental methods. On day 6 of differentiation, they were infected with 10 MOI of ST, then oxygen consumption rates (OCR) were determined in the media using a Seahorse Bioscience XF24 Flux Analyzer**.** OCR was measured in control (uninfected) macrophages (**Figure 10A**) and in macrophages at 24h post infection (**Figure 10 B**). First, basal OCR was measured at the resting stage before addition of inhibitors. Then, OCR was measured following subsequent treatment of cells with Oligomycin (inhibitor of ATP synthase), Trifluoromethoxy carbonylcyanide phenylhydrazone (FCCP) (uncoupler- that alters the mitochondrial membrane potential) and Antimycin/Rotenone (inhibitor of complexes I and III in the electron transport chain that leads to complete shutdown of mitochondrial respiration). Naïve as well as infected *Ifnar1*$^{-/-}$ and *Irf9*$^{-/-}$ BMMs displayed increased basal respiration rate in comparison to control or infected WT BMMs (**Figure 10 A-D**). Addition of Oligomycin inhibits ATP synthase which limits proton flux and prevents electron transport. As expected, OCR decreased after addition of Oligomycin in BMMs of all genotypes without and with infection **(Figure 10 A, B**). At this stage, the remaining

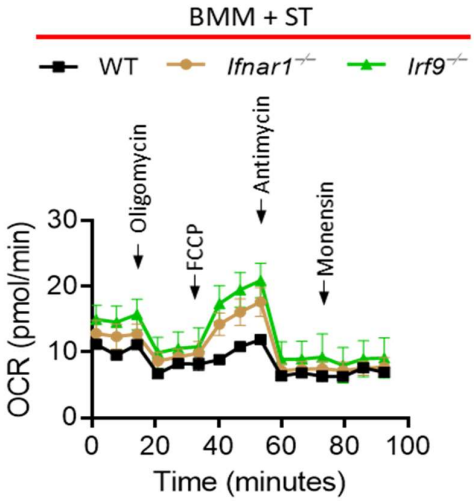
rate of mitochondrial respiration represents proton leakage, which was high in non-infected *Ifnar1*$^{-/-}$ and *Irf9*$^{-/-}$ BMMs in comparison to WT BMMs. Following infection with ST the proton leakage was low in WT and *Ifnar1*$^{-/-}$ BMMs whereas *Irf9*$^{-/-}$ BMMs still displayed increased levels of proton leakage (**Figure 10 E, F**). ATP-linked respiration, which represents the difference between basal and proton leak respiration post injection with Oligomycin was low in WT BMMs, increased in *Ifnar1*$^{-/-}$ BMMs and substantially augmented in *Irf9*$^{-/-}$ BMMs in control and ST infected cells (**Figure 10 G, H**). This suggests that there is an increased demand for ATP in the absence of type-1 IFN signaling. Next, maximal respiration was evaluated following addition of FCCP, which acts as an uncoupler by collapsing the mitochondrial membrane potential allowing free movement of electrons through the electron transport chain. Here again *Ifnar1*$^{-/-}$ and *Irf9*$^{-/-}$ BMMs displayed augmented maximal respiration in comparison to WT cells **(Figure 10 I, J)**. Spare respiratory capacity, defined as the difference between maximal respiration and basal respiration, measures the cell's ability to respond to increased energy demand. Spare respiratory capacity was higher in *Ifnar1*$^{-/-}$ and *Irf9*$^{-/-}$ BMMs compared to WT without and with infection **(Figure 10 K, L).** Finally, addition of Antimycin/Rotenone (inhibitors of complex I and III in the electron transport chain) led to determination of extra mitochondrial respiration which was still higher in uninfected *Ifnar1*$^{-/-}$ and *Irf9*$^{-/-}$ BMMs compared to WT BMMs.

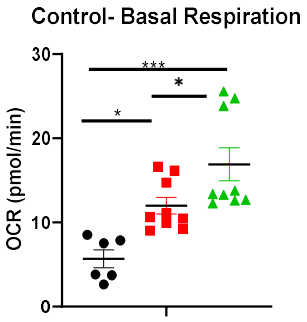
Together, these results reveal that type-1 IFN signaling downregulates mitochondrial metabolism in macrophages as *Ifnar1*$^{-/-}$ and *Irf9*$^{-/-}$ macrophages displayed enhanced oxidative phosphorylation.

(A)

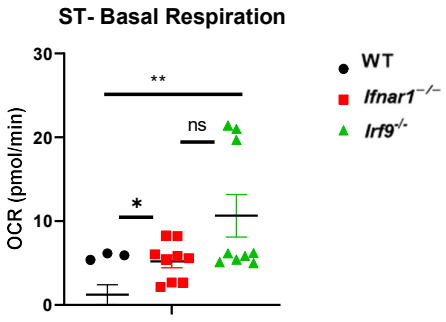

BMM Control
WT
Ifnar1−/−
Irf9−/−
Oligomycin
FCCP
Antimycin
Monensin
OCR (pmol/min)
0
10
20
30
0
20
40
60
80
100
Time (minutes)

(B)

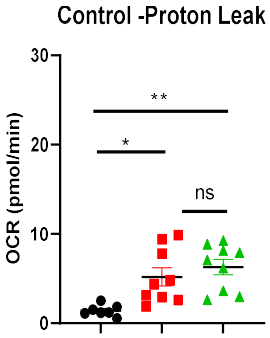

BMM + ST
WT
Ifnar1−/−
Irf9−/−
Oligomycin
FCCP
Antimycin
Monensin
OCR (pmol/min)
0
10
20
30
0
20
40
60
80
100
Time (minutes)


Control- Basal Respiration
OCR (pmol/min)
30
20
10
0
***
*
*

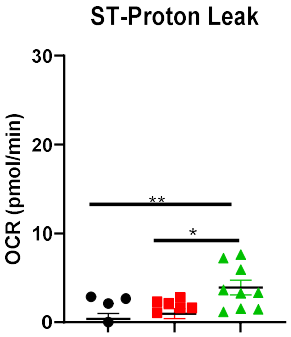
(C)


ST- Basal Respiration
OCR (pmol/min)
30
20
10
0
**
ns
*
WT
Ifnar1−/−
Irf9-/-

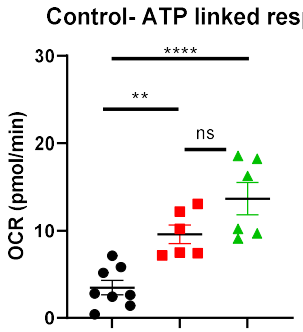
(D)


Control -Proton Leak
OCR (pmol/min)
30
20
10
0
**
*
ns

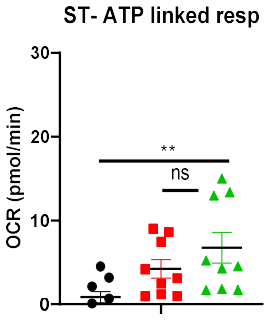
(E)


ST-Proton Leak
OCR (pmol/min)
30
20
10
0
**
*

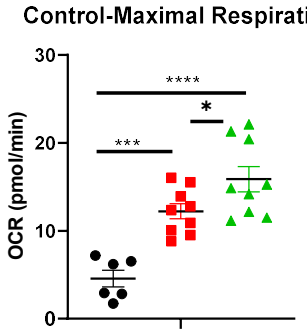
(F)


Control- ATP linked resp
OCR (pmol/min)
30
20
10
0
****
**
ns

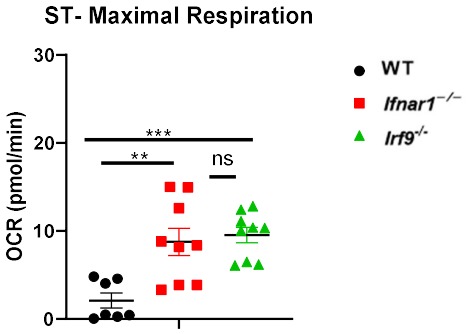
(G)


ST- ATP linked resp
OCR (pmol/min)
30
20
10
0
**
ns

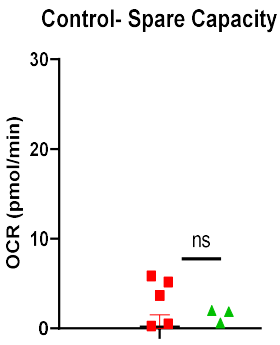
(H)


Control-Maximal Respiration
OCR (pmol/min)
30
20
10
0
****
*
***

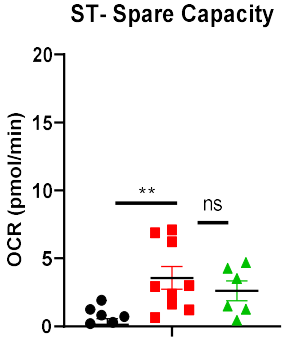
(I)


ST- Maximal Respiration
OCR (pmol/min)
30
20
10
0
***
**
ns
WT
Ifnar1−/−
Irf9-/-

(J)


Control- Spare Capacity
OCR (pmol/min)
30
20
10
0
ns

(K)


ST- Spare Capacity
OCR (pmol/min)
20
15
10
5
0
**
ns

(L)

Figure 10. *Ifnar1*$^{-/-}$ and *Irf9*$^{-/-}$ macrophages display enhanced oxidative phosphorylation.

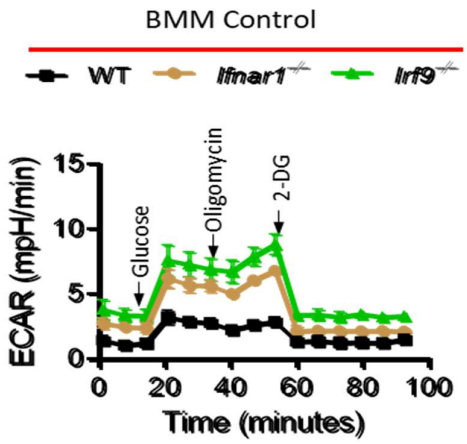
Bone marrow derived macrophages (BMMs) were generated from WT, *Ifnar1*$^{-/-}$ and *Irf9*$^{-/-}$ mice. Cells were left untreated or infected with ST at 10 MOI for 24 hours. Oxygen consumption rates (OCR) was measured before and after sequential addition of specific inhibitors of mitochondrial respiration in both non-infected **(A)** and infected **(B)** BMMs. Basal respiration **(C, D**), leak respiration **(E, F**), ATP-linked respiration **(G, H)**, maximal respiration **(I, J),** and spare capacity (**K, L**) were evaluated in non-infected and infected BMMs of WT, *Ifnar1*$^{-/-}$ and *Irf9*$^{-/-}$ mice as described in the methods section.

Data shown above are representative of three biological experiments. Statistics were done using One-way ANOVA where *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001; ns-nonsignificant.

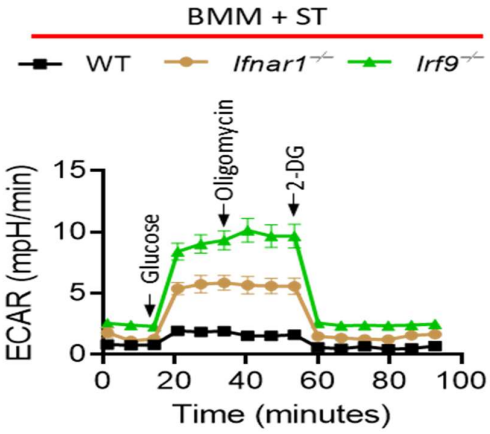
3.3.1.2 *Ifnar1*$^{-/-}$ and *Irf9*$^{-/-}$ macrophages display enhanced glycolysis

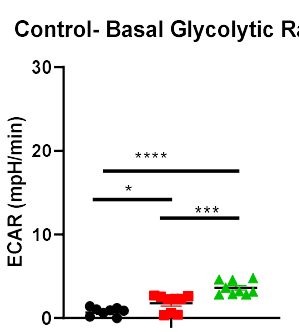
Extracellular acidification rate (ECAR) was measured in naïve and infected WT, *Ifnar1*$^{-/-}$ and *Irf9*$^{-/-}$ BMMs. Before addition of glucose, it was observed that *Irf9*$^{-/-}$ BMMs had significantly higher basal glycolytic rate compared to *Ifnar1*$^{-/-}$ and WT cells (**Figure 11 A, B)**. Post infection with ST, both *Ifnar1*$^{-/-}$ and *Irf9*$^{-/-}$ cells displayed higher basal glycolytic rate compared to WT with substantial increase in *Irf9*$^{-/-}$ cells (**Figure 11 A-D**). Glycolytic rate after glucose addition was significantly higher in non-stimulated *Irf9*$^{-/-}$ cells compared to *Ifnar1*$^{-/-}$ and WT cells. Post infection with ST, *Ifnar1*$^{-/-}$ and *Irf9*$^{-/-}$ cells displayed increased glycolytic rate compared to WT **(Figure 11 E, F)**. Since Oligomycin inhibits ATP synthase and blocks the ETC, cells undergo maximal rate of glycolysis. *Ifnar1*$^{-/-}$ and *Irf9*$^{-/-}$ BMMs displayed maximal ECAR as compared to WT cells with or without infection **(Figure 11 G, H).** Addition of 2DG inhibits glycolysis through competitive binding; hence ECAR is decreased in all groups of cells without and with infection (**Figure 11 A, B**). The non-glycolytic acidification rate post addition of 2DG is the same as basal glycolytic rate, hence overall it suggests that *Ifnar1*$^{-/-}$ and *Irf9*$^{-/-}$ display enhanced glycolysis.

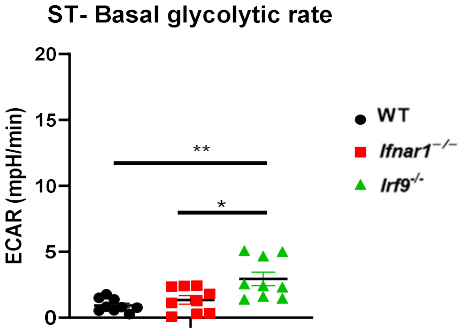
(A)


BMM Control
WT
Ifnar1−/−
Irf9−/−
ECAR (mpH/min)
Glucose
Oligomycin
2-DG
Time (minutes)

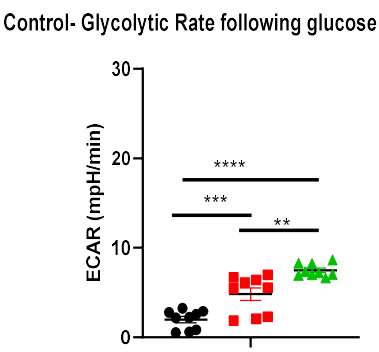
(B)

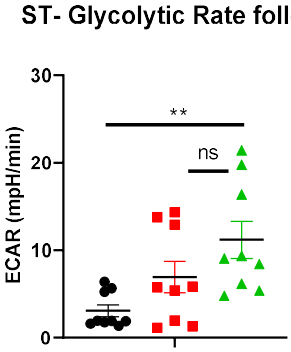

BMM + ST
WT
Ifnar1−/−
Irf9−/−
ECAR (mpH/min)
Glucose
Oligomycin
2-DG
Time (minutes)


Control- Basal Glycolytic Rate
ECAR (mpH/min)
30
20
10
0
****
*
***

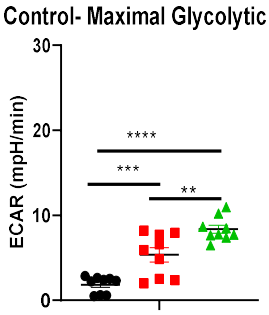

ST- Basal glycolytic rate
ECAR (mpH/min)
20
15
10
5
0
**
*
WT
Ifnar1−/−
Irf9-/-

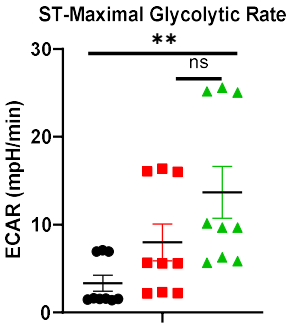
(C) **(D)**


Control- Glycolytic Rate following glucose addition
ECAR (mpH/min)
30
20
10
0
****
***
**


ST- Glycolytic Rate following glucose addition
ECAR (mpH/min)
30
20
10
0
**
ns

(E) **(F)**


Control- Maximal Glycolytic Rate
ECAR (mpH/min)
30
20
10
0
****
***
**


ST-Maximal Glycolytic Rate
ECAR (mpH/min)
30
20
10
0
**
ns

(G) **(H)**

Figure 11. *Ifnar1*$^{-/-}$ and *Irf9*$^{-/-}$ macrophages display enhanced glycolysis.

Bone marrow derived macrophages (BMMs) were generated from WT, *Ifnar1*$^{-/-}$ and *Irf9*$^{-/-}$ mice. BMMs were infected with ST at 10 MOI for 24 hours. Extracellular Acidification rate (ECAR) was measured in both non-infected **(A)** and infected **(B)** BMMs. Basal glycolytic rate **(C, D)**, glycolytic rate after glucose addition **(E, F)** and maximal glycolytic rate **(G, H)** were evaluated in non-infected and infected BMMs of WT, *Ifnar1*$^{-/-}$ and *Irf9*$^{-/-}$ mice.

Data shown above are representative of three biological experiments. Statistics were done using One-way ANOVA where *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001; ns-nonsignificant.

3.3.2 Impact of type-I interferon signaling on the expression of inflammatory cytokines by macrophages.

3.3.2.1 Both *Ifnar1*$^{-/-}$ and *Irf9*$^{-/-}$ macrophages displayed reduced secretion of the anti-inflammatory cytokine IL-10.

As described previously type-I interferon receptor *(Ifnar1)* deficient mice display enhanced resistance against ST. Since inflammatory cytokines enhance the antimicrobial functions of macrophages and promote rapid control of pathogens (Scharton KT, Afonso LC et al 1995) we were interested in evaluating the impact of type-1 IFN signaling on the expression of cytokines by macrophages. Metabolism also plays a key role in the response to infection, including cytokine production (Galván-Peña and O'Neill, 2014). Furthermore, it has been reported that increased metabolism results in enhanced cytokine production. We therefore measured the expression of pro- and anti- inflammatory cytokines by WT, *Ifnar1*$^{-/-}$ and *Irf9*$^{-/-}$ macrophages following infection with ST. Bone marrow derived macrophages were generated from WT, *Ifnar1*$^{-/-}$ and *Irf9*$^{-/-}$ mice as described in experimental methods. On day 6 of differentiation, they were infected with 10 MOI of ST, and cytokines were measured in the supernatants collected at 6 hours (for TNF-α) and 24 hours (for IL1β, IL-12, IL-10 and IL-6) post infection. The secretion of pro-inflammatory cytokine IL-1β by *Ifnar1*$^{-/-}$ and *Irf9*$^{-/-}$ macrophages was increased in comparison to WT cells **(Figure 12A).** Furthermore, *Irf9*$^{-/-}$ macrophages secreted much higher levels of IL-1β in comparison to *Ifnar1*$^{-/-}$ macrophages **(Figure 12A).** The levels of TNF-α were elevated in *Ifnar1*$^{-/-}$ macrophages compared to WT and *Irf9*$^{-/-}$ macrophages **(Figure 12B)**. *Ifnar1*$^{-/-}$ and *Irf9*$^{-/-}$ macrophages secreted significantly reduced levels of IL-10, and moderately reduced secretion of IL-12 **(Figure 12 C, D).**

Furthermore, IL-6 levels were also higher in *Ifnar1*$^{-/-}$ and *Irf9*$^{-/-}$ macrophages when compared to WT **(Figure12 E).**

Overall, type-I IFN did appear to modulate cytokine expression by macrophages but there was no modulation of pro- versus anti- inflammatory cytokines. While *Irf9*$^{-/-}$ macrophages secreted increased levels of some pro-inflammatory cytokines such as IL-1 β and IL-6, they also secreted reduced levels of other inflammatory cytokines such as TNFα and IL-12. Furthermore, *Irf9*$^{-/-}$ macrophages expressed reduced levels of the anti-inflammatory cytokine IL-10.

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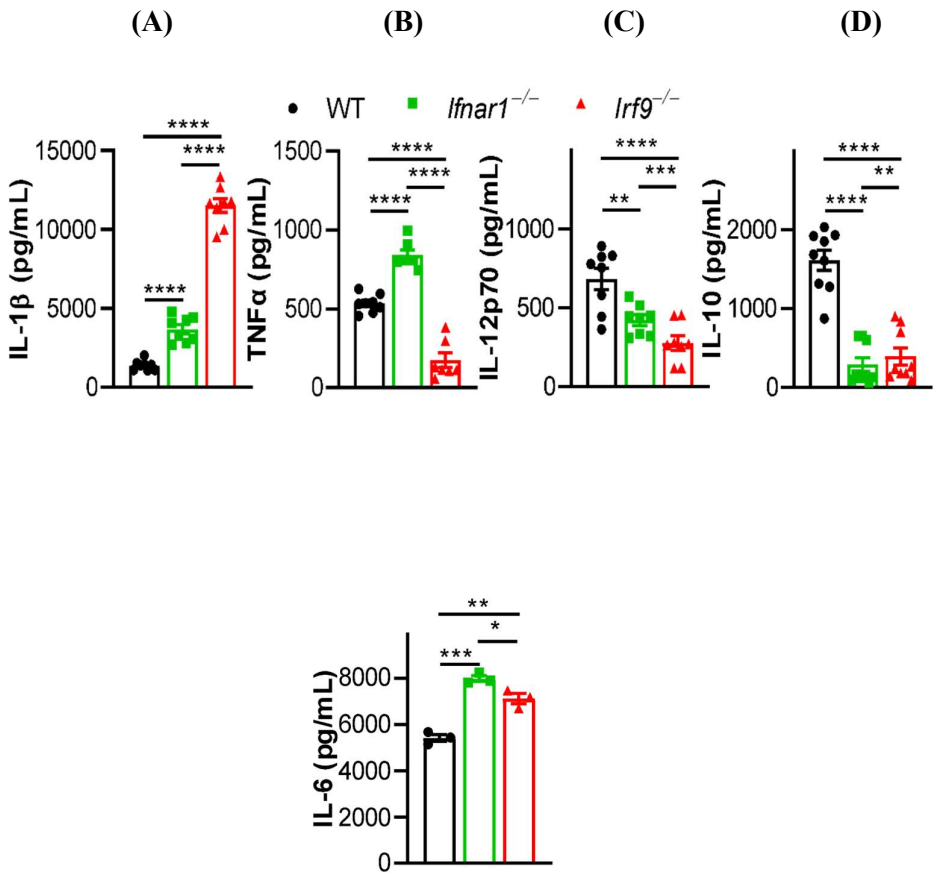

(A)
(B)
(C)
(D)
WT
Ifnar1−/−
Irf9−/−
IL-1β (pg/mL)
TNFα (pg/mL)
IL-12p70 (pg/mL)
IL-10 (pg/mL)
IL-6 (pg/mL)
(E)

Figure 12. Modulation of cytokine expression by type-1 IFN signaling.

Bone marrow derived macrophages were generated from WT, *Ifnar1*$^{-/-}$ and *Irf9*$^{-/-}$ mice as described in experimental methods. On day 6 of differentiation, they were infected with 10 MOI of ST. The expression of various cytokines in the supernatants of infected macrophages was quantified by ELISA. Graphs **(A, C, D and E)** indicate the expression of IL1β, IL-12, IL-10 and IL-6 (respectively) measured in the supernatants collected 24 hours post infection. Graph **(B)** shows the levels of TNF-α measured in the supernatants collected 6 hours post infection with ST.

Representative data is pooled from a minimum of 4 experiments, and each performed in triplicates and shown as mean±s.e.m. Statistical analysis was done by one way ANOVA, using GraphPad Prism 8 software; where *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

3.3.2.2 *Stat2*$^{-/-}$ macrophages display a cytokine secretion pattern that is similar to *Irf9*$^{-/-}$ macrophages.

As described previously *Stat2*$^{-/-}$ and *Irf9*$^{-/-}$ mice show enhanced resistance to ST infection which was much greater than that observed in WT mice. STAT2 and IRF9 exacerbate bacterial infection, while STAT1 promotes bacterial control, even though all the three form the ISGF3 transcriptional complex. Therefore, it was important to evaluate whether there are any similarities between the cytokine expression patterns of *Stat2*$^{-/-}$ and *Irf9*$^{-/-}$ macrophages. We measured the expression of various cytokines in the macrophages of WT and *Stat2*$^{-/-}$ mice following infection with ST. Bone marrow derived macrophages were generated from these mice as described in experimental methods and infected with 10 MOI of ST on day 6 of differentiation. Levels of IL-1β were significantly enhanced in *Stat2*$^{-/-}$ macrophages compared to WT **(Figure 13A)**, whereas the levels of TNF-α IL-12 and IL-10 were reduced in *Stat2*$^{-/-}$ macrophages compared to WT **(Figure 13 B, C and D).**

Overall, the cytokine production by *Stat2*$^{-/-}$ macrophages exhibited similar cytokine expression pattern as *Irf9*$^{-/-}$ macrophages, as there was increased secretion of IL 1β and reduced secretion of IL-10 in both *Irf9*$^{-/-}$ and *Stat2*$^{-/-}$ macrophages.

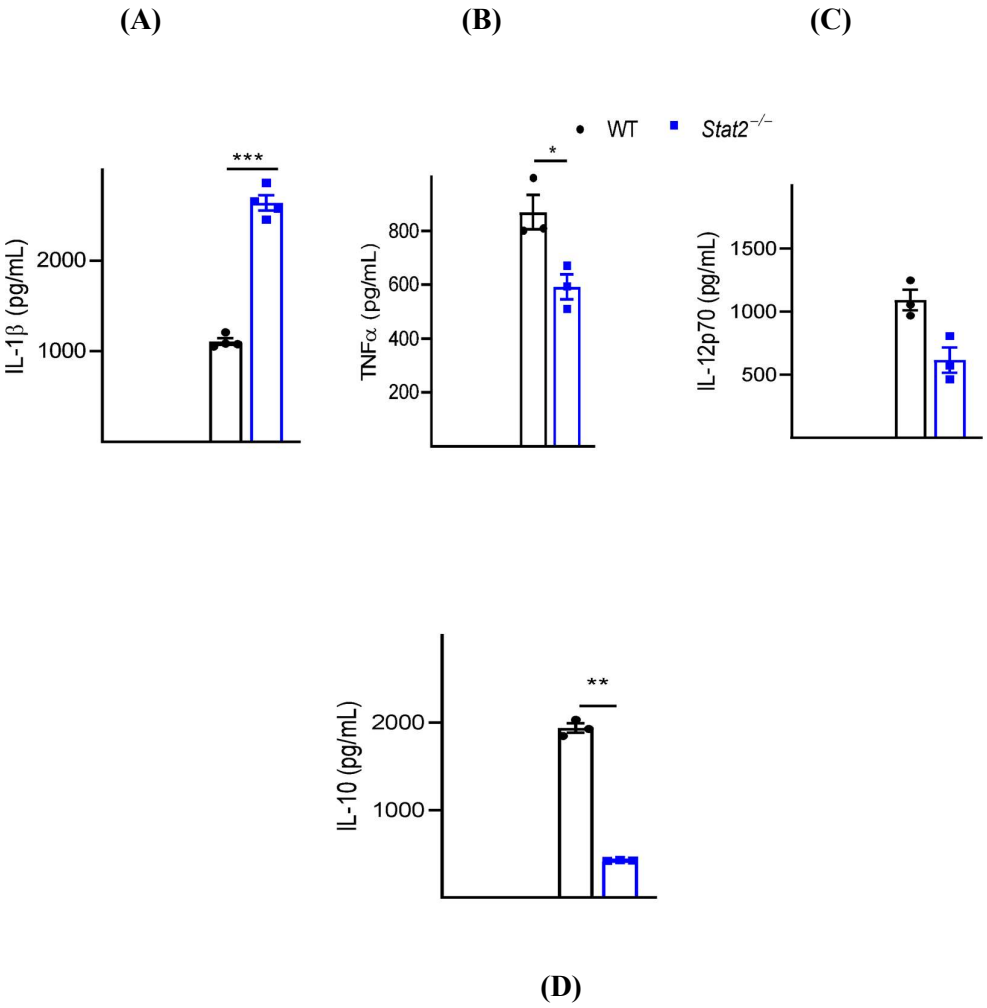

(A)
(B)
(C)
WT
Stat2−/−
***
*
IL-1β (pg/mL)
2000
1000
TNFα (pg/mL)
800
600
400
200
IL-12p70 (pg/mL)
1500
1000
500
**
IL-10 (pg/mL)
2000
1000
(D)

Figure 13. *Stat2*$^{-/-}$ macrophages exhibited similar cytokine profile as *Irf9*$^{-/-}$ cells.

Bone marrow derived macrophages were generated from WT and *Stat2*$^{-/-}$ mice as described in experimental methods. On day 6 of differentiation, they were infected with 10 MOI of ST. The expression of various cytokines in the supernatants of infected macrophages was quantified by ELISA.

Bar graph **(A, C, and D)** indicates the expression of IL1β, IL-12 and IL-10 respectively measured in the supernatants collected 24 hours after infection and TNF-α **(B)** measured in the supernatants collected 6 hours after infection.

The data is representative of one experiment of at least two similar experiments, each performed in triplicates and depicted as mean±s.e.m. Statistical significance was calculated by unpaired student's t-test using GraphPad Prism 8 software; where *$p<0.05$;**$p<0.01$; ***$p<0.001$; ****$p<0.0001$.

.

3.3.2.3 The secretion of IL1β is increased and IL-10 is decreased in *Stat1*$^{-/-}$ macrophages.

Our lab has previously observed that *Stat1*$^{-/-}$ mice are highly susceptible to ST infection, implying that STAT1 plays a protective role against ST. As described previously the bacterial burden was increased in *Stat1*$^{-/-}$ mice in comparison to WT mice (Figure 5). We therefore decided to measure the expression of key pro and anti-inflammatory cytokines by WT and *Stat1*$^{-/-}$ BMMs following infection with ST. Macrophages were cultured as described in experimental methods and infected with 10 MOI of ST on day 6 of differentiation. Interestingly, the cytokine secretion pattern of *Stat1*$^{-/-}$ BMMs was similar to *Stat2*$^{-/-}$ and *Irf9*$^{-/-}$ macrophages in that these macrophages secreted lower levels of IL-10, TNFα and IL-12 but higher levels of IL-1β **(Figure 14 A-D)**.

Overall**,** *Stat1*$^{-/-}$ macrophages displayed higher levels of IL1β compared to WT, and significantly reduced levels of IL-10. Interestingly, since the CFU is high in *Stat1*$^{-/-}$ mice and the levels of IL1β is also higher than WT, this suggests that the decreased CFU observed in *Ifnar1*$^{-/-}$ mice can't be due to increase in the levels of IL1β.

Moreover, the production of IL-10 was significantly reduced in *Stat1*$^{-/-}$ which is similar to that observed in *Ifnar1*$^{-/-}$mice, but *Ifnar1*$^{-/-}$is resistant whereas *Stat1*$^{-/-}$ is susceptible. Altogether, this data indicated that the enhanced resistance of *Ifnar1*$^{-/-}$ can't be due to decreased IL-10 levels.

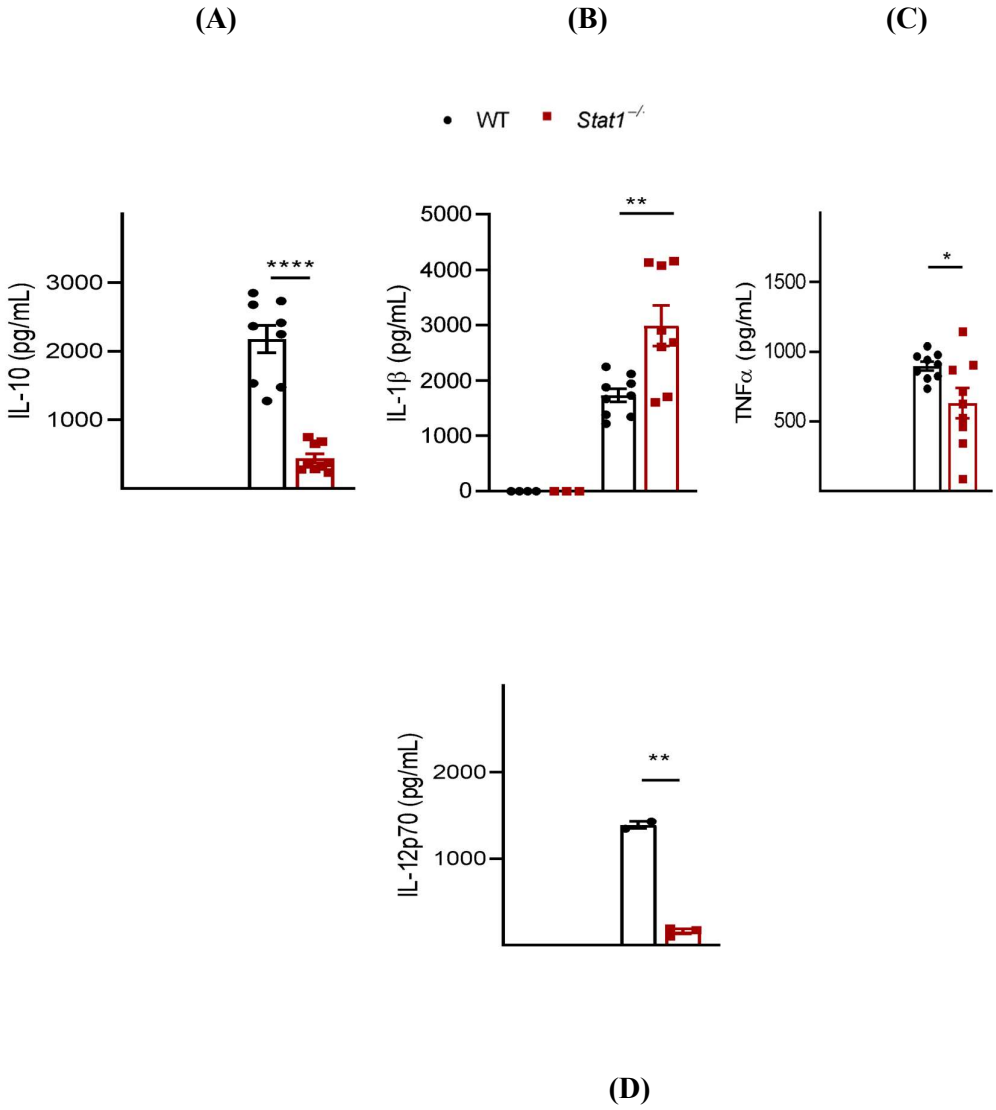

(A)
(B)
(C)
WT
Stat1−/−
IL-10 (pg/mL)
3000
2000
1000
****
IL-1β (pg/mL)
5000
4000
3000
2000
1000
0
**
TNFα (pg/mL)
1500
1000
500
*
IL-12p70 (pg/mL)
2000
1000
**
(D)

Figure 14 Secretion of IL1β is increased and IL-10 is decreased in *Stat1*$^{-/-}$ macrophages.

Bone marrow derived macrophages were generated from WT and *Stat1*$^{-/-}$ mice as described in experimental methods. On day 6 of differentiation, they were infected with 10 MOI of ST. The expression of various cytokines in the supernatants of infected macrophages was quantified by ELISA.

Bar graph **(A, B and D)** indicates the expression of IL-10, IL1β and IL-12 respectively measured in the supernatants collected 24 hours after infection and TNF-α **(B)** measured in the supernatants collected 6 hours after infection.

The data is representative of one experiment of at least two similar experiments, each performed in triplicates and depicted as mean±s.e.m. Statistical analysis was done by one way ANOVA, using GraphPad Prism 8 software; where *$p<0.05$; **$p<0.01$; ***$p<0.001$; ****$p<0.0001$.

3.3.2.4 STAT1 through type-I interferon receptor promotes the expression of inflammatory cytokines.

As described previously *Stat1*$^{-/-}$*Irf9*$^{-/-}$ exhibited significantly higher bacterial burden than *Ifng*$^{-/-}$*Irf9*$^{-/-}$ (Figure 5). Furthermore, the double deficiency of *Stat1* and *Irf9* resulted in a very high bacterial burden following a challenge with ST which was much higher than WT mice. Abrogation of *Ifn*-γ in *Irf9*$^{-/-}$ mice resulted in the escalation of the bacterial burden to WT levels (Figure 5) suggesting that the loss of STAT1 is more detrimental than *Ifn*-γ in *Irf9*$^{-/-}$ mice. This suggests that STAT1 signaling downstream of IFNAR1 mediates a protective response against ST, whereas the other two subunits of ISGF3 (STAT2 and IRF9) exacerbate the infection. To further investigate the mechanisms responsible for the differences in the bacterial burden of these mice, we measured the expression of key pro inflammatory (IL1β, TNF-α, IL-12) and anti-inflammatory cytokines (IL-10) by *Stat1*$^{-/-}$ *Irf9*$^{-/-}$ and *Ifnγ*$^{-/-}$*Irf9*$^{-/-}$ macrophages, following infection with ST.

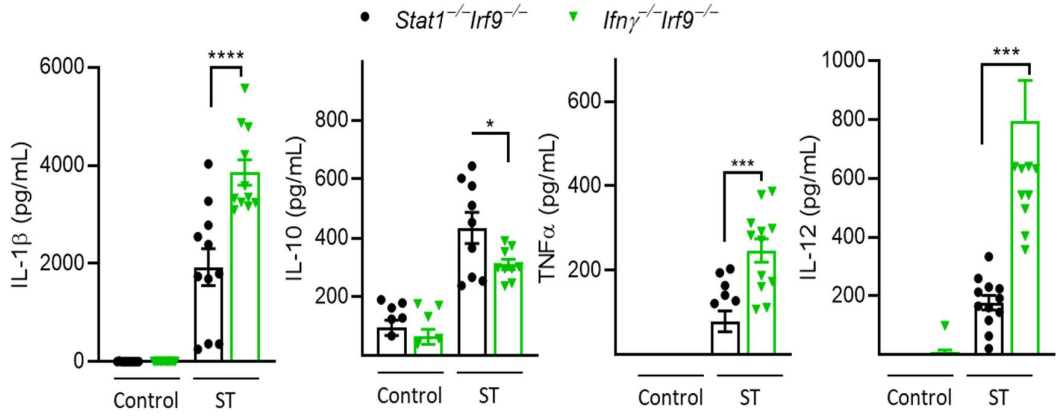
Bone marrow derived macrophages were generated from *Stat1*$^{-/-}$*Irf9*$^{-/-}$ and *Ifnγ*$^{-/-}$*Irf9*$^{-/-}$ as described in experimental methods. On day 6 of differentiation, they were infected with 10 MOI of ST. The expression of various cytokines in the supernatants of infected macrophages was quantified by ELISA. The results revealed that *Ifnγ*$^{-/-}$*Irf9*$^{-/-}$ cells expressed substantially higher levels of IL-1β when compared to *Stat1*$^{-/-}$*Irf9*$^{-/-}$ cells **(Figure 15A)**. As described previously, levels of IL1β were significantly enhanced in *Irf9*$^{-/-}$ macrophages (Figure12); this was abrogated by inactivation of STAT1; i.e. in *Stat1*$^{-/-}$*Irf9*$^{-/-}$ cells. Interestingly, the increased level of IL1β in the *Irf9*$^{-/-}$ macrophages was not normalized upon *Ifn*-γ deficiency, this suggests that STAT1 promotes IL1β secretion in *Irf9*$^{-/-}$ cells independent of *Ifn*-γ signalling. *Ifnγ*$^{-/-}$*Irf9*$^{-/-}$ macrophages secreted moderately reduced levels of IL-10 compared to *Stat1*$^{-/-}$*Irf9*$^{-/-}$ macrophages **(Figure 15 B)**. In fact,

Irf9$^{-/-}$*Ifnγ*$^{-/-}$ secreted significantly higher levels of TNF-α and IL-12 in comparison to

Stat1$^{-/-}$*Irf9*$^{-/-}$ cells **(Figure 15 C, D).**

Taken together, these results indicate that in *Irf9*$^{-/-}$ macrophages STAT1 promotes the expression of IL1β, TNF-α and IL-10 through a mechanism that does not involve IFNγ. *Ifnγ*$^{-/-}$*Irf9*$^{-/-}$ macrophages secreted significantly higher levels of pro-inflammatory cytokines in comparison to *Stat1*$^{-/-}$*Irf9*$^{-/-}$ macrophages which corelates with the lower bacterial burden observed in these mice.

Overall analysis of the cytokine expression pattern of double knockouts mice indicated that STAT1 plays a key role in promoting the secretion of pro-inflammatory cytokines through ISGF3, to facilitate bacterial control and the role of STAT2 in promoting cytokine expression through ISGF3 is minimal.


Stat1−/−Irf9−/−
Ifnγ−/−Irf9−/−
IL-1β (pg/mL)
6000
4000
2000
0
****
Control
ST
IL-10 (pg/mL)
800
600
400
200
*
Control
ST
TNFα (pg/mL)
600
400
200
***
Control
ST
IL-12 (pg/mL)
1000
800
600
400
200
***
Control
ST

(A) **(B)** **(C)** **(D)**

Figure 15. STAT1 through type-I interferon receptor promotes the expression of inflammatory cytokines

Bone marrow derived macrophages were generated from *Stat1*$^{-/-}$*Irf9*$^{-/-}$ and *Ifnγ*$^{-/-}$*Irf9*$^{-/-}$ mice as described in experimental methods. At day 6 of differentiation, they were infected with 10 MOI of ST. The expression of various cytokines in the supernatants of infected macrophages was quantified by ELISA.

Bar graph **(A, B and D)** indicate the expression of IL1β, IL-10 and IL-12 respectively measured in the supernatants collected 24 hours after infection and TNF-α **(C)** measured in the supernatants collected 6 hours post infection.

Data is pooled from a minimum of 4 experiments, each performed in triplicates and presented as mean±s.e.m. Statistical analysis was done by unpaired student's t-test using GraphPad Prism 8 software; where *p<0.05;**p<0.01; ***p<0.001; ****p<0.0001.

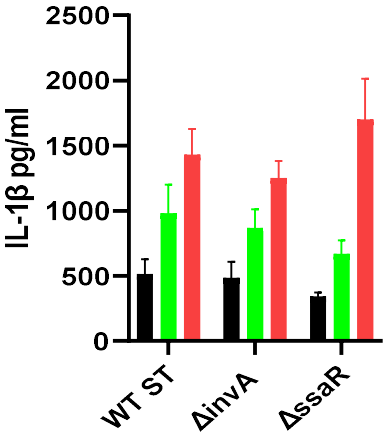
3.3.2.5 Cytokine expression by *Irf9*$^{-/-}$ macrophages is not modulated by the virulence islands of ST.

As described previously, *Salmonella* expresses several virulence genes that are essential for modulating host immune responses. The major virulence genes of *Salmonella* are clustered into two main *Salmonella* pathogenicity islands (SPI) SPI-1 and SPI-II, which encode virulence factors for assembly of the type three secretion system (T3SS) and effector proteins secreted through the T3SS (Siriken, 2013). Infection with ST with an inactive *invA* gene have a dysfunctional SPI-I T3SS, which results in a failure to infect epithelial cells and lack of infection of hosts following an oral challenge. In contrast to SPI-I, SPI-II genes promote bacterial survival and replication within infected cells (Abrahams and Hensel, 2006). Disabling of SPI-II results in a highly attenuated infection. *ssaR* is one of the key regulatory proteins in the SPI-II T3SS and inactivation of this effector protein results in a highly attenuated, non-lethal infection which is rapidly cleared by the host (Waterman and Holden, 2003). Our lab has previously reported that systemic infection of mice with the SPI-1 mutant *invA*, which fails to infect non phagocytic cells, induces a lethal infection, whereas the *ssaR* mutant of ST which is incapable of surviving within the infected cells is cleared rapidly by the host (Sad et al., 2008). We evaluated the impact of SPI-I and SPI-II on cytokine expression by macrophages. Bone marrow derived macrophages were generated from WT, *Ifnar1*$^{-/-}$ and *Irf9*$^{-/-}$ mice as described in experimental methods and were infected with 10 MOI of the following strains of ST- WT ST, Δ*invA* ST (defective SPI-I), Δ*ssaR* ST (defective SPI-II). We measured the expression of pro- and anti- inflammatory cytokines by WT, *Ifnar1*$^{-/-}$ and *Irf9*$^{-/-}$ macrophages at 6 hours (TNF-α) and 24 hours (IL1β, IL-12 and IL-10) post infection with WT ST, Δ*invA* ST and Δ*ssaR* ST. The secretion of pro-inflammatory

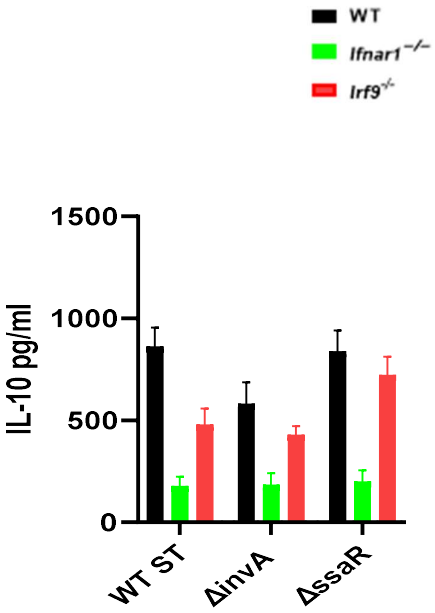
cytokine IL1β by *Ifnar1*$^{-/-}$ and *Irf9*$^{-/-}$ macrophages was enhanced in comparison to WT cells; the secretion of IL1β was increased substantially in *Irf9*$^{-/-}$ macrophages upon infection with WT ST. Similar trend was observed upon infection with Δ*invA and* Δ*ssaR* strain of ST **(Figure 16 A**). This suggests that the secretion of IL1β was not dependent on SPI-I or SPI-II, and the amplification of IL1β in *Irf9*$^{-/-}$ macrophages was also not related to these virulence hubs. *Ifnar1*$^{-/-}$ macrophages expressed reduced levels of IL-10 in comparison to WT and *Irf9*$^{-/-}$ macrophages, and this was observed in cells infected with WT, Δ*invA and* Δ*ssaR* ST (**Figure 16 B**). Similarly, the expression of IL-12 or TNF-α did not appear to be modulated by SPI-I or SPI-II (**Figure 16 C, D**).

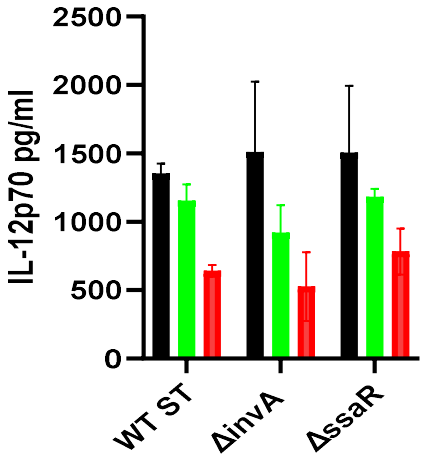
Collectively, this data suggests that the cytokine expression by macrophages is not influenced by the SPI-I and SPI-II genes and the impact of IRF9 on the expression of cytokines by macrophages is also not impacted by these virulence hubs.

(A)

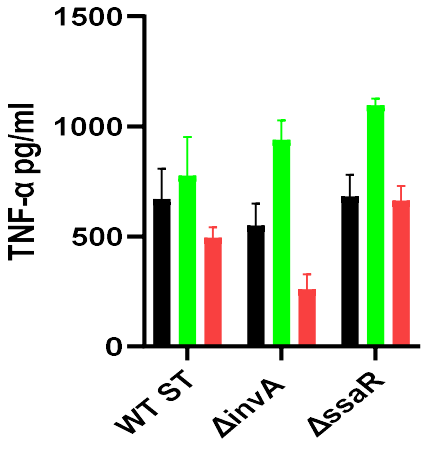

2500
2000
1500
1000
500
0
IL-1β pg/ml
WT ST
ΔinvA
ΔssaR

(B)


WT
Ifnar1 −/−
Irf9 -/-
1500
1000
500
0
IL-10 pg/ml
WT ST
ΔinvA
ΔssaR


2500
2000
1500
1000
500
0
IL-12p70 pg/ml
WT ST
ΔinvA
ΔssaR

(C)


1500
1000
500
0
TNF-α pg/ml
WT ST
ΔinvA
ΔssaR

(D)

Figure 16. Cytokine expression by *Irf9*$^{-/-}$ macrophages is not modulated by the SPI-I and SPI-II genes.

Bone marrow derived macrophages were generated from WT, *Ifnar1*$^{-/-}$ and *Irf9*$^{-/-}$ mice as described in experimental methods. On day 6 of differentiation, they were infected with 10 MOI of various strains of ST (WT ST, Δ*invA* ST (defective SPI-I) and Δ*ssaR* ST (defective SPI- II). The expression of various cytokines in the supernatants of infected macrophages was quantified by ELISA.

Bar graphs **(A, B and C)** indicate the expression of IL1β, IL-12 and IL-10 (respectively) measured in the supernatants collected at 24 hours post infection and TNF-α **(D)** measured in the supernatants collected 6 hours after infection.

Representative data is pooled from a minimum of 4 experiments, each performed in triplicates.

3.3.3 Impact of type-I interferon signaling on the induction of cell death of macrophages.

3.3.3.1 Type-1 IFN deficient macrophages undergo slightly reduced cell death

As described previously type-1 IFN signaling appears to modulate cytokine expression by macrophages, but there was no modulation of pro- versus anti- inflammatory cytokines. Although *Ifnar1*$^{-/-}$ macrophages secreted moderately reduced level of IL-12 relative to wild-type macrophages, there was a much higher expression of IL-1β in *Ifnar1*$^{-/-}$ macrophages. Furthermore *Ifnar1*$^{-/-}$ and *Irf9*$^{-/-}$ macrophages expressed reduced levels of the anti-inflammatory cytokine IL-10. Therefore, we tested whether IFNAR1 signaling had an impact on the cell death of macrophages in response to ST.

Inflammasome signaling has been shown to be the dominant pathway that induces cell death of macrophages following infection with ST (Bergsbaken et al.,2009; Lamkanfi and Dixit,2014;). Inflammasome signaling results in processing of the IL-1β and induction of cell death by pyroptosis (Broz et al, 2010; Mariathasan et al., 2004). Induction of pyroptotic cell death results in the release of intracellular bacteria which are then rapidly eliminated by neutrophils. Hence pyroptosis is an important protective mechanism (Broz and Monack, 2011). We therefore measured the impact of type-I IFN signaling on the induction of cell death of macrophages. Bone marrow derived macrophages were generated from WT, *Ifnar1*$^{-/-}$ and *Irf9*$^{-/-}$ macrophages and infected with a range of MOIs (5,10 and 50) of ST *in vitro*. Cell viability was measured at 24 hours post infection using the neutral red assay as described in experimental methods. Neutral red dye accumulates in the lysosomes of live cells; therefore, absorbance in this assay is directly proportional to the number of viable cells. Little to no death was observed upon infection of WT macrophages with 5 MOI of ST, whereas infection with 10 MOI of ST resulted in

increased cell death in WT macrophages (**Figure 17**). However, *Ifnar1*$^{-/-}$ and *Irf9*$^{-/-}$ cells displayed a moderately reduced magnitude of cell death at 10 MOI of ST in comparison to WT macrophages (**Figure 17**). When the bacterial dose was increased to 50 MOI the magnitude of cell death increased in all the three groups of macrophages and no difference in the magnitude of cell death was observable (**Figure 17**).

Taken together, type-I interferon deficient macrophages displayed a slightly reduced cell death at lower bacterial doses which is counterintuitive to the protective role cell death mechanisms in bacterial control. It is conceivable that the reduced cell death may allow macrophages to express cytokines for prolonged periods and control infection better. Furthermore, our results reveal that *Ifnar1*$^{-/-}$ and *Irf9*$^{-/-}$ macrophages undergo reduced cell death but express increased levels of IL-1β, which reveal the opposing roles of type-1 IFN signaling on key outcomes of inflammasome signaling; cell death and IL-1β secretion.

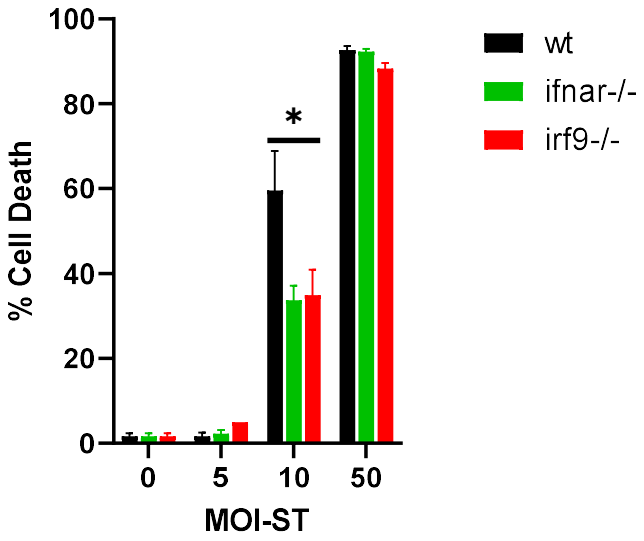

100
80
60
40
20
0
% Cell Death
*
0
5
10
50
MOI-ST
wt
ifnar-/-
irf9-/-

Figure 17 Type-I interferon deficient macrophages displayed a slightly reduced cell death at lower bacterial doses.

Bone marrow derived macrophages were generated from WT, *Ifnar1*$^{-/-}$ and *Irf9*$^{-/-}$ mice as described in the experimental methods. On day 6 of differentiation, the cells were infected with 5, 10 and 50 MOIs of ST for 24 hours. Cell death was then assessed by neutral red (NR) assay.

Graphs show the percentage of cell death (% Cell Death). NR optical densities (ODs) were normalized to the unstimulated control (cells with R8 medium only).

Representative data of one experiment of four similar experiments is shown, and each experiment was performed in triplicates and presented as mean±s.e.m. Statistical analysis was calculated by one-way ANOVA, using GraphPad Prism 8 software; where *$p<0.05$.

3.3.3.2 SPI-I and SPI-II promote cell death of macrophages.

As described previously, the major virulence genes of *Salmonella* are clustered into two main *Salmonella* pathogenicity islands (SPI) SPI-1 and SPI-II (Abrahams and Hensel, 2006). SPI-I promotes invasion into epithelial cells and SPI-II promotes intracellular replication of ST in all cell types. Proliferation of ST within macrophages is important for the pathogenicity of ST (Hansen-Wester and Hensel,2001; Hume et al.,2017; Santos et al.,2003). Our lab has previously reported that systemic infection of mice with the SPI-1 mutant *invA*, which fails to infect non phagocytic cells, induces a lethal infection, whereas the *ssaR* mutant of ST which is incapable of surviving within the infected cells is cleared rapidly by the host (Sad et al., 2008).

As described previously, the secretion of IL1β and other pro- and anti- inflammatory cytokines was not dependent on SPI-I or SPI-II, and the amplification of IL1β in *Irf9*$^{-/-}$ was also not related to these virulence hubs, which suggested that cytokine expression by *Irf9*$^{-/-}$ macrophages is not modulated by the SPI-I and SPI-II genes. As cell death has been an essential mechanism of virulence of S. Typhimurium, we therefore evaluated the type-1 IFN signaling impacts cell death of macrophages that is dependent on the SP-1 and SP-II. WT, *Ifnar1*$^{-/-}$ and *Irf9*$^{-/-}$ macrophages were generated as described in experimental methods. On day 6 of differentiation macrophages were infected *in vitro* with WT, Δ*invA* (defective SPI-I) and Δ*ssaR* (defective SPI-II) strains of ST and their viability was measured at 24 hours post infection by a neutral red assay as described in experimental methods. It was observed that the cell death of macrophages is mainly dependent on the SPI-I virulence apparatus since inactivation of *invA* resulted in a drastic

reduction of cell death which became undetectable in *Ifnar1*$^{-/-}$ and *Irf9*$^{-/-}$ macrophages (**Figure 18**). When WT macrophages were infected with the Δ*ssaR* strain of ST there was ~50% reduction of cell death in comparison to cells infected with WT ST. This suggests that SPI-II also promotes cell death of macrophages. Interestingly, the cell death induced by the Δ*ssaR* strain of ST was undetectable in *Ifnar1*$^{-/-}$ and *Irf9*$^{-/-}$ macrophages (**Figure 18**).

Since SPI-II is fully functional in the *invA* mutant of ST, and the *invA* mutant induced poor cell death these results suggest that the genes that are exported through the SPI-II play a secondary role in the induction of cell death of macrophages. When cells were infected with the *ssaR* mutant of ST there was a 50% reduction of cell death, which indicates that SPI-II does play a role in cell death of macrophages. In both cases there was reduction of cell death in *Ifnar1*$^{-/-}$ and *Irf9*$^{-/-}$ macrophages, which suggests that IFNAR1 signaling promotes both SPI-I- and SPI-II-dependent cell death.

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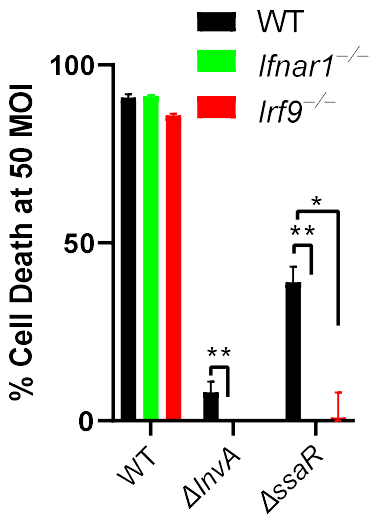
WT
Ifnar1−/−
Irf9−/−
% Cell Death at 50 MOI
100
50
0
**
**
*
WT
ΔInvA
ΔSsaR

Figure 18 Type-I interferon signaling promotes SPI-I and SP-II dependent cell death.

Bone marrow derived macrophages were generated from WT, *Ifnar1*$^{-/-}$ and *Irf9*$^{-/-}$ mice as described in the experimental methods. On day 6 of differentiation, they were infected with 50 MOI of various strains of ST (WT ST, Δ*invA* ST and Δ*ssaR* ST). Cell death was then assessed by neutral red (NR) assay at 24 hours post infection.

Graphs display % cell death. NR optical densities (ODs) were normalized to the unstimulated control (cells with R8 medium only)

Representative data of one experiment of four similar experiments is shown, and each experiment was performed in triplicates and presented as mean±s.e.m. Statistical analysis was done by one-way ANOVA; *$p<0.05$; **$p<0.01$.

4. DISCUSSION

4.1 Role of ISGF3 complex in promoting susceptibility to bacterial infection.

Salmonella is a flagellated rod-shaped, gram-negative intracellular bacterium (Coburn et al., 2007; Eng et al., 2015; Wang, M. et al 2020). *Salmonella enterica* and *Salmonella bongori* are the two most studied species of *Salmonella. Salmonella enterica* can infect a wide range of hosts and cause different types of diseases in them and is sub-classified based on genome sequence analysis into 6 different kinds of sub-species (Reeves et al., 1989).

Infections caused by *Salmonella enterica* serovars remain a major problem for public health all over the world. Broadly *Salmonella* serovars Typhi and Paratyphi are humans-restricted pathogens that can lead to systemic diseases (Hume et al., 2017). According to the most recent estimates as per the report of the 7th TB strategy summit, 13-21 million cases of typhoid fever occur annually worldwide (World Health Organization [WHO], 2022).

It has been known that the immune response to *Salmonella* Typhi is impaired due to the expression of virulence associated capsular polysaccharide, this homopolymer covers the bacterial surface, which impairs complement activation and neutrophil recruitment. This allows the spread of bacteria into the bloodstream and infect organs including spleen. (Keestra-Gounder et al., 2015).

Whereas *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium*)* is a non-typhoidal strain (NTS) of *Salmonella*, it can cause different diseases in broad variety of hosts. Mice are not susceptible to *Salmonella* Typhi (Santos et al., 2001). *Salmonella* Typhimurium (ST) can evade the intestinal epithelial barrier and cause inflammation, that

makes it a widely used model for studying human typhoidal infection (Coburn et al., 2007; Haraga et al., 2008; Santos et al., 2001). Interestingly, studies have shown that disease outcomes vary among different strains of mice. For example, C57BL/6J is one of the susceptible strains of mice in which ST causes a systemic disease, that resembles human typhoid fever (Santos et al., 2001), whereas resistant strains of mice can develop a chronic infection (Broz et al., 2012). In these strains of mice, the innate resistance to *Salmonella* can be attributed to a functional natural resistance associated macrophage protein 1, NRAMP-1 (Searle, S et al., 1998). A mutation in this gene causes susceptibility to infection due to an accumulation of ions in the phagosome which in turn promotes the intracellular replication of the bacteria (Vidal et al., 1995). Most of the experiments performed in my thesis involved the infections with *S.* Typhimurium.

Exacerbation of bacterial infections has often been observed in patients following a recent viral infection, a phenomenon termed as superinfection (Feasey et al., 2012). Viral infections induced high levels of type-I interferons, which in turn promote control of the infection, and the expression of high levels of type-I interferons have been considered as a prime candidate for bacterial superinfection (Almand et al., 2017; Lee et al., 2015). Therefore, scrutinizing the role of type-1 IFN in bacterial pathogenesis is extremely necessary. It has also been reported that invasive non-typhoidal strains (iNTS) such as *S.* Typhimurium are often associated with immune suppression due to burden of HIV which leads to fatality in HIV infected patients in African populations. (Carden et al., 2015; Gilchrist et al., 2015; Feasey et al., 2012). Hence, to meet the rising demand for identification of novel therapeutic targets, the first and foremost step is to closely examine the interplay between viruses and bacteria (Carden et al., 2015). Presently there are no efficient vaccines against *Salmonella.*

The role of type-I interferon is well recognized in the context of a viral infection; however, their role in bacterial infections is not clear (Boxx and Cheng, 2016). In my thesis we aimed to understand the role of type-1 IFNs in bacterial pathogenesis, and scrutinize the mechanism adopted by various components of type-1 interferon signaling, especially ISGF3 complex in response to *Salmonella* Typhimurium.

It has been reported that *Chlamydia trachomatis* induces type-1 IFNs that play protective function and protect the host against bacterial infection, thus promoting resistance (de la Maza et al., 1985). It was also reported that type-I IFNs can protect the host against infections caused by *Streptococci*, *E. coli* and *Streptococcus pneumoniae* (Mancuso et al., 2007).

In contrast, type-I IFN was shown to have a detrimental role during infection with various intracellular bacteria such as *Listeria monocytogenes* and *Mycobacterium tuberculosis* (Mancuso et al., 2007). During infection of mice with *Listeria monocytogenes* type-1 IFN was shown to be detrimental to host survival, which correlated with increased cell death of host cells. (Auerbuch et al., 2004; O' Connell et al., 2004). Similar results were observed with *Mtb* (Mancuso et al., 2007). Overall, these studies indicated that the role of type-1 IFN signaling in bacterial pathogenesis may depend on the unique host-pathogen interactions.

Salmonella Typhimurium induces type-1 IFN signaling through its interaction with TLR4 (Gilchrist et al., 2015). Previous work in the lab has shown that *Ifnar1*$^{-/-}$ mice are less susceptible to *S.* Typhimurium. Moreover, it was also reported that *S.* Typhimurium-induced pyroptosis and necroptosis, helps in dissemination of bacteria (Robinson et

al.,2012). Results presented in my thesis agrees that type-I IFN signaling results in poor control of ST and is detrimental to host survival (**Figure 2 A, B**).

The current work aims to delve into the role of ISGF3 complex in promoting susceptibility to bacterial infection. Engagement of the IFNAR1 results in the assembly of the trimeric transcription factor ISGF3 that is composed of STAT1, STAT2 and IRF9, which results in the transcription of interferon stimulated genes (Aaronson and Horvath, 2002). Therefore, understanding the role of these transcription factors in the pathogenesis of bacterial infection is important as each transcription factor of the complex provides a distinct, complementary function (De Weerd et al., 2013).

Studies performed in the context of viral infections focused on fundamental differences in the roles of each of the transcription factors of the ISGF3 complex. Experiments performed in mouse models for lymphocytic choriomeningitis virus (LCMV) infection, suggested that both STAT2 and IRF9 play a key role in limiting the virus spread, however only the STAT1- deficient mice caused lethal disease (Hofer et al., 2012). Overall, this study indicated a STAT1 has its unique anti-viral mechanism as compared to STAT2 and IRF9 (Hofer et al., 2012).

We decided to scrutinize the role of type-1 IFNs in bacterial pathogenesis, and understand the mechanism adopted by various components of type-1 interferon signaling, especially the ISGF3 complex in response to *Salmonella* Typhimurium. Work done in this thesis uncovered that *Stat2*$^{-/-}$ mice and *Irf9*$^{-/-}$ mice show enhanced resistance to ST infection which was much greater than that observed in *Ifnar1*$^{-/-}$ mice (**Figure 3B and 4**). *Irf9*$^{-/-}$ mice displayed reduced bacterial burden in the spleens in comparison to WT mice and restated the phenotype of *Ifnar1*$^{-/-}$ (**Figure 3A**). Previous results in the lab indicated that *Stat1*$^{-/-}$ mice are highly susceptible to infection with ST and undergo rapid fatality. We indicated

that STAT1 signaling after engagement of IFNAR1 as well as IFNGR had opposing effects. We showed that *Stat1*$^{-/-}$ mice had significantly increased bacterial burden **(Figure 5)** which overall suggested that STAT1 plays a protective role in disease pathogenesis. Our results with double knockout strains (*Stat1*$^{-/-}$*Irf9*$^{-/-}$ and *Ifng*$^{-/-}$*Irf9*$^{-/-}$) strengthened our findings. Double deficiency of STAT1 and IRF9 resulted in a very high bacterial burden when compared to WT following a challenge with ST. Abrogation of IFN-γ in *Irf9*$^{-/-}$ mice resulted in the escalation of the bacterial burden to WT levels **(Figure 5).** In nutshell, STAT1 signaling downstream of IFNAR mediates a protective response against ST.

4.2. Role of cell subset modulation in providing enhanced resistance against ST infection

Salmonella infection occurs through the oral route which breaks the gut epithelial barrier and bacteria get internalized, into APCs such as macrophages and dendritic cells that rapidly disseminates bacteria through the lymphatics and systemic sites (Haraga et al., 2008; Kaur and Jain, 2012; Monack et al., 2004).

Since my current work revealed that type-1 IFN signaling impairs the control of ST at an early time point (day 3 post infection) **(Figure 2A)**, the impact of type-I IFN is detectable at an early stage and considering the fact that the development of acquired immune response against phagosomal pathogens such as ST is delayed (Luu et al., 2006; Srinivasan et al., 2004), it is likely that the innate immune cells such as monocytes promote heightened resistance of *Ifnar1*$^{-/-}$ mice against a challenge with ST. Studies have shown that early control of *S.* Typhimurium is dependent on the induction of innate immune response (Jones BD, Falkow S 1996), moreover it has been reported that subsequent T cell responses to *S.* Typhimurium are not engaged early during infection (Albaghdadi H, Robinson N

2009). Therefore, the next aim of our thesis was to evaluate the cell type in *Ifnar1*$^{-/-}$ mice that is responsible for conferring the enhanced resistance against ST.

Our lab has also previously reported that *Ifnar1*$^{-/-}$ mice have significantly higher numbers of splenic monocytes/macrophages in comparison to WT. We thus depleted monocytes and neutrophil in our mice and observed that the depletion of monocyte/macrophage lineage resulted in complete abrogation of the resistance, whereas neutrophil depletion alone did not have an impact on the resistance of *Ifnar1*$^{-/-}$ mice **(Figure 6A, B).** Moreover, depletion of lymphocyte lineage indicated that lymphoid cell subsets do not confer enhanced resistance to *Ifnar1*$^{-/-}$ mice against ST infection. Our findings agreed with the previous work done in our lab which demonstrated that depletion of NK cells did not result in an increased bacterial burden in *Ifnar1*$^{-/-}$ mice, indicating that the enhanced resistance of *Ifnar1*$^{-/-}$ is not related to the activity of NK cells.

It has been reported that impaired immune resistance as observed in chronic infections, allows pathogen persistence (Altare,F., et al., 1998; Divangahi et al., 2008). HSCs give rise to all immune cell subsets, and it has been reported that the manipulation of HSCs by pathogens can influence the host defense to infection (Szade, K et al., 2018). After injury cells are induced to proliferate, to quickly re-establish homeostasis. Research is still ongoing to find out which specific signaling mechanisms promote the activation of HSCs (Khan, N et al., 2020) However, understanding of the HSC response to chronic infection, e.g., *Salmonella*, is not clear (Scumpia, P. O et al.,2010).

Hematopoietic stem cells (HSCs) get activated and meet the peripheral demand for immune cells during infection or stress by undergoing expansion and differentiation into more lineage-restricted progenitors, primarily within the bone marrow (BM) (Essers et al., 2009; Hartner et al., 2009; Sato et al., 2009). Our results revealed that monocytes/macrophages conferred protection in *Ifnar1*$^{-/-}$ mice, which corroborated with previously reported work

from our lab showing an increased number of macrophages in *Ifnar1*$^{-/-}$ (Robinson, N et al.,2012). We were curious to check whether the deficiency of IFNAR1 results in a modulation of myeloid cell numbers in the bone marrow compartment, which is the main site of generation of immune cells (Zhu and Emerson, 2002). No modulation was observed in the bone marrow of naïve *Ifnar1*$^{-/-}$ **(Figure 7),** suggesting that IFNAR1 signaling do not seem to play a role in regulating the differentiation of HSCs and progenitors at steady state conditions.

Moreover, studies have shown that infection-specific changes in hematopoiesis may restrict or promote generation of specific lineages to centrally bias the overall systemic immune response (Khan et al., 2020). Therefore, our next aim was to determine the impact of type-1 IFN signaling on hematopoiesis during ST infection specifically, as our results indicated that *Ifnar1*$^{-/-}$ mice were less susceptible to ST. We found that the HSCs (LSK counts) were reduced post infection in *Ifnar1*$^{-/-}$ and *Irf9*$^{-/-}$ mice in comparison to WT mice. Interestingly, ST-induced an increase in the number of MPs, while there was a very subtle increase in CMPs in *Ifnar1*$^{-/-}$ and *Irf9*$^{-/-}$ mice as compared to WT mice. Differentiation of CMPs to GMPs was also more biased in *Ifnar1*$^{-/-}$ and *Irf9*$^{-/-}$ mice resulting in an increase in GMP proportions. These results suggested that type-I IFN signaling inhibits the myelopoiesis of myeloid progenitors, which in turn restricts the availability of monocytes and macrophages in the periphery, which explains why there are increased numbers of macrophages in *Ifnar1*$^{-/-}$ mice **(Figure 8).**

The observed data correlated with previous studies wherein *Mtb* alters host cell proliferation (Cumming et al., 2017; Huang et al., 2018; Pisu et al., 2020), including modulation of bone marrow stem cell differentiation in a type-I IFN-dependent manner (Khan et al., 2020). Recent study performed by Khan and colleagues reported that *Mtb* infected *Ifnar1*$^{-/-}$ mice exhibited significant increase in CMPs and GMPs and no difference

in CLPs as compared to WT mice, suggesting that *Mtb* limits myelopoiesis and impairs trained immunity in a type-I IFN-dependent manner (Khan et al., 2020). Moreover, to further illustrate the specific role of type-1 IFN in hematopoiesis, they used a model of systemic type-1 IFN production through the intraperitoneal administration of the TLR3 synthetic viral ligand polyinosinic:polycytidylic acid (poly(I:C)). They found that similar to *Mtb*, poly(I:C) decreased the ST-HSC/MPPs and increased the CMPs while decreasing CLPs in *Ifnar1*$^{-/-}$ mice (Khan et al., 2020; Mitroulis et al., 2018). Overall, all effects observed were type-I IFN dependent, suggesting that type-I IFN signaling inhibits myelopoiesis.

Another study was also performed by Marieke A G Essers in 2009, to investigate whether long-term (chronic) activation signaling pathway leads to a decrease in HSC activity. In this study the mixed chimaeras of WT and *Ifnar1*$^{-/-}$ mice were treated with poly(I:C) and the number of phenotypic HSCs were determined. They suggested that there was a marked competitive disadvantage of HSCs expressing IFNAR1 (Essers, M.A et al.,2009). Overall, this study indicated that long-term activation of type I-IFN signaling pathway does not simply eliminate functional HSCs, but rather markedly compromises their proliferation and differentiation (Essers, M.A et al.,2009).

Above studies provided insight into the multiple signaling pathways that can play a key role in the regulation of HSCs following activation (Baldridge et al., 2010; Essers, M.A et al., 2009) and in the generation of innate memory responses by epigenetic modulation (Kaufmann et al.,2018). One such study suggested that memory like responses from myeloid cells (trained immunity) is driven by epigenetic imprinting in HSCs which can be transferred to BM macrophages (Kaufmann et al.,2018; Mitroulis et al.,2018) and this imprinting overcomes the limitation of short life span of innate immune cells that may limit the protective response against chronic infections (Kaufmann et al.,2018; Mitroulis et

al.,2018). We observed enhanced differentiation of CMPs to GMPs in the bone marrow of infected *Ifnar1-/-* and *Irf9-/-* mice as compared to WT mice. This resulted in a significant increase in GMP counts, and the LSK numbers were low, suggesting that there may be involvement of epigenetic modulation, which can be investigated in the future.

Cell death programs also play a critical role in HSC lineage outcomes (Kanayama et al., 2017). Cell death can be one of the key mechanisms by which HSC and progenitor populations promote lineage biasing. Studies have shown that type-1 IFN signaling induces cell death especially in myeloid progenitors. One such study suggested that tumor necrosis factor (TNF) promotes cell death in myeloid progenitors, and it prevents HSC necroptosis (Yamashita and Passegue, 2019). This indicated that there are safeguard mechanisms in HSCs that are absent in more committed myeloid progenitors. Thus, we investigated whether the increase in the number of macrophages in *Ifnar1*$^{-/-}$ mice is because of reduced cell death in the myeloid progenitors in these mice. Overall, a reduced degree of cell death was observed in myeloid progenitors particularly in CMPs of infected *Ifnar1*$^{-/-}$ and *Irf9*$^{-/-}$ mice (**Figure 9).** Nevertheless, it is important to note that *Ifnar1*$^{-/-}$ macrophages undergo reduced cell death by the non-canonical inflammasome and necrosome pathways; however alternate pathways of cell death can compensate and induce cell death in *Ifnar1*$^{-/-}$ mice. Additionally, our lab has previously reported that when the inflammasome and necrosome pathways of cell death are disabled this does not result in increased macrophage numbers in the spleen (Robinson et al.,2012). Taken together, these results suggest that an increased number of macrophages in *Ifnar1*$^{-/-}$ mice may not be attributable to the reduced cell death of myeloid progenitors of these mice.

4.3 Impact of type-I interferon signaling on macrophage function.

4.3.1 Metabolism in macrophages

Since metabolism plays a key role in survival and function of cells, it has been reported that macrophages rely on both mitochondrial oxidative phosphorylation and glycolysis pathways (O'Neill et al., 2016). When our body encounters an infection, it needs to generate energy quickly and hence macrophages rely preferentially on glycolysis for a quick source of energy (O'Neill et al., 2016). This is achieved by deviating the carbon in metabolic intermediates to macromolecular biosynthesis that leads to enhanced export of lactate, and the resulting glycolytic flux can be quantified by measuring (ECAR) extracellular acidification rate. On the other hand, macrophages tend to resort to mitochondrial oxidative phosphorylation (OXPHOS) for a slower but sustained energy supply in the form of ATP; this process involves transfer of electrons to Oxygen. Oxygen consumption rate (OCR) is thus measured to quantify flux through the electron transport chain (ETC) (O'Neill et al., 2016). Overall mitochondrial respiration is more efficient as it generates more energy.

Like any other cell type in the body, metabolism must operate efficiently. The connections between immunometabolic changes and proliferation in macrophages are often neglected (O'Neill and Pearce, 2016). It is very important for immune cells to have a robust mitochondrial phosphorylation and glycolysis as they play a key role in cell proliferation. Recent evidence indicates that, in innate immune cells, signaling occurs downstream of pattern-recognition receptors (PRRs) and cytokine receptors, which in turn affects metabolism and fate and function of the cell (O'Neill and Pearce, 2016).

Studies with purified bacterial components support the simplified model that macrophages maintain a balance between glycolysis and OXPHOS (i.e., when one increases, the other

decreases), but macrophage metabolism during infections with live pathogens displays much more complex patterns (Ayres, 2020; Eisenreich et al., 2019; Gleeson and Sheedy, 2016; Russell et al., 2019).

There have been contradictory reports about the role of type-1 interferon in metabolism. Some reports revealed that type-1 interferon promotes metabolism whereas other reports indicate that type-1 IFN dampens these metabolic pathways. A recent study was carried out by Olson and colleagues to establish the relationship between type-I IFN and macrophage metabolism in the context of *Mycobacterial* infection. They found that type-I IFN downregulates glycolysis and mitochondrial respiration in BMMs following infection with *Mtb* (Collins et al., 2015; Manzanillo et al., 2012; Wassermann et al., 2015; Watson et al., 2015). They revealed that upon measuring glycolytic capacity and mitochondrial health during *Mtb* infection, *Ifnar1*$^{-/-}$ BMMs showed increased glycolysis and maximal respiration (Cumming et al., 2018). Furthermore, they also confirmed that type-I IFN decreases macrophage metabolism in vivo during an aerosol infection with *Mtb*. They isolated alveolar macrophages (AMs) by bronchoalveolar lavage, they showed that infected *Ifnar1*$^{-/-}$ mice displayed significantly increased mitochondrial capacity compared to either *Ifnar1*$^{-/-}$ AMs from uninfected mice or WT AMs from infected mice (Olson et al., 2021).

Moreover, to further interrogate the signaling pathway that could explain the metabolic changes during infection, Olsen and colleagues also performed RNA seq in WT and *Ifnar1*$^{-/-}$ mice post infection with live *Mtb* at 24 hours. They observed that addition of IFN-β lead to significant downregulation of the genes related to metabolic response, especially related to the two key glycolytic enzymes - Glucose-6 phosphate isomerase (GPI) and Aldolase C (ALDOC) in *Ifnar1*$^{-/-}$ macrophages (Olson et al., 2021).

We investigated the impact of type-1 IFN signaling on glycolysis and oxidative phosphorylation in bone marrow-derived macrophages. Our results also suggested that there was an increased demand for ATP in the absence of type-1 IFN signaling. Moreover *Ifnar1*$^{-/-}$ and *Irf9*$^{-/-}$ macrophages displayed enhanced oxidative phosphorylation and glycolysis suggesting that type-1 IFN signaling downregulates metabolism in macrophages **(Figure 10).** Interestingly, our preliminary RNA Seq data performed in ST infected WT, *Ifnar1*$^{-/-}$ and *Irf9*$^{-/-}$macrophages also revealed that the expression of Aldolase C and GPI was upregulated in *Ifnar1*$^{-/-}$ and *Irf9*$^{-/-}$macrophages compared to WT (Data not shown).

Overall, our results correlated with the studies performed by Olson and colleagues and indicated that type-I IFN acts as a master regulator of macrophage metabolism which downregulates mitochondrial metabolism and glycolysis in macrophages. Future studies exploring the association of type-I IFN-mitochondrial function could contribute to pathogenesis across a range of diseases and understanding these dynamics might help in explaining how type-I IFN augments vaccination strategies.

4.3.2 Impact of type-I interferon signaling on the expression of inflammatory cytokines by macrophages

Metabolism has been known to play a key role in response to infection, including cytokine production (Galván-Peña and O'Neill, 2014). It has been reported that increased metabolism results in enhanced cytokine production (O'Neill et al., 2016).

Both immune and non-immune cells of the body recognize the structural motifs known as microbial pathogen associated molecular patterns (PAMPS) (Akira et al., 2006). Interaction of PRRs with PAMPs followed by downstream signaling leads to recruitment of immune cells at the site of infection, along with the secretion of inflammatory mediators called cytokines (Baskic et al.,2017). Excessive secretion of these inflammatory

cytokines can have detrimental effects to the host (Cavaillon,2001; Dinarello,2000). Timely resolution of this pro-inflammatory response is therefore very important. (Coondoo, 2011; Zhang and An, 2007). Therefore, IL-10 and transforming growth factor (TGF- β) acts as immunoregulatory molecules and suppress this response (Iyer and Cheng 2012; Moore et al., 2001).

We measured the expression of various pro- and anti- inflammatory cytokines by WT, *Ifnar1*$^{-/-}$ and *Irf9*$^{-/-}$ macrophages following infection with ST. We found that *Ifnar1*$^{-/-}$ and *Irf9*$^{-/-}$ macrophages secreted significantly reduced levels of the anti-inflammatory cytokine IL-10 and moderately reduced levels of the pro-inflammatory cytokine IL-12. However, the levels of TNF-α were slightly elevated in *Ifnar1*$^{-/-}$ macrophages compared to WT and *Irf9*$^{-/-}$ macrophages. Traditionally, it has been observed that a decrease in the levels of anti-inflammatory cytokine IL-10 causes a corresponding increase in the secretion of the pro-inflammatory cytokines to restore homeostasis (Iyer and Cheng, 2012; Moore et al., 2001). Studies with *Listeria monocytogenes* indicated that overexpression of IL-10, was directly correlated with susceptibility to infection, and inhibition of IL-10 lead to increased resistance to infection (Roque et al.,2007). Another study showed that IL-10$^{-/-}$ mice suffered from chronic enterocolitis and were characterized by increased levels of TNF-α, IL-12 and IL-6 (Kühn et al., 1993). However, we could not find a pro- or anti- inflammatory bias in *Ifnar1*$^{-/-}$ and *Irf9*$^{-/-}$ macrophages in terms of their cytokine production. Considering that the IL-10 levels were not drastically reduced in *Ifnar1*$^{-/-}$ macrophages, it is possible that the modulation of cytokines could be due to other mechanisms at play.

Additionally in our work we observed that the *Stat2*$^{-/-}$ macrophages displayed a cytokine expression pattern that was similar to *Irf9*$^{-/-}$ macrophages as there was reduced secretion of IL-10 in both *Irf9*$^{-/-}$ and *Stat2*$^{-/-}$ macrophages. As mentioned before *Stat1*$^{-/-}$ mice are highly susceptible to ST infection, and STAT1 plays a protective role against ST whereas *Stat2*$^{-/-}$

, *Irf9*$^{-/-}$ and *Ifnar1*$^{-/-}$ mice show enhanced resistance to ST infection. Interestingly, the cytokine secretion pattern of *Stat1*$^{-/-}$ macrophages was similar to *Stat2*$^{-/-}$ and *Irf9*$^{-/-}$ macrophages in a way that these macrophages secreted lower levels of IL-10, TNF-α and IL-12 but higher levels of IL-1β. Since the production of IL-10 was significantly reduced in *Stat1*$^{-/-}$ macrophages which is similar to that observed in *Ifnar1*$^{-/-}$ macrophages, but *Ifnar1*$^{-/-}$ mice is resistant whereas *Stat1*$^{-/-}$ mice are susceptible to infection. These results indicate that the enhanced resistance of *Ifnar1*$^{-/-}$ mice cannot be attributed to decreased IL-10 levels. Taken together, we concluded that type-1 IFN modulates cytokine expression by macrophages, but this may not be responsible for increased resistance of *Ifnar1*$^{-/-}$ mice.

4.4 Role of type-1 IFN signaling on the induction of cell death.

Engagement of inflammasomes, leads to induction of pyroptotic cell death, this has been shown to be mediated by activation of caspases, which then cleaves the pro- IL-1β and pro-IL-18 which leads to further release of pro- inflammatory cytokines (Broz et al.,2012b; Kayagaki et al.,2013).

Traditionally, cytokines that have the secretory signal at the N terminal of the nascent peptide are exported out of the cell, through the golgi apparatus (Galione, A.,2018). In comparison, the IL-1 family of cytokines is a unique family of cytokines, as IL-1β lacks the secretory signal peptide, and thus undergo “nonconventional” secretion, and must be processed through inflammasome signaling, that involves activation of caspase-1 (Lamkanfi and Dixit.,2014).

S. Typhimurium is a virulent pathogen that induces rapid cell death. It has been previously reported that *Ifnar1*$^{-/-}$ macrophages are less susceptible to *S.* Typhimurium induced cell death than WT macrophages (Robinson et al., 2012). Type-1 IFN signaling has been known to play diverse role in pathway that is responsible for induction of cell death in

macrophages (Guarda et al.,2011; Henry et al.,2007). Interplay between inflammasome signaling and type-1 IFN signaling were discussed in studies involving infection with gram-negative *Francisella tularensis,* it was suggested that type-1 IFN positively regulates inflammasome signaling during infection with *Francisella tularensis* (Henry et al.,2007). Our results also indicate that *Ifnar1*$^{-/-}$ and *Irf9*$^{-/-}$ macrophages undergo slightly reduced cell death as compared to WT macrophages **(Figure 17).** However, we observed that the secretion of pro-inflammatory cytokine IL-1β by *Ifnar1*$^{-/-}$ and *Irf9*$^{-/-}$ macrophages was increased in comparison to WT cells **(Figure 12A)**. As mentioned before the CFU is high in *Stat1*$^{-/-}$ mice and the levels of IL1β are also higher than WT. This suggests that the decreased CFU observed in *Ifnar1*$^{-/-}$ mice cannot be attributed to increased IL1β levels. Overall, our results reveal the opposing roles of type-I IFN signaling on key outcomes of inflammasome signaling; cell death and IL-1β secretion. This raised our curiosity about what mechanism may be at play that could explain increased secretion of IL1β by *Ifnar1*$^{-/-}$ and *Irf9*$^{-/-}$ macrophages despite reduced cell death. Perhaps, this is related to an autophagy-based unconventional secretion mechanism in the golgi apparatus that promotes secretion of the proteins that cannot enter the conventional export pathway due to the absence of secretory signals that would bring them into the ER/golgi secretory pathway (Davis et al, 2011). It is conceivable that this mechanism may be modulated by IFNAR1 signaling, which can be investigated in the future.

Salmonella expresses several virulence genes that are essential for modulating host immune responses. The major virulence genes of *Salmonella* are clustered into two main *Salmonella* pathogenicity islands (SPI) SPI-1 and SPI-II (Abrahams and Hensel, 2006). The impact of SPI-1 and SP-II on cell death and cytokine expression pattern was evaluated in this thesis. SPI-I promotes invasion into epithelial cells and SPI-II promotes intracellular replication of ST in all cell types (Sad et al., 2008). Inactivating mutation in *invA* gene of

ST results in a dysfunctional SPI-I T3SS, which results in a failure to infect epithelial cells and lack of infection of hosts following an oral challenge. In contrast to SPI-I, SPI-II genes promote bacterial survival and replication within infected cells (Abrahams and Hensel, 2006). Upon evaluating the impact of SPI-I and SPI-II on cytokine expression by macrophages, our results indicate that the cytokine expression by macrophages is not influenced by the SPI-I and SPI-II genes the secretion of IL1β was also not dependent on SPI-I or SPI-II. Our results revealed that the cell death of macrophages is mainly dependent on the SPI-I virulence apparatus. Interestingly, our results revealed that SPI-II also promotes cell death of macrophages, as the cell death induced by the Δ*ssaR* strain of ST was undetectable in *Ifnar1*$^{-/-}$ and *Irf9*$^{-/-}$ macrophages. Overall, these results suggest that type-I IFN signaling promotes both SPI-I and SPI-II dependent cell death. IL1β secretion was not dependent on SPI-I or SPI-II whereas SPI-I and SPI-II promoted cell death of macrophages. Altogether these results suggest that IL1β secretion is independent of cell death in our model.

5. CONCLUSION

This thesis provides insights into different mechanisms opted by type-1 interferons in the pathogenesis of *Salmonella* Typhimurium. The results obtained correlated with previous work done in our lab that *Ifnar1*$^{-/-}$ mice display better survival and lower bacterial burden post infection in comparison to WT. This suggests that type-1 IFN signaling is detrimental to host survival, which results in poor control of ST. More importantly results presented indicate that the downstream transcription factors of IFNAR1 signaling do not have similar roles. So, it was of utmost importance to study the mechanism opted by various components of type-1 IFN signaling. It was observed that STAT1 promotes protective response against ST whereas the downstream transcription factors, STAT2 and IRF9 promotes susceptibility against ST Further studies done in our two double knockouts (*Stat1*$^{-/-}$*Irf9*$^{-/-}$ versus *Ifng*$^{-/-}$ *Irf9*$^{-/-}$) mice to find out whether the protective role of STAT1 was through type-I or type-II IFN pathway revealed that double deficiency of *Stat1* and *Irf9* resulted in a very high bacterial burden following challenge with ST which is much higher than WT, whereas abrogation of IFNγ in *Irf9*$^{-/-}$ mice resulted in escalation of the bacterial burden to WT levels. Hence, based on these results we concluded that STAT1 signaling through IFNAR1 pathway mediates a protective response against ST and this is antagonized by STAT2 and IRF9**.**

Interestingly, my work revealed that type-1 IFN signaling impairs the control of ST early time point (day 3 post infection) this suggested that it is indeed modulation of innate immunity that is responsible for enhanced resistance of *Ifnar1*$^{-/-}$ mice. My results suggested that deletion of monocyte/macrophage lineage resulted in abrogation of resistance in *Ifnar1*$^{-/-}$ mice. Therefore, it was concluded that monocyte-macrophage lineage is responsible for conferring the enhanced resistance against ST. I also observed that type-1 IFN signaling inhibited the myelopoiesis of myeloid progenitors, which restricted the availability of monocytes and macrophages in the periphery, thereby explaining why the number of macrophages are increased in these mice. I also observed a reduced degree of cell death in myeloid progenitors, particularly in CMPs of infected *Ifnar1*$^{-/-}$ and *Irf9*$^{-/-}$ mice. However, alternate pathways of cell death can compensate and induce cell death in *Ifnar1*$^{-/-}$ mice, therefore, it was concluded that enhanced number of macrophages in *Ifnar1*$^{-/-}$ mice cannot be attributable to the reduced cell death of myeloid progenitors of these mice. Overall, I concluded that the increased number of monocytes/macrophages promote heightened resistance of *Ifnar1*$^{-/-}$ mice against a challenge with ST.

The next aim was to evaluate the various protective functions of macrophages that promote control of intracellular bacteria. Firstly, I investigated the impact of type-1 IFN signaling on glycolysis and oxidative phosphorylation in bone marrow-derived macrophages of WT, *Ifnar1*$^{-/-}$and *Irf9*$^{-/-}$mice post infection with ST. I observed that there was an increased demand for ATP in the absence of type-I IFN signalling. The glycolysis and oxidative phosphorylation in *Ifnar1*$^{-/-}$and *Irf9*$^{-/-}$ macrophages was enhanced in comparison to WT cells. Overall, these results suggested that type-I IFN acts as a master regulator of macrophage metabolism which downregulates mitochondrial metabolism and glycolysis in macrophages. It has been previously reported that metabolism also enhances the secretion of IL-1β and other inflammatory cytokines which are protective against bacterial infection, therefore conferring that *Ifnar1*$^{-/-}$and *Irf9*$^{-/-}$mice can fight infection better**.**

Secondly, to evaluate the impact of type-I IFN signaling on the expression of inflammatory cytokines by macrophages, I measured the expression of various pro- and anti-inflammatory cytokines by WT, *Ifnar1*$^{-/-}$ and *Irf9*$^{-/-}$ macrophages following infection with ST. However, a clear-cut pattern of a pro- or anti- inflammatory bias in *Ifnar1*$^{-/-}$ and *Irf9*$^{-/-}$ macrophages was not detected. Taken together, these results suggest that while type-1 IFN modulates cytokine expression by macrophages this may not be responsible for increased resistance of *Ifnar1*$^{-/-}$ mice. Moreover, analysis of cytokine expression pattern of double knockouts revealed that *Ifng*$^{-/-}$*Irf9*$^{-/-}$ secreted significantly higher levels of pro-inflammatory cytokines such IL-1β, TNF-α and IL-12 than *Stat1*$^{-/-}$*Irf9*$^{-/-}$ macrophages which corelated with the lower bacterial burden in these mice. In conclusion, the role of STAT2 in promoting cytokine expression through ISGF3 is minimal and STAT1 plays key role in promoting the secretion of pro-inflammatory cytokines which correlated with enhanced bacterial control**.**

Interestingly, my results also indicate that *Ifnar1*$^{-/-}$ and *Irf9*$^{-/-}$ macrophages undergo slightly reduced cell death but express increased levels of IL-1β. This suggested that type-I IFN has opposing roles on the two key outcomes of inflammasome signaling, cell death and IL-1β secretion. Experiments involving Δ*invA* and Δ*ssaR* strains of ST also indicated that the cytokine expression by macrophages is not influenced by the SPI-I and SPI-II genes, and secretion of IL1β was not dependent on SPI-I or SPI-II. Since the induction of cell death in *Ifnar1*$^{-/-}$ and *Irf9*$^{-/-}$ macrophages was both SP-I and SPI-II dependent, these results suggest that IL-1β may be secreted through the "secretory autophagy" pathway that maybe

modulated by IFNAR1 signaling. Understanding this modulation can be a part of future investigation.

Collectively, these results highlight the role of type-I IFN signaling in modulating innate immune response to *Salmonella* infection. We revealed that IFNAR1-deficient monocytes/macrophages promote protection against ST. Type-I IFN plays a key role in the regulation of different cellular processes such as myelopoiesis, mitochondrial respiration and glycolysis. I found that the downstream transcription factors of IFNAR1 signaling do not have similar roles. STAT1 through IFNAR1 signaling, promotes enhanced expression of pro-inflammatory cytokines and protection against ST whereas STAT2 and IRF9 through IFNAR1 signaling antagonized this protection and inhibited the protective regulatory mechanisms of macrophages such as myelopoiesis, mitochondrial respiration and glycolysis **(Figure 19).**

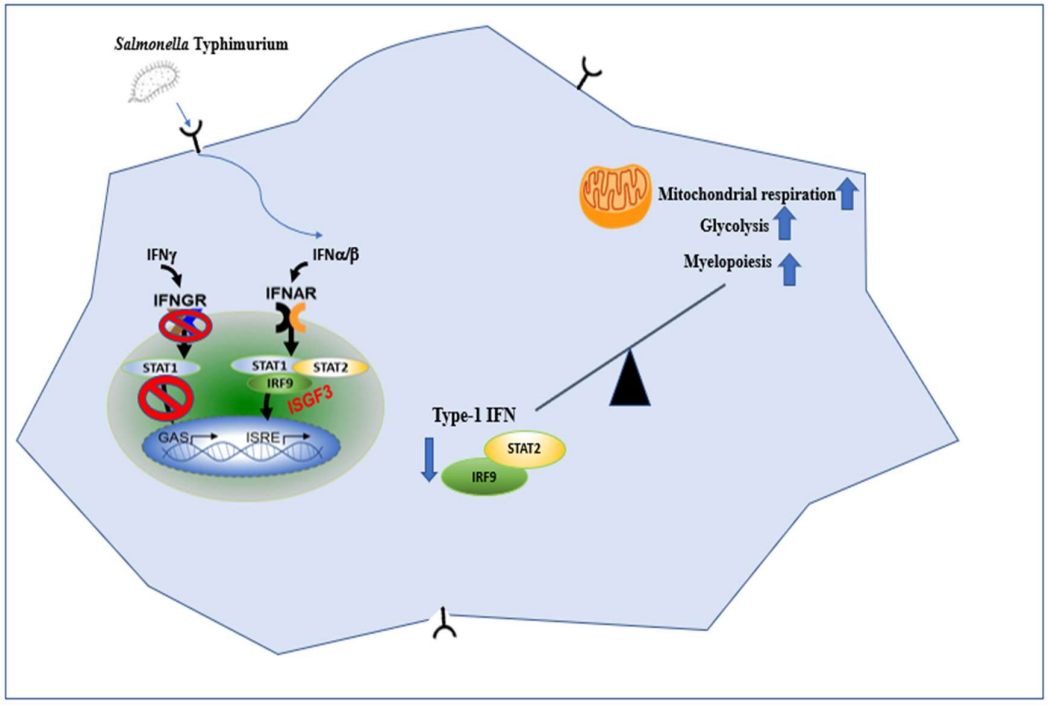
Salmonella Typhimurium
IFNγ
IFNα/β
IFNGR
IFNAR
STAT1
STAT1
STAT2
IRF9
ISGF3
GAS
ISRE
Mitochondrial respiration
Glycolysis
Myelopoiesis
Type-1 IFN
STAT2
IRF9

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Figure-19. Model depicting the role of type-1 IFN signalling in the pathogenesis of *Salmonella* Typhimurium.

Model represents that monocyte-macrophage lineage is responsible for the control of ST. Type-I IFN signaling through STAT1 promotes protection against ST, whereas type-I IFN signaling through STAT2 and IRF9 antagonize this protection, suggesting that, the three transcription factors downstream of IFNAR1 have different impacts in mounting an innate immune response against ST. Moreover, STAT2 and IRF9 through type-1 IFN signaling inhibits myelopoiesis, mitochondrial respiration and glycolysis, hence compromising the protective functions of macrophages.

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CURRICULUM VITAE

PRIYA VERMA

Educational experience:

1. **Graduate Student, MSc (Immunology)**
   2020-2022
   University of Ottawa, Ottawa Canada

   Thesis Title- Evaluation of the role of type-1 interferon signaling in the pathogenesis of *Salmonella* Typhimurium

   Expertise: Innate immune responses, *Salmonella* pathogenesis, Macrophage function, Hematopoiesis, Metabolism

   Lab skills: Trained in performing various lab skills such as -Western Blotting, Biochemical assays such as ELISA, Bradford assay, cell death assays, handling mice, performing in vivo experiments with infected mice, Flow cytometry, Cell culture techniques, Seahorse assay.

   Certifications: Workplace Hazardous Materials information System (WHIMIS), Respect in the workplace, Principles of Biosafety, Lab safety and autoclave safety, Animal Safety certified.

2. **Post Graduate Diploma- Quality Assurance & Regulatory Affairs**
   2016-2017
   AAPS (Academy of Applied and Pharmaceutical Science Inc.) Toronto, ON

   Expertise and skills:

   - Demonstrate good documentation practices as per GMP/cGMP
   - Experienced in writing 20 standard operating procedure including instrument calibration
   - Trained on conducting self-inspection, internal audit and preparation for FDA audits
   - Prepared and presented various presentation on ICH, GMP, 21CFR and quality management
   - Conducted routine wet chemistry tests for pharmaceutical product analysis
   - Performed test on USP dosage units to determine hardness, friability, viscosity, uniformity of dosage units and limit tests to identify the heavy metals and inorganic impurities content

- Performed dissolution tests for dissolution profile of USP for immediate/delayed release tablets
- Prepared various reagents and solutions including TS (test solution), VS (Volumetric solution), HPLC mobile phase and dissolution media

Work experience:

1. **Derma Sciences Ltd**
   (2017)
   Toronto, ON

   - Demonstrate good GMP Skills
   - Packaging of Total Contact Casting system following GMP.
   - Well Trained on proper gowning as per Health and Safety Guidelines

2. **Symbiotech Pharma lab Ltd**.
   (2011-2012)
   Indore, India

   - Good Knowledge about pre-clinical and clinical drug categories
   - Excellent knowledge in the field of Regulatory Affairs – Natural Health Products, Biologics/Biotechnology Products and Generic Drugs.
   - Practiced submission of Class 2 Application for Medical Devices.
   - Wrote and reviewed OOS, deviation reports, change control and CAPAs
   - Wrote 20 SOPs for quality assurance – Drug and Food industries.
   - Excellent understanding of ICH part 210& 211, FDA, USFDA, 21 CFR and Health Canada guidelines
   - Practiced laboratory safety and WHMIS certified
   - Prepared NDA &ANDA document for submission.
   - Well Trained on proper gowning as per Health and Safety Guidelines

Academic qualification:

1. **Master of Science in Microbiology and Immunology** – BMI, Faculty of medicine, University of Ottawa.
2. **Postgraduate Diploma in Quality Assurance and Regulatory Affairs in Food, Pharma cosmetics -** Academy of Applied Pharmaceutical Science, Toronto.

3. **Bachelor of Pharmacy -** RGPV university, India.

Skills:

1. Time management and strong interpersonal skills.
2. Excellence in computer skills -MS Office, MS-Word and MS-Excel, along with strong presentation skills.
3. Well versed with research based softwares such as GraphPadPrism, WAVE software for seahorse assay, FlowJo, Image J and SoftMax Pro.

Community and voluntary services:

1. Involved in volunteer work with Blood Donation camps.
2. Took active participation in pharma fest and pharma week as a senior volunteer.
3. Secured second prize from university for academic proficiency in Bachelor of Pharmacy.
4. Secured outstanding performance certificate in high school.

Extracurricular Interests:

1. Spiritual speaker.
2. Passionate about cooking.
3. Tarot Card Reading.