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INDUCTION OF ANTI-MICROBIAL IMMUNE RESPONSES IN SEVERELY IMMUNODEFICIENT HOSTS BY IFNβ-EXPRESSING SALMONELLA ENTERICA SEROVAR TYPHIMURIUM CORRELATES WITH EFFICIENT ACTIVATION OF MACROPHAGE EFFECTORS

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United Arab Emirates University

College of Medicine and Health Sciences

Department of Microbiology

INDUCTION OF ANTI-MICROBIAL IMMUNE RESPONSES IN SEVERELY IMMUNODEFICIENT HOSTS BY IFNγ-EXPRESSING *SALMONELLA ENTERICA* SEROVAR TYPHIMURIUM CORRELATES WITH EFFICIENT ACTIVATION OF MACROPHAGE EFFECTORS

Mohammed Achraf Al-Sbiei

This thesis is submitted in partial fulfilment of the requirements for the degree of Master of Medical Sciences (Microbiology & Immunology)

Under the Supervision of Professor Basel K. Al-Ramadi

April 2015

Declaration of Original Work

I, Mohammed Achraf Al-Sbiei, the undersigned, a graduate student at the United Arab Emirates University (UAEU), and the author of this thesis entitled "*Induction of anti-microbial immune responses in severely immunodeficient hosts by IFN-γ-expressing Salmonella enterica serovar typhimurium correlates with efficient activation of macrophage effectors*", hereby, solemnly declare that this thesis is an original research work that has been done and prepared by me under the supervision of Professor Basel K. al-Ramadi, in the College of College of Medicine and health Sciences at UAEU. This work has not been previously formed as the basis for the award of any academic degree, diploma or a similar title at this or any other university. The materials borrowed from other sources and included in my thesis have been properly cited and acknowledged.

Student's Signature_____ Date _____

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Abstract

Susceptibility to infection by the intracellular bacterial pathogen, *Salmonella enterica* serovar Typhimurium (*S. typhimurium*), is controlled by many genes of innate and adaptive immunity. One of the most critical genes is *IFN-γ* and mice deficient in IFN-γ synthesis are highly susceptible to Salmonella. Previously, we demonstrated that mice deficient in MyD88, an adaptor that regulates TLR signaling, are susceptible to Salmonella infection. In the current study, we compared immune responses in mice deficient in IFN-γ or MyD88 with wild-type controls following infection with an attenuated strain of *S. typhimurium* (designated BRD509E) or a recombinant derivative engineered to express murine IFN-γ (GIDIFN). Infection studies with BRD509E or GIDIFN revealed that the latter strain was significantly less virulent in immunodeficient mice than BRD509E and correlated with decreased bacterial loads in systemic organs. Enhanced responsiveness was due to GIDIFN strain's ability to activate effector macrophages, as shown by increased synthesis of inflammatory cytokines and anti-microbial effector molecules, including NO. Gene expression profiling by qPCR demonstrated stronger induction of key inflammatory modulators by GIDIFN in macrophages of immunodeficient animals. These findings suggest that immunotherapeutic approaches using attenuated bacterial strains expressing immunomodulatory genes is more efficacious and offers a superior safety profile even in severely immunodeficient hosts.

Keywords:

IFNγ; Innate immunity; *Salmonella* infection.

Title and Abstract (in Arabic)

تحريض الإستجابات المناعية المضادة للميكروبات في نموذج حيواني منقوص المناعة, عند تعرضه لبكتيريا السالمونيلا المنتجة للإنتفيرون جاما (IFN-γ). هذه الإستجابة مرتبطة بتفعيل كفاءة الخلايا البلعومية (Macrophages) .

قابلية الإصابة من قبل بكتيريا السالمونيلا التيفية الفأرية (*S. typhimurium*)،تعتمد على العديد من جينات المناعة الفطرية والتكيفية. واحد من الجينات الأكثر أهمية هو IFN-γ والفئران الغير قابلة لانتاج IFN-γ هم عرضة للإصابة ببكتيريا السالمونيلا. في السابق أثبتنا أن الفئران منقوصة MyD88، و هو محول ينظم إشارات مستقبل الخلية (TLR)، هم أيضا معرضون للإصابة بنفس الجرثومة. في الدراسة الحالية قارنا الاستجابات المناعية في الفئران ذوات نقص في IFN-γ أو MyD88 مع نوع مضعف من سلالة بكتيريا السالمونيلا التيفية الفأرية (تسمى BRD509)أو نوع آخر مشتق من السلالة السابقة مدعمة جينيا لإنتاج IFN-γ (تسمى GIDIFN).و كشفت النتائج أن إصابة الفئران ذوات النقص في IFN-γ بالبكتيريا القابلة لانتاج IFN-γ كانت أقل ضررا من النوع الآخر، و قد كان مرتبطا بانخفاض في أعداد البكتيريا المتواجدة في الأعضاء الداخلية للفئران. وقد ربطت هذه النتائج بسبب قدرة سلالة GIDIFN على تفعيل الخلايا البلعومية (Macrophages) من خلال زيادة إفراز افرازالسيتوكينات الالتهابية (Inflammatory cytokines) والجزيئات المضادة للميكروبات، بما في ذلك النيتريك أوكسيد (NO). تشير دراسة التعبير الجيني (qPCR) أثبت أن الخلايا البلعومية (Macrophages) لديها إستجابة أقوى لبكتيريا- GIDIFN من خلال تحويل نشاط هذه الخلايا بصورة أقوى إلى تفاعل مضاد للجراثيم في الفئران ذوات النقص في الجهاز المناعي.

وتشير هذه النتائج إلى أن النهج باستخدام هذه السلالة من البكتيريا القابلة على تغيير نمط الإستجابة المناعية للمرضى الذين يعانون من نقص في الجهاز المناعي تعطي نتائج أكثر كفاءة و سلامة.

الكلمات المفتاحية:

الإنتفيرون جاما، المناعة الفطرية، مرض السالمونيلا

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I would like to thank my committee for their guidance, support and assistance throughout my preparation of this thesis, especially Dr. Eyad Elkord. My special thanks goes to my lab colleagues particularly Mr. Yassir for helping me in performing most of the experiments done in this work. In addition I would like to thank all my friends in the college of medicine who restored my motivation to finish this work whenever I was down.

Special thanks go to my parents, brothers, and sister who helped me along the way.

Dedication

To my beloved parents and family

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List of Abbreviations

Ab	Antibody
ADCC	Antibody-Dependent Cellular Cytotoxicity
Ag	Antigen
APC	Antigen-Presenting Cell
BCG	Bacillus Calmette Guerin
BSA	Bovine Serum Albumin
CD#	Cluster of Differentiation
CFU	Colony Forming Unit
CpG	Oligodeoxynucleotides Carrying Unmethylated CpG Motifs
DNA	Deoxyribonucleic Acid
FSC	Forward Scatter
i.p.	Intraperitoneal
IFN	Interferon
IL	Interleukin
iNOS	Inducible Nitric Oxide Synthase
IRAK	IL-1 Receptor-Associated Kinase
LPS	Lipopolysaccharide
M cell	Microfold Cell
mAb	Monoclonal Antibody
mg	Milligram
MHC	Major Histocompatibility Complex

ml	Milliliter
MyD88	Myeloid Differentiation Protein-88
NK	Natural Killer
NLR	NOD-Like Receptor
NO	Nitric Oxide
Nramp1	Natural Resistance Associated Macrophage Protein 1
NTS	Non-Typhoidal Salmonellosis
PAMP	Pathogen Associated Molecular Pattern
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PRR	Pattern Recognition Receptor
RNA	Ribonucleic Acid
SCV	Salmonella-Containing Vacuole
SEM	Standard Errors of Mean
SSC	Side Scatter
STAT	Signal Transducer and Activator of Transcription
T3SS	Type-3 Secretory System
Th1	T helper Cell Type 1
TLR	Toll-Like Receptor
TNF	Tumor Necrosis Factor
TRAF	Tumor Necrosis Factor Receptor-Associated Factor
WT	Wild-Type

Chapter 1: Introduction

Salmonella is an intra-macrophage pathogen that is transmitted through the oral route, causing a localized gastroenteritis, or a systemic typhoid fever or bacteremia. Salmonella colonizes the macrophages, and replicates inside the so-called Salmonella containing vacuole (SCV). This capacity is regulated by the Salmonella pathogenicity island (SPI), an important virulence factor. Immunity to Salmonella is dependent on IFN-γ, a cytokine with powerful macrophage-activating properties that acts to induce their microbicidal potential. Moreover, IFN-γ functions to shift the antibody response to Th1-mediated isotypes, which includes IgG2a and IgG3, which are critical for enhanced bacterial opsonization and phagocytosis as well as antibody-dependent cellular cytotoxicity (ADCC). Proper development of a Th1 cell-mediated immune response and production of appropriate antibodies are required for the efficient control of primary Salmonella infections.

1.1. Classification of Salmonella

Salmonella are facultative intracellular bacteria that belong to the Enterobacteriaceae family, a group that contains other gram-negative, medically important bacteria like Shigella and *E. coli*. The Salmonella's genus consists of two species, *S. enterica* and *S. bongori*, the latter being an opportunistic pathogen and restricted to cold blooded animals (Euzéby, 1999), making the other genus (*S. enterica*) of most interest in the study of host-parasite interactions.

S. enterica is divided into six subspecies and is further classified to serogroups based on their somatic (O) and flagellar (H) antigens (Costa et al., 2012). These subspecies are genetically similar but can be classified according to their host

tropism and whether they cause systemic or local infections. *S. enterica* serovar Typhi is a human pathogen that results in systemic infection (typhoid fever) and does not naturally, infect any other mammals. *S. enterica* serovar Typhimurium, causes a systemic infection (typhoid like disease) in mice, and a localized gastroenteritis in humans (Parry et al., 2002). Other serovars of these subspecies are also host-restricted, such as *S. Dublin* in bovine and *S. Cholerasuis* in swine (Costa et al., 2012).

Salmonella-mediated infections have a global impact. In developed nations, gastroenteritis caused by Non-Typhoidal Salmonella (NTS) is a major cause of mortality due to contaminated food, with food outbreaks having a significant impact on the healthcare system and economy of affected countries (Nyachuba, 2010). On the other hand, Salmonella has a greater impact on the developing nations of the globe. Salmonella Typhi and Paratyphi strains are transmitted from human to human due to lack of proper sanitation system and poor hygiene, resulting in systemic infections with high mortality rates (Parry et al., 2002). Moreover, in immune-deficient individuals (such as HIV patients), infections by NTS are associated with high death rates due to the rapid and systemic dissemination of the pathogens (Feasey et al., 2012).

1.2. Salmonella pathogenesis

Studying the pathogenesis of Salmonella requires the availability of an animal model that mimics the interactions occurring in humans. *S.* Typhi and *S.* Paratyphi are both human-restricted serovars that cause a systemic infection, called typhoid fever. These serovars are not pathogenic in animals; instead serovar *S. typimurium* is capable of infecting mice, causing an invasive systemic typhoid-like

disease, making it the most widely accepted model of typhoid fever in experimental animals (Hormaeche, 1979).

Salmonella bacteria encode many virulence factors that facilitate their efficient invasion in host tissue. Being a water-borne pathogen, the primary mode of transmission is through the oral route. After colonizing the lumen of the intestine **(Fig. 1)**, the bacteria start producing molecules that are injected to the epithelia lining of the mucosa in a needle like projection called type III secretion system (T3SS). This invasion machinery is regulated by genes located in a genetic locus called Salmonella pathogenicity island 1 (SPI-1). It has been shown that mutations in SPI-1 render Salmonella non-virulent (Penheiter et al., 1997). Consequent to the invasion, *Salmonella* organisms colonize host macrophages and use them for intracellular replication as well as for systemic dissemination to target organs. This phase of infection is regulated by a set of proteins encoded on SPI-2 (Salmonella pathogenicity island 2) (Salcedo et al., 2001). Upon entry into host macrophages, Salmonella organisms are taken up into a phagosome that fuses with the lysosome to form a phagolysosome, enabling the lysis of the invading bacteria (Chakravortty et al., 2002). Host macrophages also play an important role as antigen-presenting cells, displaying Salmonella-derived peptides with MHC class II molecules for recognition by $CD4^+$ T cells, thereby initiating the adaptive immune response (Mittrücker et al., 2002).

In case of Salmonella, induction of the SPI-2 encoded type III secretion system prevents the fusion of the lysosome with the phagosome, thus acting as a critical virulence factor (Cirillo et al. 1998; Pfeifer et al. 1999). The action of SPI-2-encoded proteins transforms the phagosome into a hospitable environment, named

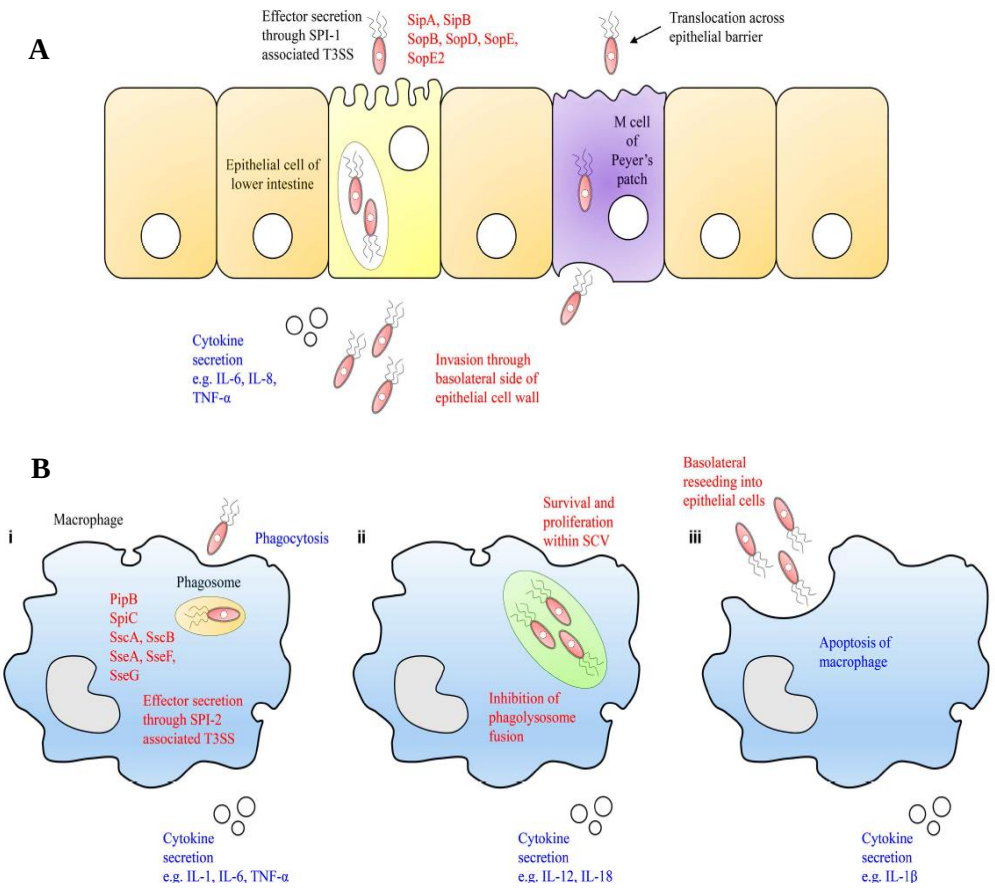

Figure 1: Salmonella pathogenesis.

Schematic illustration of the infection of epithelial cells of the lower intestine and macrophages by Salmonella is shown (A) Salmonella then translocate across M cells of Peyer's patches or actively invade epithelial cells by the secretion of effector proteins through the SPI-1 encoded T3SS-1.(B) (i) After crossing the epithelial barrier, Salmonella are engulfed by proximal macrophages that will secrete effector proteins into the cytosol of the cell via the SPI-2 encoded T3SS-2 and prevent fusion of the phagosome with the lysosome. (ii)With in the SCV, Salmonella will proliferate resulting in cytokine secretion by the macrophage. (iii) Finally, the macrophage will undergo apoptosis, and Salmonella will escape the cell to basolaterally reinvade epithelial cells or other phagocytic cells of the host innate immune system. (Reference: Hurley et al., 2014).

Salmonella-containing vacuole (SCV), which allows bacterial replication and growth (Lefebre & Galán et al., 2014). Recently, another study challenged these earlier findings by demonstrating that high bacterial loads can be achieved within phagocytic cells by SPI-2 mutant bacterial strains (Grant et al., 2012). Instead, the new data favor a model whereby SPI-2 T3SS confers the ability on the bacteria to

exit the infected macrophage and establish new foci of infection, thereby aiding in bacterial dissemination. The induction of SPI-2 effectors during the acute phase of the infection is a critical determinant of Salmonella virulence and its capacity to spread systemically into deeper host tissues. Salmonella strains carrying mutations in SPI-2 fail to cause a systemic infection (Penheiter et al., 1997).

1.3. Innate immunity to Salmonella infection

Salmonella infection occurs through the ingestion of contaminated food/water, in which Salmonella survive the stomach acidity and colonizes the intestine (Muller et al., 2009). Invasion of the intestinal epithelium is facilitated through specialized cells called M cells (Microfold cells) which functions by sampling the antigenic contents of the gut (Halle et al., 2007) This process is dependent on the SPI-1 which encodes several proteins that are injected through a needle-like complex called T3SS (type 3 secretory system). These proteins hijack the cell machinery in order to facilitate the bacterial invasion process (Jones et al., 1994; Penheiter et al., 1997). Once the bacterium reaches the lymphoid cells in Peyer's patches (PP), it gets engulfed by the phagocytes located there (Hopkins et al., 2000; Wick, 2002). Meanwhile, macrophage containing bacteria travel through the body's lymphatic system to the mesenteric lymph node (MLN) and then the bacteria at that stage starts spreading systemically to the spleen and liver (Mittrücker & Kaufmann et al., 2000).

Control of the salmonella infection is mediated by the host's different components of the innate immune system components, which must prevent the systemic spread of bacteria **(Fig. 2)**. Early recognition of Salmonella bacteria is mediated by the TLRs (Toll like receptors), a family of pattern recognition receptors

(PRRs), that recognize conserved components of Salmonella called pathogen associated molecular patterns (PAMP). To date, four TLRs have been shown to be involved in the response to Salmonella infection, including TLR2, TLR4, TLR5 and TLR9 which mediate the recognition of lipoproteins and bacterial amyloid fibers, lipopolysaccharide, flagellin, and CpG DNA, respectively (Takeuchi et al., 1999; Gewirtz et al., 2001; Feuillet et al., 2006; O'Brien et al., 1980; Vazquez-Torres et al., 2004; Hayashi et al., 2001; Tükel et al, 2009). Additionally, a different family of intracellular receptors, called NOD-like receptors (NLRs), mediates the recognition of Salmonella virulence protein products (Miao et al., 2010). Recognition of multiple bacterial products via PRRs results in the production of proinflammatory cytokines and chemokines, which, in turn, leads to the recruitment of neutrophils and monocytes to the site of recognition (intestinal lymphoid tissue). Migration of these cells forms an inflammatory foci, and results in increasing the level of TNF-α, IL-1β and nitric oxide that will arrest bacterial spread (Rydström & Wick, 2007). The inflammatory foci will lead to the production of other inflammatory cytokines such as IL-6, IL-12, IL-18 and IL-23 (Thiennimitr et al., 2012). That will have an impact on lymphocytes and NK cells, inducing the production of IFN-γ. IFN-γ activates the microbicidal activity of the phagocyte-containing bacteria, as well as the activation of a $CD4^+$ Th1 immune response that contributes to the complete clearance of the bacterium, and provides the host with protection against secondary infection through the adaptive immune response (Hormaeche et al., 1990).

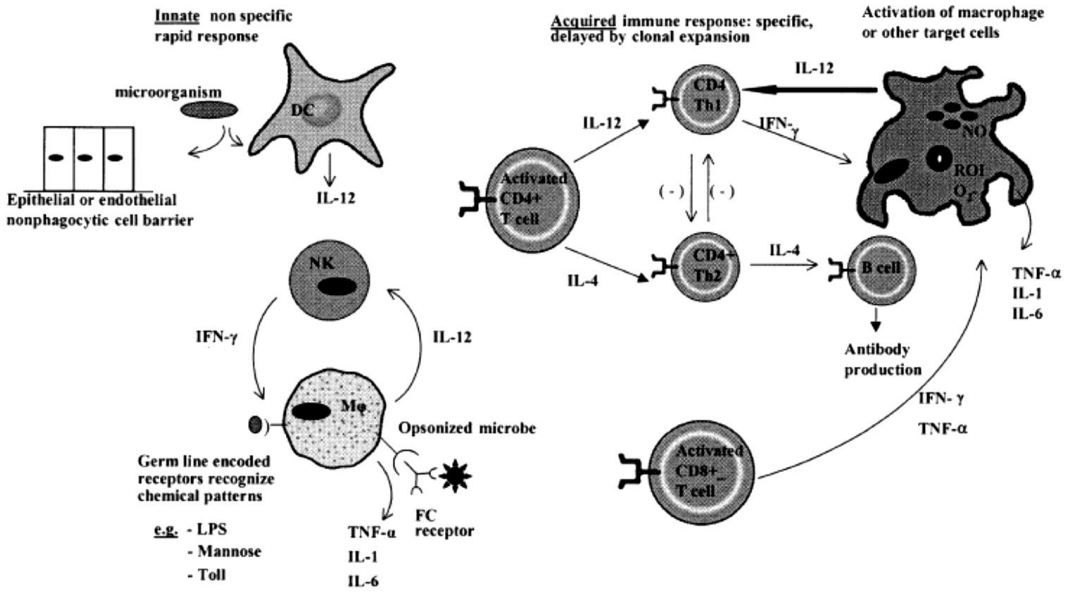

Figure 2: Innate immunity to intracellular pathogens.

A schematic presentation of the innate immune response, $CD4^+$ T cell differentiation and function of major regulatory cytokines generated in response to infection with intracellular pathogens. Effector Th1 $CD4^+$ T cells, $CD8^+$ T cells, and NK cells activate the phagocytes, through IFN-γ production, to kill the intracellular microbes via generation of toxic products such as NO and ROIs. (Reference: Ismail et al., 2002).

1.4. Animal models for Salmonella infection

Studying the mechanisms of a particular pathogen requires the availability of model that mimics the events occurring in humans. As previously stated, oral inoculation of *S. typhimurium* causes a systemic infection in mice similar to typhoid fever in humans, making it a suitable typhoid fever model (Hormaeche, 1979) . The genetic makeup of the host is decisive in determining the susceptibility to infection. By gene targeting several mouse models have been developed to study the role of different genes in Salmonella infection. Using a virulent strain of bacteria in a susceptible mouse background, leads to early mortality. In contrast, the use of attenuated strains of Salmonella allows one to dissect the complex interplay between

pathogen and host. The derivation of Salmonella strains carrying mutations in various genes of the metabolic pathway has greatly facilitated work on defining the contribution of various host genes in Salmonella infection. The best characterized Salmonella mutants have genetic mutations in genes coding for enzymes involved in the biosynthetic pathway of aromatic amino acids, such as aminobenzoic acid and dihydroxybenzoic acid. As these aromatic compounds are not normally produced in mammalian hosts, the use of bacterial strains with these genetic mutations render them avirulent (Hoiseth & Stocker 1981).

1.5. Susceptibility factors to salmonella infection

Immunity to Salmonella infection requires the synergistic effect of multiple components of the immune system. Defects in specific immune system pathways increase the host's susceptibility to Salmonella infection. The use of attenuated Salmonella strains and genetically defined animal models have greatly facilitated work on dissecting the contribution of different components of the immune system. In this section, the role of the most important contributing factors in Salmonella infection will be discussed.

1.5.1. Nramp1

Studies have identified a gene that controls bacterial replication, as an innate resistance trait for Salmonella infection, called Nramp1 gene (Natural resistance associated macrophage protein1) (Vidal et al., 1995). The Nramp1 gene encodes a transmembrane lysosomal protein that acts as a channel for divalent cations and controls intracellular microbial replication during the early phase of infection. The Nramp1-associated resistance to infection is due to an almost complete inhibition of

early bacterial growth in phagocytic cells (Plant & Glynn, 1979; Gruenheid & Gros, 2000; Forbes and Gros 2001).

Nramp1 is considered a resistance factor in Salmonella infection and functions at multiple levels to enhance the macrophage killing ability: 1) increases response to activating cytokines TNF-α and IFN-γ, 2) enhances antigen processing and presentation by increasing the expression of MHC class II molecule to facilitate the $CD4^+$ T-cell expansion, and 3) increase the early production of IFN-γ by NK cells (Soo et al., 1998). Certain inbred mouse strains, such as C57BL/6 and BALB/c, carry a mutated form of the Nramp1 gene and are at least 1000-fold more susceptible to infection with *S. typhimurium* than 129sv and C3H lineage mouse strains that carry the wild-type gene allele. Mutations in Nramp1 gene have also been associated with increased susceptibility to intracellular infections in humans (Skamene et al. 1998).

1.5.2. Toll like receptors

TLRs play an essential role in the innate immune response against Salmonella infection. C3H/HeJ mice are well known for their hyporesponsiveness to LPS and for being hypersusceptible to Gram-negative bacterial infections (O'Brien et al., 1982). The underlying genetic basis of this phenotype was shown to be a mutation in the TLR4 gene (Poltorak et al., 1998; Qureshi et al., 1999), which emphasizes the central role of LPS recognition by TLR4 in the immune response to Salmonella infection. As referred to earlier, other Salmonella PAMPs known to be recognized by TLRs include lipoprotein (TLR2), flagellin (TLR5) and CpG DNA (TLR9). Among those receptors, TLR4 appears to be the most crucial in immunity to Salmonella due to its effect on secreting proinflammatory cytokines upon recognition of Salmonella, which leads to macrophage activation and killing of the bacteria by the activation of

antimicrobial effectors such as nitric oxide (Weiss et al., 2004) . Despite the fact that C3H/HeJ mice harbor a functional Nramp1 gene, infection with an attenuated strain of *S. typhimurium* revealed a defect in controlling the growth of the bacteria when compared to mice harboring functional TLR4 gene, supporting the essential role of TLR4 in immunity to Salmonella infections (Al-Ojali et al., 2013).

1.5.3. MyD88

Myeloid differentiation protein-88 (MyD88) is an adaptor molecule for utilizing the TLR signals upon the recognition of pathogens. MyD88 recruits IL-1 receptor-associated kinase 4 (IRAK4) and TNF receptor-activated factor 6 (TRAF6), which leads to the activation of NF-κb, and eventually the expression of proinflammatory cytokines and chemokines. It has been shown that MyD88 deficiency leads to losing the protective immunity to pyogenic bacteria in humans and animals (von Bernuth et al., 2008). Furthermore, mice lacking MyD88 are highly susceptible to attenuated strain of *S. typhimurium* due to their inability to control systemic bacterial spread and growth in target organs, accompanied with delayed recruitment of phagocytic cells, and defective cytokine production (Issac et al., 2013).

1.5.4. Cytokines

Communication between immune cells is accomplished through cytokines that act on cells harboring cytokine-specific receptors and causing a particular response through the signal transduction machinery. Immunity to Salmonella infection requires the collaboration between multiple cytokines to drive the immune cells to the proper action and, eventually, control the infection. To date, many studies

in the Salmonella model have highlighted critical roles for TNF-α, IL-12, IL-18 and IFN-γ in Salmonella immunity. Defects in any of these cytokines or their receptors increase the host's susceptibility to Salmonella infection.

1.5.4.1. TNF-α

Tumor necrosis factor alpha (TNF-α) is an inflammatory cytokine that has an essential role in controlling infection of different pathogens (Mastroeni et al., 1992; Green et al., 1993). It is associated with the recruitment of mononuclear cells and induction of nitric oxide that lead to bacterial killing (Havell, 1989; Mastroeni et al., 1995). TNF-α works synergistically with IFN-γ to enhance the bactericidal activity of macrophages (Tite et al., 1991). A previous study has shown that mice with defects in TNF-α receptor expression have heightened susceptibility to virulent as well as avirulent strains of *S. typhimurium* in an oral infection model (Everest et al., 1998). Interestingly, these mice appeared to be less prone to infection-related mortality by attenuated *aroA*$^{-}$ Salmonella mutants in comparison with mice deficient in IFN-γ (Hess et al., 1996).

Another study investigated the precise role of TNF-α in macrophage phagocytic and anti-Salmonella microbicidal activity (Vázquez-Torres et al., 2001) and demonstrated that TNFRp55-deficient macrophages are unable to localize NADPH oxidase-containing vesicles to Salmonella-containing vacuoles, resulting in increased susceptibility to infection.

1.5.4.2. IL-12

Interleukin 12 (IL-12) is a heterodimeric cytokine produced primarily by macrophages, polymorphonuclear cells and dendritic cells following engagement of PRRs by PAMPs (Brunda, 1994). IL-12 mediates immunity to Salmonella through the induction of IFN-γ production by NK cells and T cells, and facilitates the development of a Th1 response (Castro et al., 1995; Manetti et al., 1993). Resistance to many intracellular pathogens, such as Leishmania, Mycobacterium and Brucella, is mediated by IL-12 (Locksley, 1993; Cooper et al., 1995; Heinzel et al., 1995; Zhan & Cheers, 1995).

In innately resistance A/J mice (normal *Nramp1*^{n}), neutralization of IL-12 via monoclonal antibodies, impaired the control of bacterial proliferation at late stage of infection with virulent *S.* Typhimurium C5 strain. This effect correlated with a significant decrease in IFN-γ levels in serum and spleen when compared with non-treated infected mice (Mastroeni et al., 1996).

Neutralization of IL-12 in susceptible BALB/c mice (mutated *Nramp1*^{d} gene) exacerbated infection with an attenuated *aroA*-deficient strain of *S. typhimurium*, with a significant increase in the bacterial burden in the spleen and liver 100-fold more than non-treated infected BALB/c. The increased susceptibility was mediated through a reduction in IFN-γ and downregulation of the expression of MHC class II and iNOS, important indicators of deficient macrophage activation. Treatment of these mice with recombinant IFN-γ restored their host resistance, reduced bacterial burden in spleen and liver, up-regulated MHC class II in macrophages and decreased the level of IL-10 which was elevated when treated with anti-IL-12. The results from

this study demonstrate the protective role of IL-12 in host defense against Salmonella infection, which is mediated by IFN-γ (Mastroeni et al., 1998).

1.5.4.3. IL-18

Interleukin-18 (IL-18) is a cytokine produced by several cell types in response to bacterial and inflammatory stimuli (Stoll et al., 1997). IL-18 works synergistically with IL-12 to promote the production of IFN-γ from T-cells, in part by IL-12-mediated induction of IL-18 receptor expression (Takeda et al., 1998). This cytokine plays an important role in defense against Salmonella bacteria, as neutralization using monoclonal antibodies lead to an increase in bacterial load in spleen and liver, coinciding with low IFN-γ levels in serum. Treatment of these mice with recombinant IL-18, reversed the neutralization effect and increased mice survival and IFN-γ production (Mastroeni et al., 1999). Finally, IL-18 is dependent on the availability of IL-12 in order to induce IFN-γ production, which was confirmed by failure of treating infected IL-12p40 knockout mice with recombinant IL-18, to increase the production of IFN-γ necessary for enhancing the survival of these mice (Dybing et al., 1999).

1.5.4.4. IFN-γ

Interferons were first discovered as physiologic agents that interfere with viral replication (Isaacs & Lindenmann, 1957). They are classified into type I (consisting of IFN–α, IFN–β, IFN–ω and IFN–τ) and type II which only consist of IFN–γ. IFN–γ is structurally unrelated to type I interferons, and binds to a specific receptor composed of two subunits (IFN-γR1 and IFN-γR2).

Signaling through the IFN-γ receptor is mediated by JAK1 and JAK2, two Janus tyrosine Kinases that transmit the signal through phosphorylation of STAT1 (signal transducer and activator of transcription) (Schindler et al., 2007). Phosphorylation of STAT1 allows dimerization of the molecule allowing it to translocate to the nucleus and act as a transcription factor responsible for the up-regulation of many IFN-γ regulated genes (Bach et al., 1997).

IFN-γ is produced in response to a microbial encounter in a temporal fashion. Depending on the phase of the immune response, two cell types are primarily responsible for IFN-γ production. NK cells are the main producers during the early innate phase of the immune response and $CD4^+$ Th1 cells during the adaptive immune response phase. The whole process begins by the recognition of a pathogen associated molecular pattern (PAMP), such as lipopolysaccharide (LPS), by a pattern recognition receptor (PRR) on macrophages and dendritic cells. This engagement triggers a cascade of downstream signaling pathway, which is crucially dependent on the adaptor molecule MyD88 (Myeloid Differentiation 88), ultimately leading to the activation of the transcription factor NF-κb. As a master regulator, NF-κb activates gene transcription of many proinflammatory cytokines, including IL-1, IL-6, IL-12, IL-18 and TNF-α. IL-12 plays a critical dual role in activating NK cells to produce IFN-γ and induces the differentiation of $CD4^+$ T cells to the Th1 pathway (Xie et al., 1997; Schwacha et al., 1998). The secreted IFN-γ, in turn, binds to its high-affinity receptors on macrophages leading to increased macrophage microbicidal activity and, hence, elimination of the invading pathogen (Mastroeni et al., 1998; Mastroeni et al., 1999; Pie et al., 1997).

1.6. Adaptive immunity to Salmonella infection

Controlling Salmonella infection at early stages is maintained by the innate immune system. However, achieving full protection and complete eradication of Salmonella at later stages of infection requires the development of a Salmonella-specific T cell response.

In primary infections with attenuated Salmonella strains in susceptible mice, $CD4^+$ $\alpha\beta TCR^+$ T cells with a Th1 phenotype mediate the clearance of the bacteria from the tissues with little or no obvious contribution of $CD8^+$ T cells (Hess et al., 1996). B cells are dispensable in primary infection with similar attenuated Salmonella strains, as mice lacking B cells were able to resolve infection with similar kinetics to wild type mice (Mcsorley et al., 2000; Mastroeni et al., 2000).

On the other hand, resistance to virulent challenge in susceptible mice requires the action of both $CD4^+$ and $CD8^+$ T cells with the additional requirement for anti-Salmonella antibodies (Mastroeni et al., 1992; Mastroeni et al., 1993). In these studies, depletion of $CD4^+$ or $CD8^+$ T cells in mice immunized with attenuated Salmonella strain, leads to the death of mice after challenge with virulent strain of Salmonella (Mastroeni et al., 1992), and the adoptive transfer of spleen cells alone from immunized to naïve mice was not sufficient for conferring protection against virulent Salmonella infection (Mastroeni et al., 1993). However, the transfer of spleen cells and serum was shown to provide immunity against challenge with virulent Salmonella infection (Mastroeni et al., 1993).

$CD4^+$ T cells contribute to protection via the production of Th1 cytokines such as IFN-γ and TNF-α, that leads to the activation of macrophages containing Salmonella (Ravindran et al., 2005). Depletion of Th1 cytokines (IFN-γ, TNF-α and

IL-12) impaired the protection of immunized mice after secondary infection with Salmonella ((Mastroeni et al., 1998; Mastroeni et al., 1992).

$CD8^+$ T cells are important for host defense against intracellular pathogens (Kaufmann et al., 1995). They kill Salmonella-infected host target cells via the release of cytotoxins (perforin 1 and granzymes), creating holes in the target-cells membrane (Harty & Bevan, 1999).

B cells have an essential role in providing protection against virulent Salmonella infection, since B cells-deficient mice challenged orally with virulent Salmonella after vaccination, failed to control the infection (Mastroeni et al., 2000). In addition, B cells also confer protection through its contribution in the expansion of anti-Salmonella Th1 cells. This was observed in a study, where total splenocytes and purified $CD4^+$ T cells isolated from Igh-$6^{-/-}$ (B cell deficient) mice after vaccination showed an impaired production of Th1 cytokines after stimulation in vitro with Salmonella antigens (Ugrinovic et al., 2003).

1.7. Role of IFN-γ in immunity to Salmonella

IFN-γ is a decisive factor in immunity to Salmonella infection. The essential function of IFN-γ is to activate macrophages and yield a protective response by enhancing antigen presentation, and skewing the T-cells towards a protective Th1 response (Shtrichman & Samuel, 2001). In innately resistant mice, depletion of IFN-γ with mAbs resulted in the loss of resistance to a virulent strain of *S. typhimurium*, and mice succumb to infection at early stage of infection (7 days), in comparison to the non-treated infected mice where they remained alive through the infection course (Nauciel & Espinasse-Maes, 1992). In susceptible C57BL/6 mice (mutated Nramp1),

oral infection with attenuated *aroA⁻/aroD⁻* mutants *S. typhimurium*, resulted in a controlled infection with all mice surviving the infection. This was attributed primarily to IFN-γ, as infecting IFN-γ-deficient mice with same bacterial dose rendered them susceptible to an attenuated strain of Salmonella. This susceptibility was marked with increased bacterial growth over time seen as lesions in liver, spleen, mesenteric lymph node and PP, as compared to a controlled bacterial growth in wild type mice. Histological analysis, showed a decrease in the cells expressing MHC class II, suggesting a defect in macrophage activation (Bao et al., 2000). This IFN-γ dependent expression of MHC class II was observed in another study where Bacillus Calmette Guerin (BCG) infection in IFN-γ knockout mice, also showed susceptibility related to the lack of macrophage activation, impaired nitric oxide production and reduced MHC class II expression (Dalton et al., 1993).

1.8. Role of IFN-γ in Salmonella persistence

In human Salmonella infection, about (1-6%) of the patients become chronic carriers, with bacteria remaining in these patients without causing any symptoms, but showing a continuous bacterial shedding in stool and urine for a long period of time that ranged from 1 year to lifetime (Levine et al., 1982). This occurs despite evidence of adequate immune responses in these carriers, including high levels of serum antibodies against the pathogen (House et al., 2001). A similar phenomenon was observed in inherently-resistant mice, such as strain 129sv with functional Nramp1, infected orally with virulent *S. typhimurium* (strain SL1344). In this model, mice were infected and monitored for up to one year for bacterial shedding in the feces (Monack et al., 2004). The findings showed that infection resulted in bacterial persistence and fecal shedding throughout the study period. This occurred despite

evidence of high levels of anti-Salmonella antibodies being present in infected mice. Examination of the systemic colonization of bacteria over the 1-year period identified the mesenteric lymph nodes as the site where bacteria persisted. Since many studies have shown the role of IFN-γ in maintaining the early phase of Salmonella infection (Nauciel & Espinasse-Maes, 1992), the role of IFN-γ in Salmonella persistence was studied. Depletion of IFN-γ in chronically infected mice (260 days post infection) by a 3-week treatment with a neutralizing mAb led to the systemic spread of bacteria to different organs. This confirmed the primary role of IFN-γ in maintaining the balance between the immune system and bacteria, by suppressing bacterial replication in infected macrophages (Monack et al., 2004).

1.9. Recombinant attenuated Salmonella

Attenuated strains of Salmonella had been greatly used in humans as vector encoded vaccines for a variety of non-Salmonella pathogens. This effect was attributed to invasive characteristics of Salmonella that elucidate B and T cell memory response with long lasting immunity (Roland & Brenneman, 2013). Attenuated Salmonella strains have been used for vaccine delivery through cloning genes encoding heterologous protective antigens, and then the Salmonella express that antigen through a stable Inducible promoter. Attenuated Salmonella has been used in one study as a vaccine for *Leishmania major* parasite, where attenuated Salmonella expressing GP63 afforded protection to susceptible BALB/c mice after oral challenge with virulent Leishmania. This protection was developed through a Th1 response and yielded a high levels of Leishmania-specific antibodies (Xu et al.,

1995). Other studies have used attenuated Salmonella engineered to express heterologous proteins of other pathogens, including viruses, bacteria and fungi. These strains were able to achieve specific immune response and render protection upon challenge with virulent pathogen (Pasetti et al., 2003).

1.10. Attenuated Salmonella expressing cytokines

The use of attenuated Salmonella as vaccine is effective when the circumstances of achieving a specific immune response towards the vaccine are met. The fate of such vaccine requires the availability of a healthy immune response. However, using an attenuated vaccine in immunocompromized hosts could lead to systemic, lethal, infections. An attenuated strain of Salmonella engineered to express a particular cytokine is one way of guiding the immune response of immunocompromized hosts to achieve the required protection.

This was first done with the construction of an attenuated strain of Salmonella that expressed the human IL-1β cytokine. The resultant strain was able to cause an immunological reaction influenced by the expressed cytokine, shown by the high levels of antibodies against the engineered human IL-1β (Carrier et al., 1992).

This was followed by the construction of attenuated Salmonella strains expressing murine cytokines, including IL-2, TNF-α, IFN-γ and MIF (Xu et al., 1998). These strains were used to boost the immunity of susceptible BALB/c mice against *Leishmania major* infection through the induction of protective Th1 responses (Xu et al., 1998). The potential of these cytokine-expressing strains in regulating anti-Salmonella immune responses in susceptible BALB/c mice was also explored (al-Ramadi et al., 2001; al-Ramadi et al., 2002). Salmonella strains expressing IL-2 or TNF-α, designated GIDIL2 and GIDTNF respectively, were

compared with parental non cytokine-expressing Salmonella strain, designated BRD509E, in terms of their influence on the anti-Salmonella immune response. The findings demonstrated that the expression of IL-2 by the GIDIL2 strain led to a rapid clearance of bacteria with decreased splenomegaly and enhanced macrophage activation through the upregulation of cell surface proteins and induction of high levels of nitric oxide synthesis. GIDIL2-vaccinated mice also developed high levels of resistance to subsequent challenge with the virulent SL1344 Salmonella strain (al-Ramadi et al., 2001; al-Ramadi et al., 2002).

Subsequent studies focused on analyzing the effect of infection with cytokine-expressing strains in immunodeficient mice with the aim of delivering compensatory tools for different immunodeficient mouse model. Given the essential role of IFN-γ in immunity against Salmonella, an attenuated strain of Salmonella was engineered to express murine IFN-γ, designated GIDIFN (Xu et al., 1998). In vitro studies have shown the ability of GIDIFN to induce a strong immune response demonstrated by the production of TNF-α, IL-6, IL-12p40 and NO, with specific targeting of macrophages leading to the upregulation of several activation markers and surface costimulatory molecules. These effects were much more pronounced than with the use of GIDIL2 or the parental, non-cytokine-expressing, BRD509E strain (Fernandez-Cabezudo et al., 2009). Furthermore, the potential of GIDIFN was tested *in vivo* in different immunocompromized murine models. GIDIFN was able to confer a protective response to mice with a deficiency in TLR4, an essential molecule for the recognition of LPS component of the bacteria (Al-Ojali et al., 2013). This effect correlated with a more efficient control of bacterial proliferation in systemic target organs, presumably through the increased production of proinflammatory cytokines IL-6 and IL-12 (Al-Ojali et al., 2013). Vaccination of

these susceptible mice with GIDIFN also provided protection against challenge with virulent Salmonella that was significantly higher than in mice immunized with BRD509 (Al-Ojali et al., 2013). GIDIFN was also able to enhance the anti-bacterial response in mice deficient in CD154 protein, an essential molecule for induction of type 1 cytokines and antibody isotype switching response (al-Ramadi et al., 2006; Al-Ojali et al., 2012). This was demonstrated by reduced bacterial load in target organs and increased mouse survival, which was mechanistically linked with an enhanced proinflammatory cytokine response, including IL-6, IL-12, TNF-α and IFN-γ (Al-Ojali et al., 2012). In addition, GIDIFN was able to afford protection to $CD154^{-/-}$ mice when challenged with lethal Salmonella (Al-Ojali et al., 2012).

A previous study demonstrated that mice deficient in IFN-γ regained their resistance after injecting them with recombinant IFN-γ (Mastroeni et al., 1998). So we hypothesized that it might be possible to compensate for the deficiency of IFN-γ in IFN-$\gamma^{-/-}$ mice through the use of the GIDIFN Salmonella strain. This is the premise on which the studies conducted in this project were carried out.

Chapter 2: Materials and Methods

2.1 Materials

2.1.1. Summary of materials used and suppliers

Materials	Company
SS Agar	Mast Group
Ampicillin	Sigma
Streptomycin	Sigma
Microscopic slides	Fischer Scientific
7-AAD Viability Staining Solution	e-Bioscience
PBS (-$CaCL_2$ – $MgCl_2$)	GIBCO
BSA	Sigma
RPMI 1640 medium (-L glutamine)	HyClone
Ethanol	Carlo Erba
H_2SO_4	Readel-de Haen
Trypan blue solution (0.4%)	Sigma
Thioglycolate	Sigma-Aldrich
Trypticase soy broth	Oxoid
24 well tissue culture plates	Becton Dickenson
96 well Microtest U bottom culture plates	Becton Dickenson
96 well Maxisorp Nunc Immuno plate	Thermo Scientific

Table 1: Summary of materials used and supplies

2.1.2. Summary of antibodies used

Antibody	Conjugate	Company	Catalogue	Dilution used
Armenian hamster anti-mouse CD3	FITC	e-Bioscience	11-0031-85	1:100
Rat anti-mouse CD11b	APC-eFluor780	e-Bioscience	47-0112-82	1:500
Rat anti-mouse CD19	PE-cy7	BD Pharmingen	552854	1:200
Rat anti-mouse Gr-1	PE	BD Pharmingen	553128	1:500
Armenian Hamster IgG1 Isotype Control	Alexa Fluor488	e-Bioscience	12488883	1:200
Rat IgG2b,k Isotype Control	APC-eFluor780	e-Bioscience	47-4031-82	1:200
Rat IgG2a,k Isotype Control	PE-cy7	e-Bioscience	25-4321-82	1:200
Rat IgG2b,k Isotype Control	PE	e-Bioscience	12-4031-82	1:200
Anti-mouse CD16/CD32	-	e-Bioscience	14-0161-85	1:100

Table 2: Summary of antibodies used

2.1.3. List of primers used for RT-PCR

Gene	Source	Assay I.D	Reporter dye	Quencher
HPRT	Applied biosystem	Mm01545399-ml	FAM	NFQ
iNOS	Applied biosystem	Mm00440502-m1	FAM	NFQ
S100A9	Applied biosystem	Mm00656925-m1	FAM	NFQ

Table 3: Summary of primers used for RT-PCR

2.1.4. Mice

C57BL/6 mice were purchased from Harlan Olac (Bicester, UK). All strains were bred at the animal facility in the College of Medicine and Health Sciences, UAE University and used at 8–12 weeks of age. Mice received rodent chow and water ad libitum. All studies involving animals were carried out in accordance with, and after approval of, the animal research ethics committee of the College of Medicine and Health Sciences, UAE University.

2.2. Methods

2.2.1. Bacterial strains and growth condtions

BRD509E is an attenuated, *aroA*$^{-}$*/aroD*$^{-}$ mutant strain derived from SL1344 with an LD50 of 5 x10^{6} CFUs per mouse when administered i.p. to susceptible mice. For the current study, a derivative of BRD509E expressing the empty nirB plasmid vector, which was developed in al-Ramadi laboratory, was used. The latter strain

behaves indistinguishably from BRD509E except for the fact that it acquired resistance to ampicllin, which is encoded by the nirB plasmid, and can, therefore, be easily selected, transfectant of the BRD509E strain, designated GIDIFN, in which the murine IFN-γ gene is expressed, was also used. The expression of IFN-γ is under the control of the anaerobic growth-induced nirB promoter, and cytokine expression was maintained by selection in 100 μg/ml ampicilin.

Aliquots of frozen bacteria were routinely plated on Salmonella Shigella (S.S) agar (Oxoid, Basingstoke, United Kingdom) in the presence of ampicillin and grown overnight at 37°C. Five to ten CFUs were cultured overnight in Trypticase Soy broth and then diluted 1:10 in fresh medium and grown for a further 2-3 hr at 37 °C with shaking. For induction of cytokine expression, bacterial colonies of the GIDIFN strain were grown under anaerobic conditions overnight at 37°C in T-Soy broth containing ampicillin and 4 mg/ml glucose in a closed screw-cap container. The concentration of bacterial suspensions was estimated from spectrophotometer readings at 600nm wavelength using the following formula:

OD_{600} *of 0.1 =* $1.2x10^{8}$ *CFUs/ml*

Appropriate dilutions of log-phase bacterial suspensions were prepared in pyrogen-free phosphorus-buffered saline (PBS) (Sigma Chemical Co., St. Louis, Mo.) and administered i.p. in 0.5 ml volume per mouse. Bacterial doses were confirmed by CFU plate counts.

2.2.2. Enumeration of bacteria in organ homogenates

To determine liver bacterial load, the organs were removed aseptically, individually weighed and homogenized in 1 ml of cold sterile saline in an Ultra-

terrax T25 tissue homogeniser (Janke and Kunkle, Staufenim Breisgau, Germany). A 100 µl aliquot of the homogenate, or an appropriate dilution, was plated on SS agar plates in the presence or absence of ampicillin, and viable CFUs were determined after an overnight incubation.

2.2.3. Peritoneal cells preparation

Thioglycolate (4%) was injected to groups of mice 3 days before processing day. After sacrificing mouse,10 ml of cold Ca^{2+}, Mg^{2+} free physiological saline was injected into the exposed peritoneal cavity. Peritoneal fluid obtained from individual mice was withdrawn through the anterior abdominal wall with a 20-gauge needle. Then cells were spun down at 1200 rpm for 5 minutes at 4^{o}C. Cells were counted on a hemocytometer and cell viability was determined by trypan blue dye.

2.2.4. Spleen cell preparation

Single cell suspensions were prepared by gently teasing the spleen between frosted ends of microscope slides. Cells were spun down at 1200rpm for 5 minutes at 4^{o}C. The spleen cell suspension was depleted of red blood cells by incubation in RBC lysis buffer (8.3NH_4Cl, 1g $KHCO_3$ 1.3ml of 5%EDTA dissolved in 1L distilled water) (4 ml/spleen + 6 ml of PBS) for 5 minutes, after which they were spun down at 1200 rpm for 5 minutes and suspended in 1xPBS. Cells were counted on a hemocytometer and cell viability was determined by trypan blue dye.

2.2.5. Peritoneal cell culture

Peritoneal cells from thioglycolate-injected mice were harvested and pooled from several similarly-treated mice per group. Cells were seeded in 24-well plates

($2x10^6$ cells/well) in 5% antibiotic-free RPMI medium and incubated for 2 hours at 37^oC. Log phase BRD509 or GIDIFN were prepared at different MOIs. Cells were incubated with bacteria for 1 hour to allow bacterial invasion, after which cells were treated with 200 μg/ml of gentamicin for 30 minutes to kill all remaining extracellular bacteria. Then medium was replaced with a fresh medium containing 20 μg/ml of gentamicin, and cells were incubated for 4 hours, and cell-free supernatants were collected and kept at -20°C. Trizol (0.5 ml) was added to the cells in each well collected and stored at -80°C for RNA extraction.

2.2.6. Nitric oxide determination

Production of NO was measured by the accumulation of NO_2^- in culture supernatants using the Griess reaction. Cells were cultured under the indicated conditions for 4 hours and then cell-free culture supernatants were collected and frozen at -20°C until analysed. Nitrite content was determined by mixing 50μl of culture supernatant with an equal volume of Griess reagent (0.05% N-1-napthylethylenediamide hydrochloride, 0.5% sulphanilamide in 2.5 % phosphoric acid) and incubated at room temperature for 5 to 10 minutes. Nitrite concentration was quantified using $NaNO_2$ as the standard and expressed as the micromolar concentration of NO_2^- per 2×10^6 PECs after 4 hours of incubation. The absorbance at 562 nm was measured in an automated microplate reader.

2.2.7. Flow cytometry

Spleen or peritoneal cell suspensions were prepared from normal or infected mice. Cells were re-suspended in staining buffer (PBS/1% FCS/0.1% NaN_3) at a concentration of 0.5 x 10^6 cells/well in U-bottom 96-well plate (BD) and incubated

with 50 µl/well of anti-mouse CD16/CD32-specific mAB (clone 2.4G2) for 30 minutes at 4^{o}C to block FcγRIII/II sites in order to avoid nonspecific binding. The plate was then centrifuged at 750 rpm for 3 minutes at 4^{o}C. After decanting the buffer, cells were stained with appropriate dilutions, as mentioned in directly conjugated monoclonal antibodies (mAbs; all purchased from eBioscience or BD or Biolegend) in a total volume of 100 µl/well and analyzed by 6-color FACS. In all staining groups, 7-AAD dye (eBioscience) was included in order to exclude non-viable cells from the analysis. All antibodies were pre-titrated in preliminary experiments and used at saturating concentrations. Cells were incubated with a mixture of appropriately diluted mAbs at 4^{o}C for 30 minutes followed by two wash cycles with staining buffer. The first wash was with 100 µl/well of staining buffer where the cells were mixed well with the pipette and centrifuged. In the second wash, 200 µl/well of staining buffer was added and mixed well followed by centrifugation. Cells were finally re-suspended in 200 µl/well of staining buffer. Data was collected on 30,000 cells using BD FACSCantoII and analyzed by BD FACSDiva software. Gating strategies is shown in **Fig.3**. First, we exclude dead cells by gating on the 7AAD negative cells (P2 gate) **(Fig.3 *A*)**. Then, we analyzed the immunophenotype of these viable cells based on the expression of different cell markers **(Fig.3 *B*).**

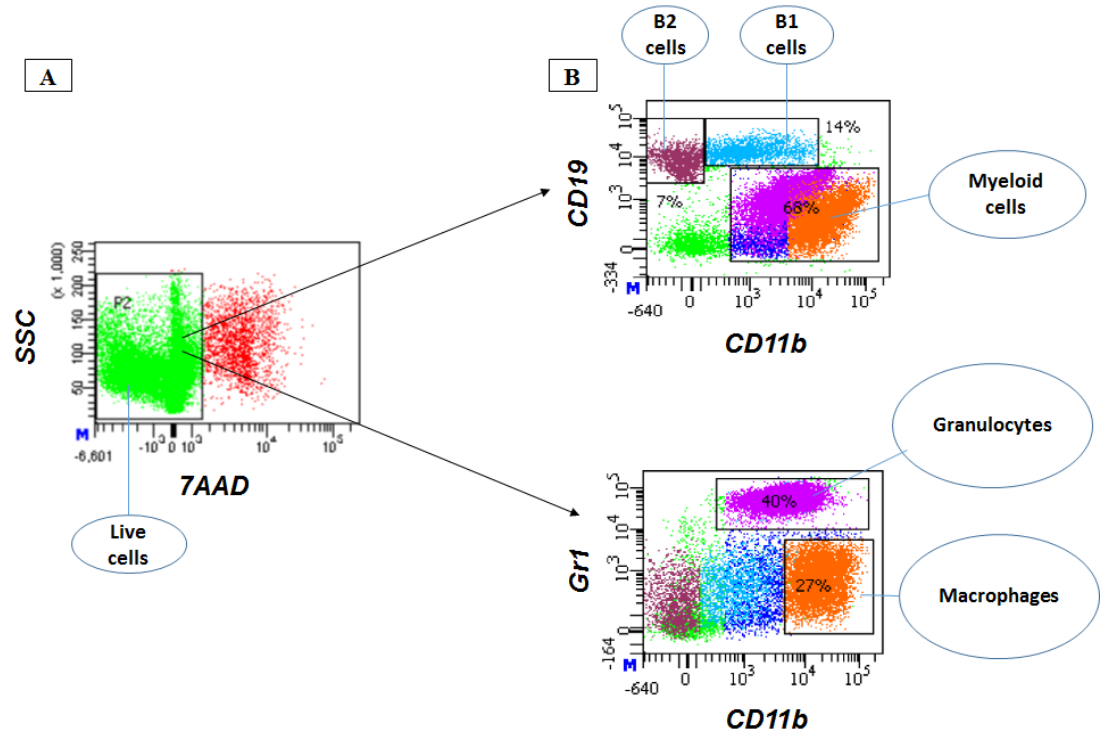

Figure 3: Flow cytometric analysis gating strategies.

Illustration of the gating strategies for flow cytometric analysis. (A) Dot plot showing SSC vs. 7AAD staining. Gate P2 indicates viable (7AAD-negative) cells. (B) Immunophenotyping of viable PECs based on the expression of different cell surface markers (CD11b, Gr-1, and CD19).

2.2.8. RNA extraction using Trizol

RNA was extracted from peritoneal cells by the Trizol method. Cells (2-5 x 10^6 per sample) were pelleted and re-suspended in 1 ml Trizol (Invitrogen) following which 200µl of chlorophorm was added and mixed well. Once cells were spun down at 14000 rpm for 10 minutes, 3 phases are obtained from which the top clear RNA layer (~500µl) was transferred to a new tube. Equal volume of 2-propanol (~500µl) was then added and mixed well to precipitate the RNA. Tubes were spun again, supernatant was discarded and 500 µl of 70% ethanol added. On flicking the tubes, RNA was observed as a white pellet. Tubes were spun again, supernatant removed

and RNA was finally suspended in 20-40µl nuclease free water and stored in -80^{0}C. The quality and quantity of RNA was determined using the Nanodrop ND-1000 spectrophotometer (Thermo Scientific, Waltham, MA).

2.2.9. Reverse Transcription Reaction

RNA was reverse transcribed using TaqMan reverse transcription reagent (Applied Biosystem #N8080234). Each master mix reaction contained 10x RT buffer, 25mM $MgCl_2$, deoxy NTPs mixture (2.5mM), random hexamers (50µM), RNAase inhibitor (20U/µl) and MultiScribe RT enzyme. The master mix was aliquoted into separate PCR tubes. RNA was added (1µg/10µl per reaction) and total volume was made up to 50µl with nuclease-free water. The one step RT-PCR reaction was on GeneAmp PCR System 2700 (Applied Biosystem), under the following conditions: hexamer incubation for 10 minutes at 25^{0}C, reverse transcription at 48^{0}C for 30 minutes and reverse transcription inactivation at 95^{0}C for 5 minutes. The samples were held at 4^{0}C for a maximum for 1 hour until the sample could be removed and stored at –20^{0}C.

2.2.10. Real time PCR reactions

The real time PCR was performed using TaqMan gene expression assay as specified below and amplified using the 7500 Real Time PCR System (Applied Biosystems). Each 20µl PCR reaction contained 10µl of 2xTaqMan Universal Master Mix (Applied Biosystems #4440047), 1 µl of 20x TaqMan assays Mix (Applied Biosystems #4331182), 2 µl cDNA and 7 µl nuclease-free water. A negative PCR reaction was also carried out using only the reaction mixture without

cDNA in order to ensure that there was no DNA contamination. The thermal cycling conditions were as follows: 95^0C for 10 minutes (Inactivation of Reverse Transcriptase and activation of TaqMan polymerase), 95^0C for 15 seconds (denaturation of dsDNA) and 60^0C for 1 minute (annealing/extension-fluorescent data collected during this step) for a total of 40 cycles with the threshold set as 0.2. Data was analyzed using the Ct values for each sample that were in duplicates. Results were normalized to HRPT (Hypoxanthine-guanine phosphoribosyl transferase) and the mRNA fold change was determined using the following equation:

Fold change= $2[^{\Delta Ct\ (infected)}]/2[^{\Delta Ct\ (control)}]$,

where ΔCt (infected) = threshold cycle (Ct) for target gene after infection – Ct for HPRT after infection and ΔCt(control) = Ct for target gene saline treated – Ct for HPRT saline treated.

The control used was uninfected wild-type (C57BL/6).

2.2.11. Statistical Analysis

Statistical significance was analyzed using Student's unpaired t-test, Mann-whitney test, or one way ANOVA with Tukey's multiple comparison test using the statistical program of GraphPad Prism software (San Diego, CA). Differences between experimental groups were considered significant when p values were <0.05.

Chapter 3: Results

3.1. Susceptibility to infection by attenuated Salmonella

Differential susceptibility of immunodeficient mice to Salmonella infection normally involves an intricate interplay between cells as well as soluble factors of the innate and adaptive immune systems (al-Ramadi et al., 2006). Given the facultative intracellular growth nature of the pathogen, macrophages and Th1 lymphocytes play critical roles in host protection. One of the most important Th1-produced cytokines for macrophage activation is IFN-γ. Importantly, this proinflammatory cytokine upregulates anti-microbial (microbicidal) activity of host macrophages through induction of different effectors, such as ROS and NO. Moreover, the induction of the T lymphocyte differentiation pathway towards Th1 cells is dependent on IL-12, a product secreted primarily by macrophages following stimulation by microbial ligands through TLRs. Most TLR signaling pathways depend on MyD88 adaptor protein for functionality, hence this molecule's central importance in innate immune responses. It is clear, therefore, that both innate and adaptive immune systems cooperate in the response to Salmonella infection.

To assess the importance of various components of the immune system in Salmonella infection, two genetically modified mouse strains were used; the first with a deficiency in MyD88 expression, referred to as $MyD88^{-/-}$ (Adachi et al., 1998) and the second carrying a deficiency in IFN-γ synthesis, $IFN\gamma^{-/-}$. Using an attenuated, double auxotrophic, strain of *S. typhimurium*, designated BRD509E (al-Ramadi et al., 2001; al-Ramadi et al., 2004), we compared the course of infection in wild type control (C57BL/6 mice) and the two immunodeficient mouse strains. The LD_{50} of

BRD509E in wild-type mice is >2x10^6/mouse when given i.p. (al-Ramadi et al., 2006). For the current study, the bacterial dose used for infection ranged from 300 to 1000 CFUs per mouse and the host survival data for the three mouse strains is illustrated in **Figure 4**. As expected, no infection-related mortality was observed in C57BL/6 mice. However, MyD88$^{-/-}$ mice infected with BRD509E exhibited an overall survival of 35% over the 60-day observation period. This suggests that the LD_{50} dose is <1 x 10^3 per mouse, representing ~2000-fold increase in susceptibility to infection compared to wild-type mice. A similar dose BRD509E inoculation in IFNγ$^{-/-}$ mice was associated with a significantly higher mortality with 100% of the mice succumbing to infection by day 28 ($p < 0.0001$). Median survival of BRD509-infected MyD88$^{-/-}$ and IFNγ$^{-/-}$ mice was 51 days and 19.5 days, respectively (**Fig. 4**).

Next, we assessed the differential susceptibility of MyD88$^{-/-}$ and IFNγ$^{-/-}$ mice to infection by a genetically-engineered transfectant of BRD509 in which murine IFN-γ is expressed under the control of a prokaryotic promoter (strain GIDIFN) (Xu, et al. 1998). Using a similar dose to BRD509E (verified dose was 300 CFUs/animal), infection with the GIDIFN strain resulted in markedly decreased mortality in both immunodeficient mouse strains (**Fig. 4**). For MyD88$^{-/-}$ mice, 90% of animals survived the infection with GIDIFN in contrast to the 35% of mice that survived after BRD509 inoculation ($p = 0.0003$). In further studies, increasing the dose of GIDIFN to 1x10^5 CFUs/mouse led to 60% overall survival (Al-Ojali S. and al-Ramadi BK, unpublished data), suggesting that the LD_{50} for GIDIFN in MyD88$^{-/-}$ mice was >1x10^5 CFUs/mouse, which represents >300-fold increase in resistance to infection compared to the BRD509E Salmonella strain.

An essentially similar picture emerged when IFNγ$^{-/-}$ mice were infected with the GIDIFN bacterial strain (**Fig. 4**). The right-sided shift observed in the survival curve signifies decreased mortality in GIDIFN-infected mice in comparison to those infected with BRD509E (p <0.0001). Despite the increase in mean survival from 19.5 to 26.5 days in animals injected with GIDIFN, the level of resistance to Salmonella infection did not match what was observed in similarly treated MyD88$^{-/-}$ mice. It is therefore clear that notwithstanding the fact that IFNγ$^{-/-}$ mice are significantly more susceptible to Salmonella infection than MyD88$^{-/-}$ mice, infection with a strain of bacteria expressing the immunoregulatory cytokine IFNγ led to a marked decrease in the level of susceptibility to Salmonella infection.

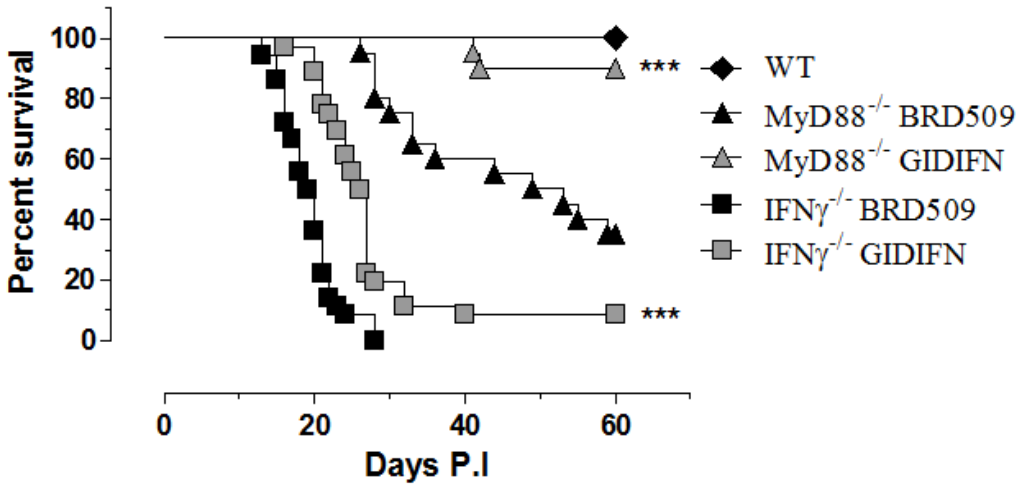

Figure 4: Relative survival of WT vs. MyD88$^{-/-}$ vs. IFNγ$^{-/-}$ deficient mice following infection with BRD509E or GIDIFN Salmonella strain.

WT and MyD88$^{-/-}$ were infected with 10^3 CFUs/mouse, while IFNγ$^{-/-}$ were infected with 300 CFUs/mouse. Survival was scored for up to 60 days after inoculation. Asterisks denote statistically significant differences between BRD509E and GIDIFN-infected mice (*p ≤0.05, **p ≤0.01, ***p ≤0.0001). The results are representative of 6 independent experiments.

3.2. Bacterial loads in systemic organs

We next assessed the extent of bacterial loads in target organs in different strains of mice following infection with BRD509E or GIDIFN. The data for MyD88$^{-/-}$ vs. WT mice comparison is shown in **Figure 5**. Infection with either BRD509 or GIDIFN strain resulted in similar bacterial growth kinetics in the target organs (liver and spleen) in both WT and MyD88$^{-/-}$ mice, except for the significantly lower GIDIFN loads observed at all-time points. In WT mice, the number of GIDIFN CFUs was 3x and 9.5x fold lower than BRD509E at days 7 and 14 post infection (**Fig. 5*A*-*B***). Strikingly, bacterial loads in MyD88$^{-/-}$ mice were significantly increased compared to their wild-type counterparts (**Fig. 5*C*-*D***). As early as day 3 post infection, the bacterial load, of either strain, recovered from MyD88$^{-/-}$ spleens or livers was >7-fold or ~17-fold higher than that seen in infected wild-type mice. The significant increase in bacterial proliferation in MyD88$^{-/-}$ mice so early after infection suggests that a defective early innate immune response may be responsible. By day 14 post infection, the bacterial CFUs of the BRD509E strain in MyD88$^{-/-}$ mice reached septic levels, with approximately 1 x 10^8 CFUs/spleen and more than 2 x 10^7 CFUs/gram of liver. This underlies the higher mortality rate among BRD509E-injected MyD88$^{-/-}$ mice that began to be manifested by 3-4 weeks post infection (**Fig. 4**). Importantly, even in a background of significant immunodeficiency, the extent of proliferation observed with the GIDIFN strain in MyD88$^{-/-}$ mice was significantly lower than that of BRD509E, remaining mostly below septic threshold levels. It appears, therefore, that the expression of IFN-γ by Salmonella facilitates their more efficient elimination, thereby leading to a decrease in host mortality. Similar findings were found when bacterial loads were compared in infected WT vs. IFNγ$^{-/-}$ mice

(**Fig. 6**). In contrast to WT mice that controlled the infection and had very low bacterial loads in the liver (<1 CFU/mg), IFNγ$^{-/-}$ mice exhibited 94-, 1900-, and 78,500-fold higher BRD509E CFUs at 7, 10 and 14 days post infection, respectively. Despite the heightened susceptibility of IFNγ$^{-/-}$ mice, bacterial liver CFUs of GIDIFN were significantly lower (1.7 to 3.7-fold) than the corresponding BRD509E loads. Thus, the enhanced survival of GIDIFN-infected mice correlates with decreased bacterial burden in systemic organs.

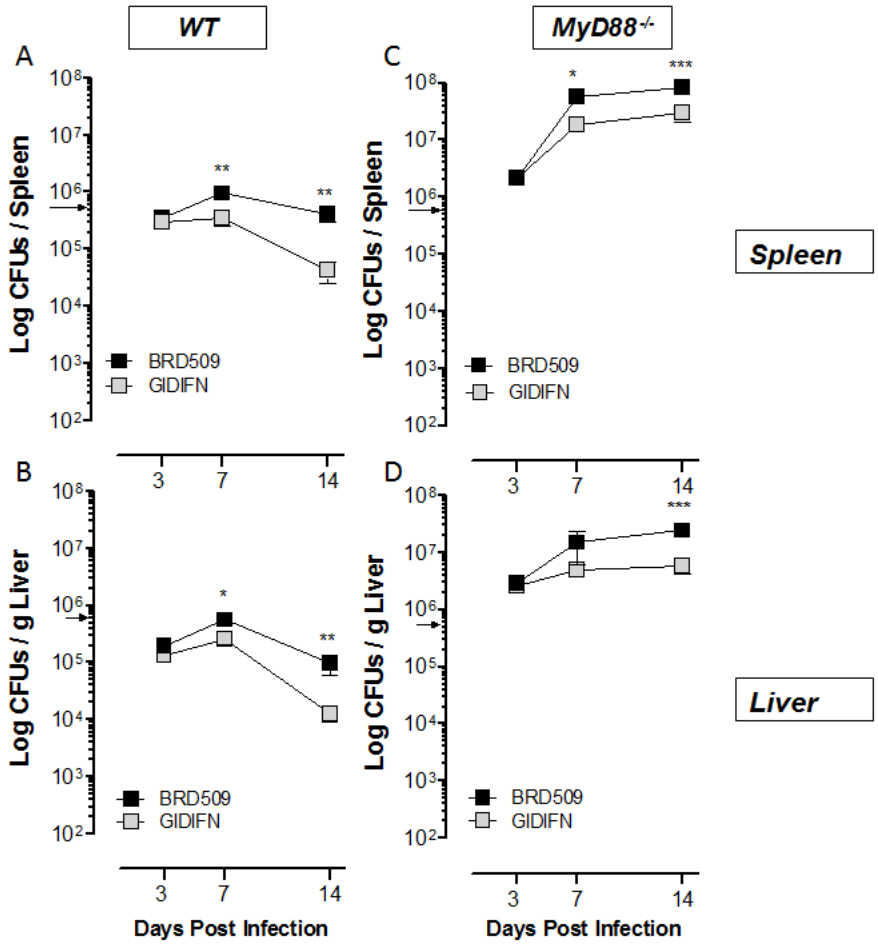

Figure 5: Rapid clearance of IFNγ-expressing Salmonella in *vivo*.

WT **(A-B)** and MyD88$^{-/-}$ **(C-D)** mice were inoculated i.p with ~0.5x 10^6 CFUs/mouse and at indicated time points were sacrificed and the bacterial load in spleens **(A/C)** and livers **(B/D)** enumerated. Each data point represents the mean ± SEM of 8-15 mice per group. Asterisks denote statistically significant differences between BRD509E and GIDIFN-infected mice (*$p \leq 0.05$, **$p < 0.01$, ***$p < 0.0001$).

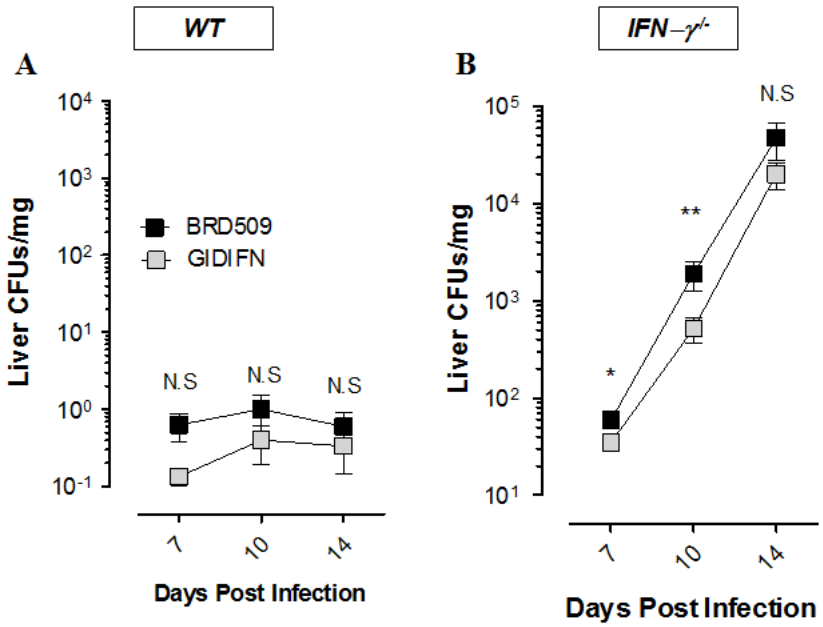

Figure 6: Decreased bacterial load of IFNγ-expressing Salmonella in *vivo*.

WT (A) and IFNγ$^{-/-}$ (B) mice were inoculated i.p with ~300 CFUs/mouse and at indicated time points were sacrificed and bacterial load in livers enumerated. Each data point represents the mean ± SEM of 3-6 mice per group. Asterisks denote statistically significant differences between BRD509E and GIDIFN infected mice (*p < 0.05; **p < 0.01; ***p < 0.001). Compiled from 4 independent experiments.

3.3. Induction of splenomegaly

The degree of splenomegaly induced by infection with BRD509E or GIDIFN in different mouse strains was assessed. The data for MyD88$^{-/-}$ vs. WT mice comparison is shown in **Figure 7.** In both mouse strains, maximum splenomegaly was observed on day 14 post infection. Despite the fact that the degree of splenomegaly was relatively greater in MyD88$^{-/-}$ mice, there was a delay in the induction of this response early in infection. This is seen when one examines splenomegaly induced on day 3 post infection in both mouse strains. While the mean spleen weight of infected mice increased by 2.5 to 3.0-fold in WT mice, the mean weights in MyD88$^{-/-}$ mice represented about 1.6-fold compared to non-infected

animals. Another consistent observation was that the degree of splenomegaly induced by GIDIFN was uniformly less than that induced by the BRD509E strain **(Fig. 7*A*-*B*).** This may well be related to the above-discussed differences in bacterial loads in target organs, including the spleen.

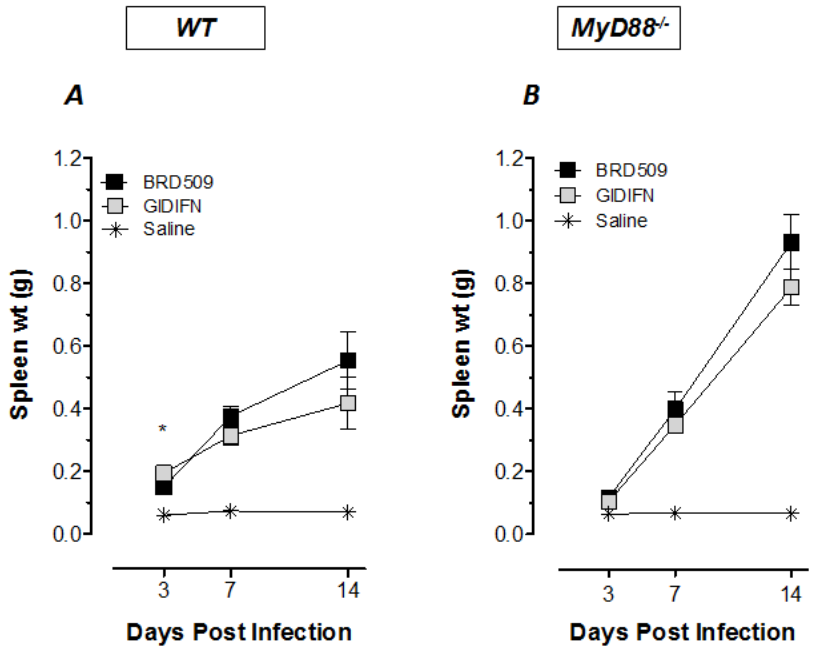

Figure 7: Infection-induced splenomegaly in Salmonella-injected mice.

Animal were inoculated with ~0.5 x 10^6 CFUs/mouse of BRD509E or GIDIFN strain. At days 3, 7 and 14, spleens were aseptically removed WT (A) and MyD88$^{-/-}$ (B) mice and weighted. Each data point represents the mean ± SEM of 5 mice per group. Asterisks denote statistically significant differences between BRD509E and GIDIFN infected mice (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

A similar picture could be observed when we compared splenomegaly in infected WT vs. IFN$\gamma^{-/-}$ mice **(Fig. 8*A*-*B*)**. In contrast to WT mice that had a controlled level of splenomegaly, BRD509-infected IFN$\gamma^{-/-}$ mice exhibited 1.3- and 2.5-fold higher splenomegaly at 10 and 14 days post infection, respectively. A similar pattern of splenomegaly was observed in GIDIFN-infected IFN$\gamma^{-/-}$ mice;

however, the extent of the increased spleen weights was in general lower (~10%) than in BRD509E-infected mice.

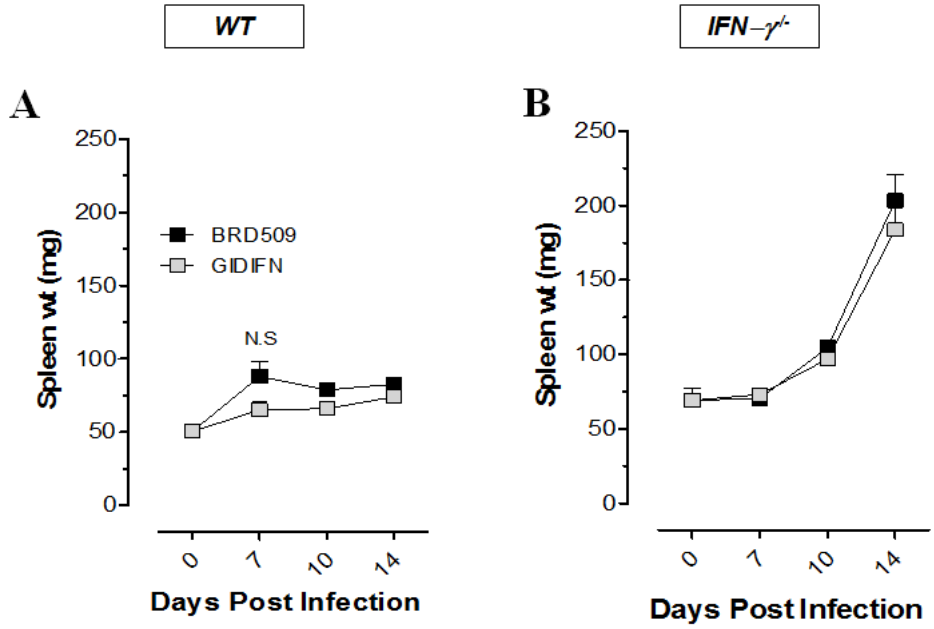

Figure 8: Infection-induced splenomegaly in Salmonella-injected mice.

Animal were inoculated with ~300 CFUs/mouse of BRD509E or GIDIFN strain. At days 7, 10 and 14, spleens were aseptically removed from WT (A) and IFN$\gamma^{-/-}$ (B) mice and weighted. Each data point represents the mean ± SEM of 3-6 mice per group. Compiled from 4 independent experiments. Asterisks denote statistically significant differences between BRD509E and GIDIFN infected mice (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

3.4. Enumeration of peritoneal cavity cellularity

Recruitment of inflammatory cells was investigated through enumeration of peritoneal cells harvested from WT and IFN$\gamma^{-/-}$ mice following infection with BRD509E or GIDIFN as shown in **Figure 9**. Infection with BRD509E results in recruitment of inflammatory cells at similar levels in both mouse strains **(Fig. 9*A*-*B*).** Interestingly, infection with GIDIFN causes influx in peritoneal cells as early as 20

hours post infection, that continues to increase in IFNγ$^{-/-}$ mice while it normalizes in WT after 48 hours post infection **(Fig. 9*A-B*).** Thus, the expression of IFN-γ by GIDIFN correlates with the enhancement in the cellularity of peritoneal cells.

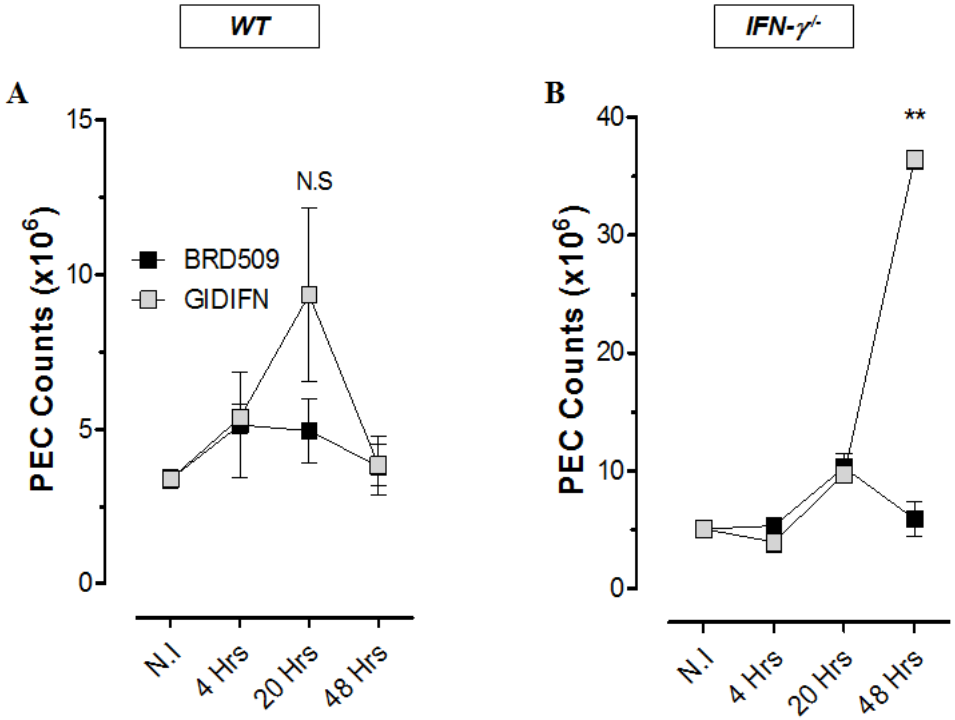

Figure 9: GIDIFN enhances the recruitment of peritoneal cells.

(A-B) WT and IFNγ$^{-/-}$ mice were infected i.p with 1x10^6 CFUs/mouse of BRD509E (A) or GIDIFN (B), and the number of total peritoneal exudate cells (PEC) was enumerated at indicated time points. Each data point represents the mean ± SEM of 3-5 mice per group.

3.5. Phenotypic analysis of recruited cells in the peritoneal cavity post inoculation with Salmonella

The ratios of different cell sub-populations in the peritoneal cavity 4 and 48 hours after infection with either BRD509E or GIDIFN were next analyzed. Peritoneal exudate cells from saline-injected mice were used as control. Specifically, the aim was to ascertain if there were any changes in cell composition in the two groups of infected animals as compared to control. Moreover, the extent and makeup of the inflammatory cell infiltrates into the peritoneal cavity was determined. The results of this analysis are shown in **Figures 10-11**.

Flow cytometric analysis using PE-cy7-conjugated CD19, APC-cy7-conjugated CD11b and PE-conjugated Gr1 mAbs is shown in **Figures 10-11**. In uninfected WT mice, four populations were observed; 20% $CD19^{+}$ $CD11b^{+}$ (B1 cells) and 62% $CD19^{-}$ $CD11b^{+}$ (myeloid cells), which represents myeloid cells that is sub-divided according to the expression of Gr1 surface antigen into: 26% $Gr1^{+}$ $CD11b^{+}$ (granulocytes) and 36% $Gr1^{-}$ $CD11b^{+}$ (macrophages) **(Fig. 10*A/D*).**

Upon infection with BRD509, the ratio of myeloid cells increases slightly (62% to 68%) at 4 hours, then increases to 92% 48 hours post-infection **(Fig. 10*G*)**. The increase in myeloid cells observed with BRD509E, is presented by the same increase of the myeloid sub-populations, with a high increase after 48 hours post infection **(Fig. 10*H-I*)**. Moreover, the increase of myeloid cells in infected mice, corresponds with decease in the ratio of B1 cells that is 6.7- 4.7- fold decrease 48 hours post-infection with BRD509E or GIDIFN, respectively **(Fig. 10*J*)**. Similar findings were observed with GIDIFN **(Fig. 10*H-J*)**. By contrast, in uninfected $IFN\gamma^{-/-}$ mice, the ratio of myeloid cells is 1.7- fold less than WT, with most of myeloid cells

comprise of macrophages (35%) compared to the granulocytes (1%), while the ratio of B1 cells remains similar (19%) **(Fig. 11*A*/*D*)**. upon infection with BRD509E, the ratio of myeloid cells increases to 60% 4 hours post-infection, and remains at similar ration after 48 hours of infection **(Fig. 11*G*)**. This increase, is mostly in the granulocytes with the ratio getting to 26%, then drops to 5.3% after 4 and 48 hours post-infection, respectively **(Fig. 11*H*)**. While the ratio of macrophages remains the same in both infection time points **(Fig. 11*I*)**. Interestingly, the ratio of myeloid cells shows 1.5- fold increase in GIDIFN-infected mice compared to BRD509 48 hours post infection. This increase is marked by the granulocytes, that is 50-fold and 9.3-fold higher than uninfected and BRD509E-infected mice, respectively **(Fig. 11*G*-*I*)**. Moreover, infection with either strains causes a decrease in the B1 cell ratios 48 hours post-infection, that is least in GIDIFN-infected mice (19% to 10% and 19% to 3.5%) **(Fig. 11*G*).**

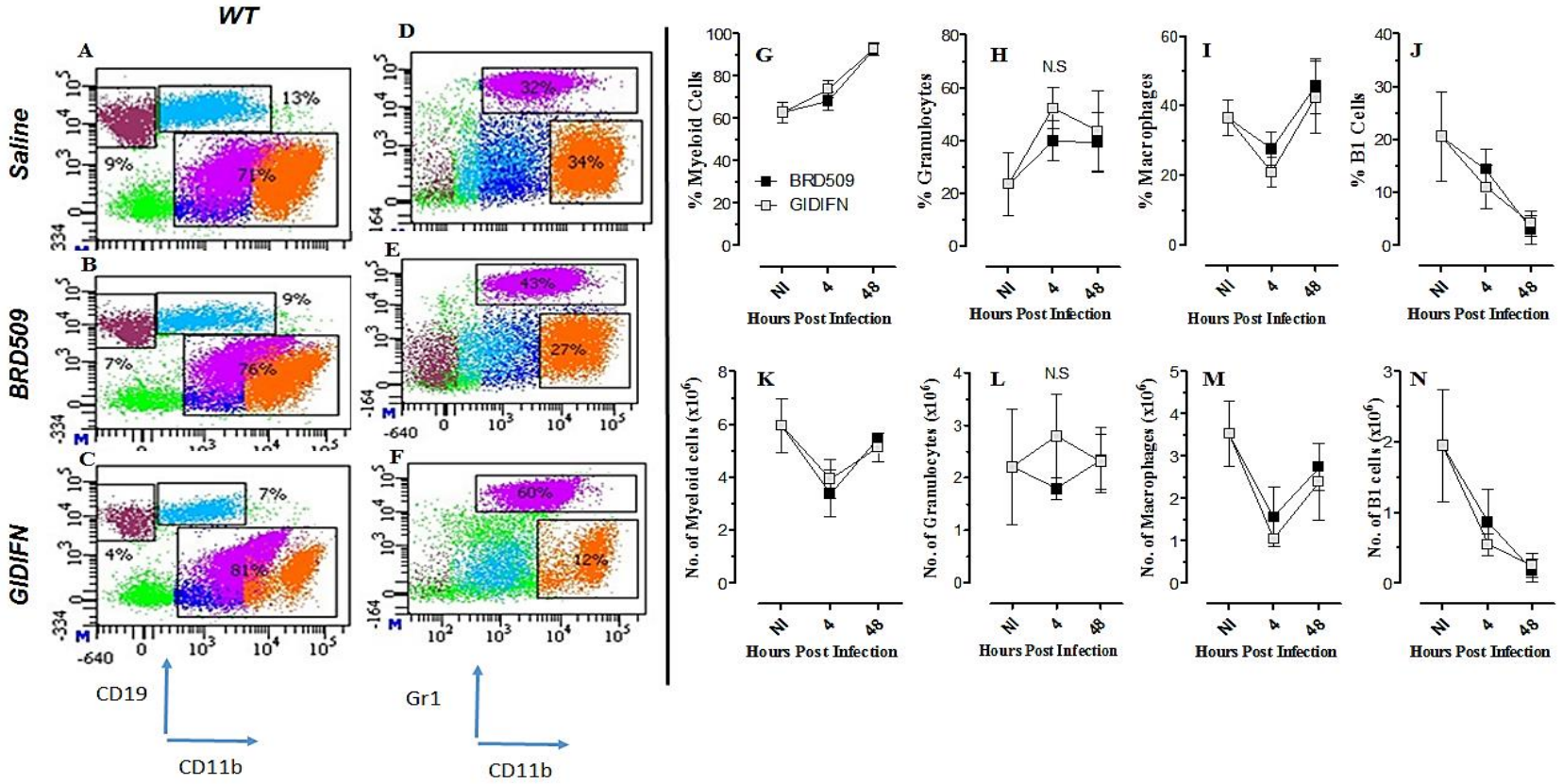

Figure 10: Flow cytometric analysis of peritoneal cells post i.p. Salmonella infection in WT mice.

Peritoneal cells were prepared from non-infected (A, D), BRD509E-infected (B, E) or GIDIFN-infected (C, F) WT mice (bacterial dose =1x10^6 CFUs/mouse). PECs were harvested 4 and 48 hours post inoculation and analyzed using mAbs specific for CD19, CD11b and Gr1 proteins. The results of analysis of 3 mice per group are shown as cell percentages (panels G-J) or absolute cell counts (panels K-N). Asterisks denote statistically significant differences between BRD509E and GIDIFN infected mice (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

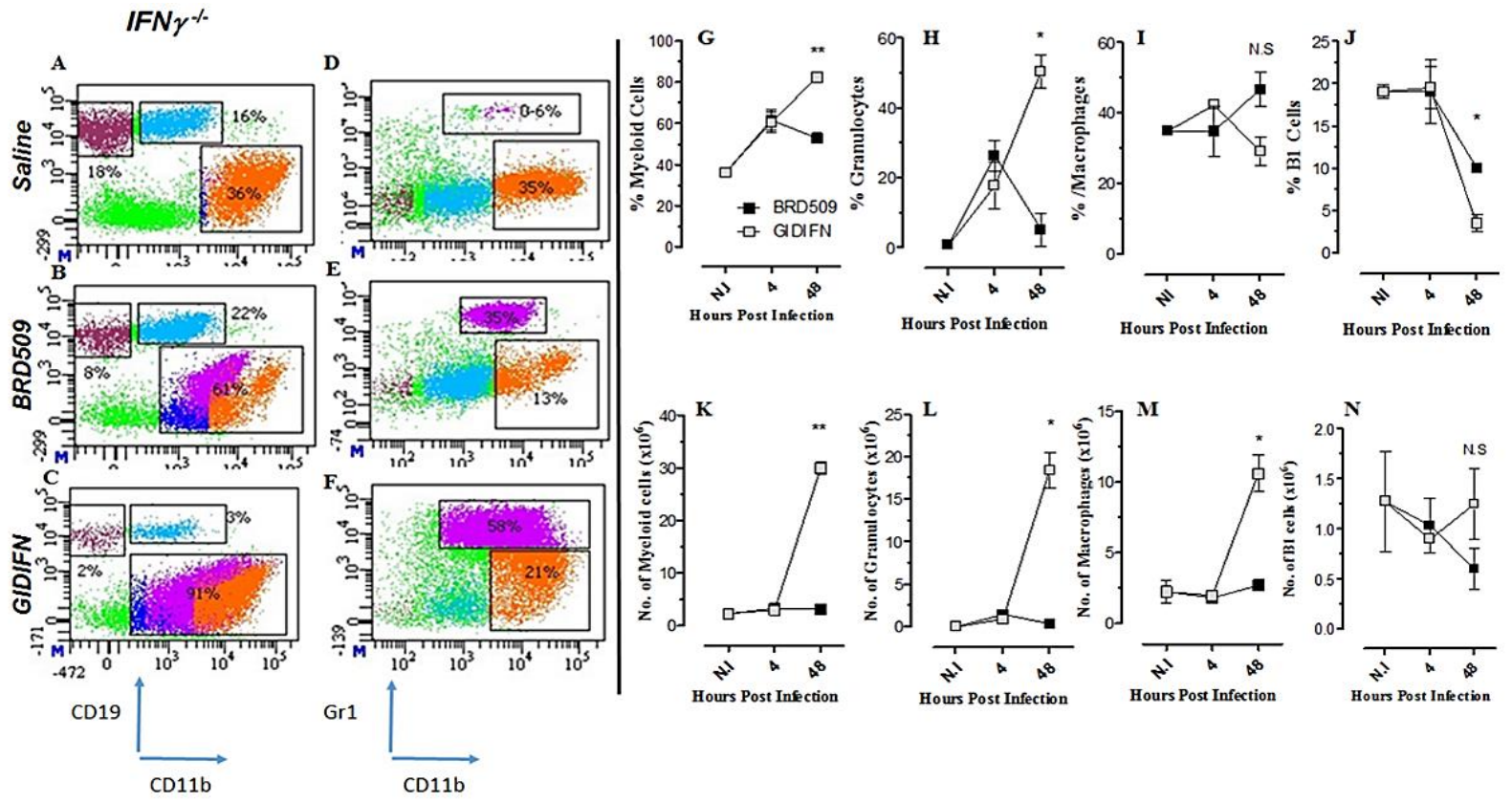

Figure 11: Flow cytometric analysis of peritoneal cells post i.p. Salmonella infection in IFN$\gamma^{-/-}$ mice.

Peritoneal cells were prepared from non-infected (A, D), BRD509E-infected (B, E) or GIDIFN-infected (C, F) WT mice (bacterial dose =1x10^6 CFUs/mouse). PECs were harvested 4 and 48 hours post inoculation and analyzed using mAbs specific for CD19, CD11b and Gr1 proteins. The results of analysis of 3 mice per group are shown as cell percentages (panels G-J) or absolute cell counts (panels K Asterisks denote statistically significant differences between BRD509E and GIDIFN infected mice (*p < 0.05; **p < 0.01; ***p < 0.001).

3.6. Phenotypic analysis of cell populations in the spleen of Salmonella infected mice

We further assessed the ratios of different cell sub-populations in systemic organ (spleen) 7 days after infection with BRD509E or GIDIFN. As in the above, spleen cells from saline-injected mice were used as control, and analysis was based on comparing the changes of cell composition in the two groups of infected mice to control mice. The results of this analysis are shown in **Figures 13-14**.

Flow cytometric analysis using FITC-conjugated CD3, PE-cy7-conjugated CD19, APC-cy7-conjugated CD11b and PE-conjugated Gr1 mAbs is shown in **Figures 13-14**. In uninfected WT mice, cells comprising the spleen were identified as follows; 50% $CD19^+$ (B cells), 30% $CD3^+$ (T cells) and 12% $CD19^-$ $CD3^-$ (myeloid cells) **(Fig. 13*A*).** Myeloid cells sub-populations are identified by their surface expression of Gr1 into: 4.4% $Gr1^+$ $CD11b^+$ (granulocytes) and 7.6% $Gr1^-$ $CD11b^+$ (macrophages) **(Fig. 13*D*)**. Upon infection with BRD509E, the ratio of myeloid cells increases 2.6-fold, with almost similar increase in the ratios of granulocytes and macrophages (3.2-fold and 2.2-fold, respectively) compared to uninfected mice **(Fig. 13*G*-*H*)**. Similar findings were observed in GIDIFN-infected mice, in the matter of fold increase to uninfected and compared to BRD509E **(Fig. 13*G*-*H*)**. Infection with BRD509E or GIDIFN cause a 1.3-fold decrease in the ratio of B cells compared to uninfected mice **(Fig. 13*J*)**. Similarities in the cell ratios observed were correlated to the spleen weight and absolute count values **(Fig. 12*A*-*B*)**.

Data obtained from $IFN\gamma^{-/-}$ mice showed similar findings, in the matter of cell ratios to uninfected mice and between the two bacterial strains **(Fig. 14*G*-*I*)**.

Nevertheless, GIDIFN-infected mice showed a 1.3-fold increase in the ratio of granulocytes compared to BRD509E **(Fig. 14*H*)**. Finally, comparison of the absolute cell counts between the WT and IFNγ$^{-/-}$, shows similarities in all cells, except for B cells which is 1.5-fold more in WT than IFNγ$^{-\backslash-}$ **(Fig. 13*K-N* & Fig. 14*K-N*)**, which correlates with the higher total spleen cell counts in WT than IFNγ$^{-\backslash-}$ mice **(Fig. 12*A-B*)**.

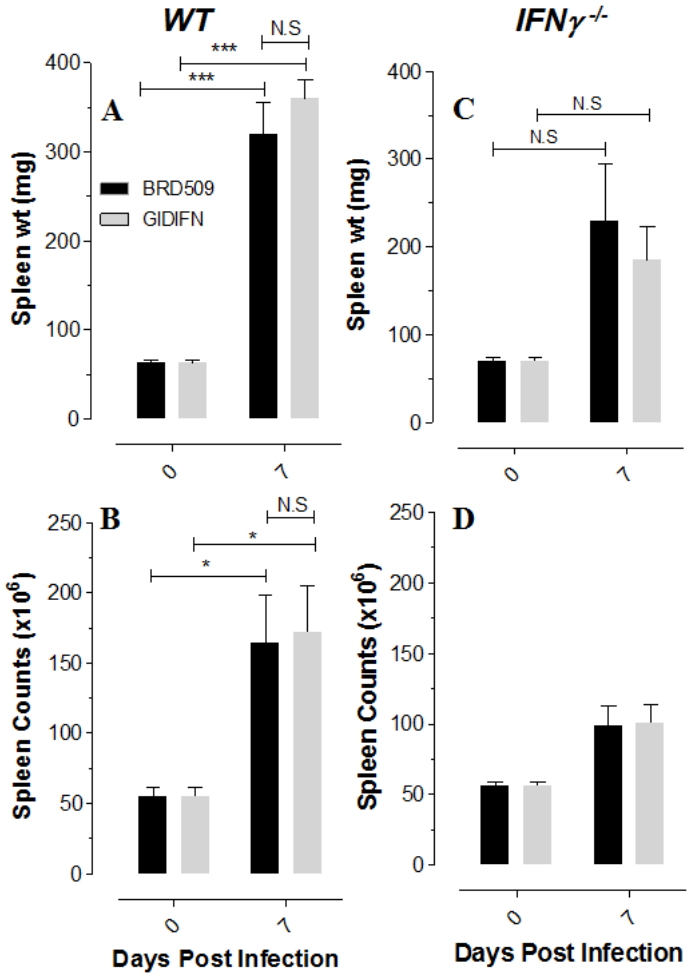

Figure 12: Infection induced splenomegaly in Salmonella-injected WT and IFNγ$^{-/-}$ mice.

Animals were inoculated with ~1x10^6 CFUs/mouse of BRD509E or GIDIFN strain, and spleens were aspectically removed at day 7 post infection. Data presented as spleen weights and absolute splenocyte count for WT (panels A-B) and IFNγ$^{-/-}$ (panels C-D). Each data point represents the mean ± SEM of 3-4 mice per group. Asterisks denote statistically significant differences between BRD509E and GIDIFN infected mice (*p < 0.05; **p < 0.01; ***p < 0.001).

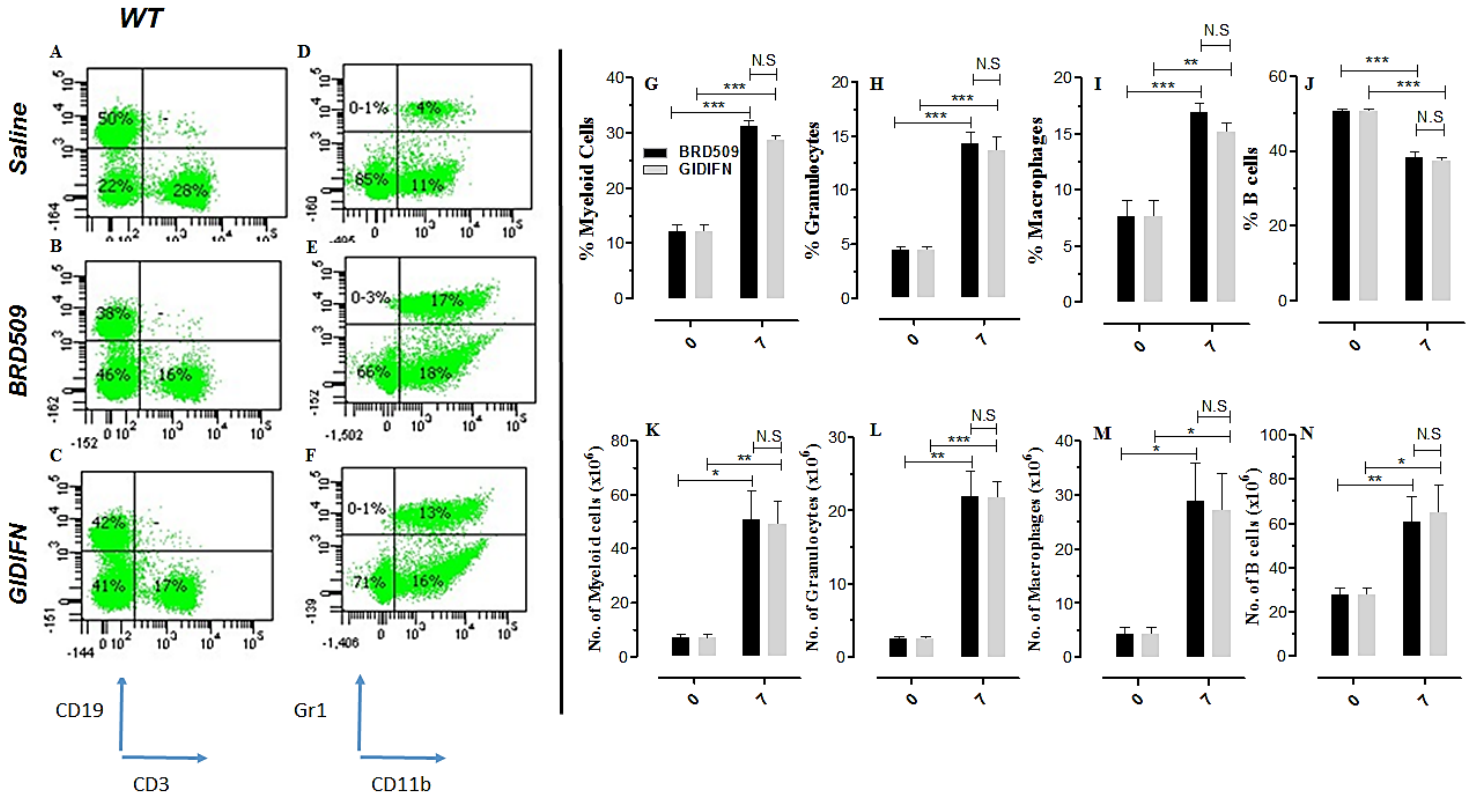

Figure 13: Flow cytometric analysis of spleen cells post Salmonella infection in WT mice.

Splenocytes were prepared from non-infected (A, D) or BRD509E-infected (B, E) or GIDIFN-infected (C, F) WT mice. ~1x10^6 CFUs/mouse was inoculated i.p and spleens were harvested after 7 days. Cells were analyzed using mAbs specific to CD3, CD19, CD11b and Gr1 proteins and compared to saline group in the form of percentage of total cells (panels G-J) or absolute count (panels K-N). Each data point represents the mean ± SEM of 3-6 mice per group Asterisks denote statistically significant differences between BRD509E and GIDIFN infected mice (*p < 0.05; **p < 0.01; ***p < 0.001).

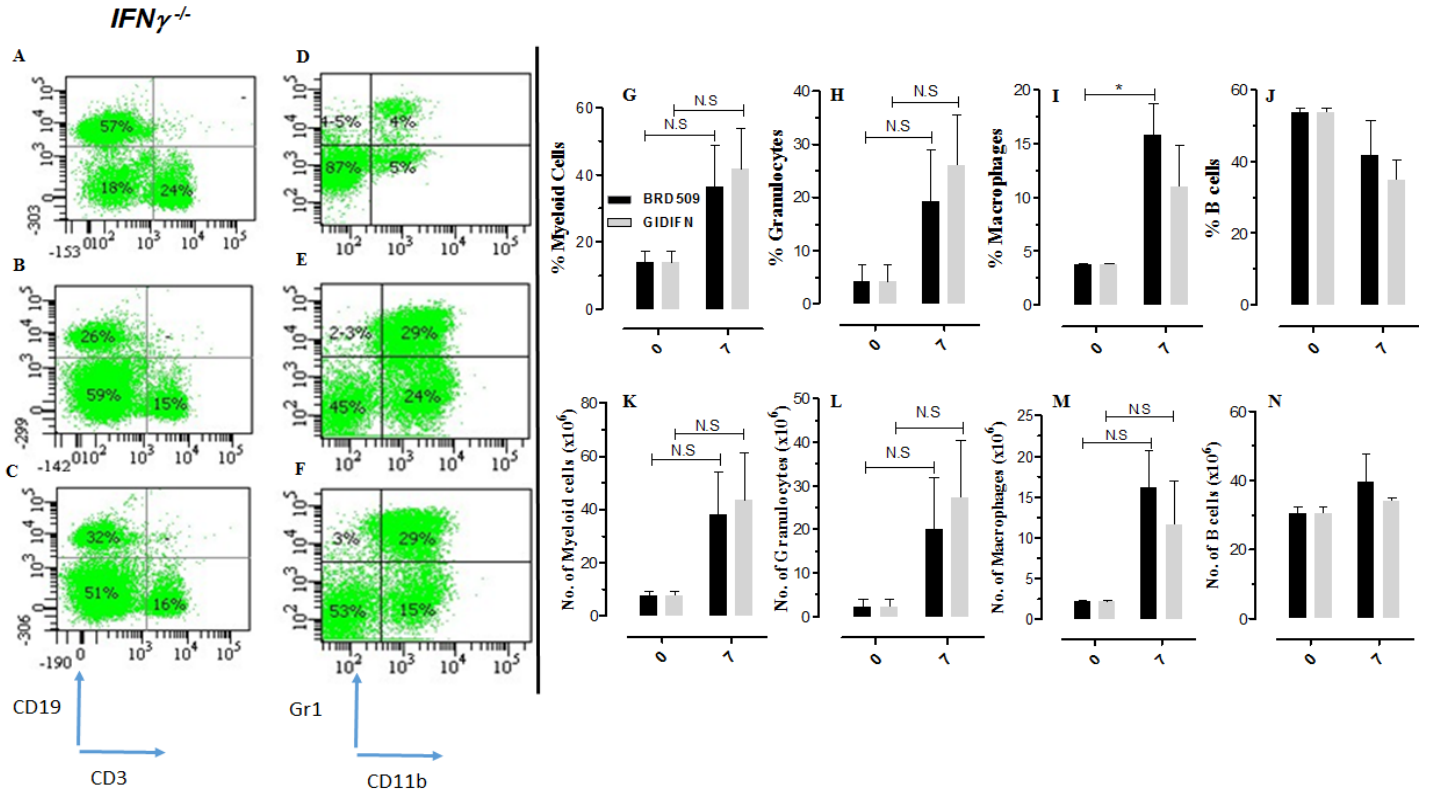

Figure 14: Flow cytometric analysis of spleen cells post Salmonella infection in IFNγ$^{-/-}$ mice.

Splenocytes were prepared from non-infected (A, D) or BRD509E-infected (B, E)or GIDIFN-infected (C, F) IFNγ$^{-/-}$ mice. 1x10^{6} CFUs/mouse was inoculated i.p and spleens were harvested after 7 days. Cells were analyzed using mAbs specific to CD3, CD19, CD11b and Gr1 proteins and compared to saline group in the form of percentage of total cells (panels G-J) or absolute count (panels K-N). Each data point represents the mean ± SEM of 3-6 mice per group. Asterisks denote statistically significant differences between BRD509E and GIDIFN infected mice (*p < 0.05; **p < 0.01; ***p < 0.001).

3.7. Antimicrobial effect of GIDIFN on ex vivo-cultured peritoneal exudate cells

We assessed the activation status of macrophages in response to Salmonella infection. Nitric oxide (NO) is an essential anti-microbial compound produced by activated macrophages in response to Salmonella infection. Thioglycolate-elicited peritoneal exudate cells were harvested from WT and IFN$\gamma^{-/-}$ mice and cultured with BRD509E or GIDIFN organisms, as described in methods section. Four hours after initiation of culture, cell-free supernatants were collected and analyzed for NO content by the Griess assay. As shown in **Figure 15**, the extent of NO production was dependent on the bacterial dose used in culture, judging by the increased NO response as the multiplicity of infection (MOI) increases. WT mice infected with BRD509E shows increasing level of NO response of 5.6mM, 8.2mM and 54.9mM corresponding to 5:1, 30:1 and 60:1 MOI, respectively **(Fig. 15*A*)**. The response elicited by GIDIFN Salmonella strain was superior to with BRD509E, with 1.9-fold and 1.3-fold increase in 30:1 and 60:1 MOI, respectively **(Fig. 15*A*)**. By contrast, NO response in IFN$\gamma^{-/-}$ mice shows similar trend in the increased level in correspondence to MOI, with GIDIFN Salmonella strain showing superiority in level of NO response observed as a 1.8-fold and 2-fold increase compared to BRD509 at 30:1 and 60:1 MOI, respectively **(Fig. 15*B*)**.

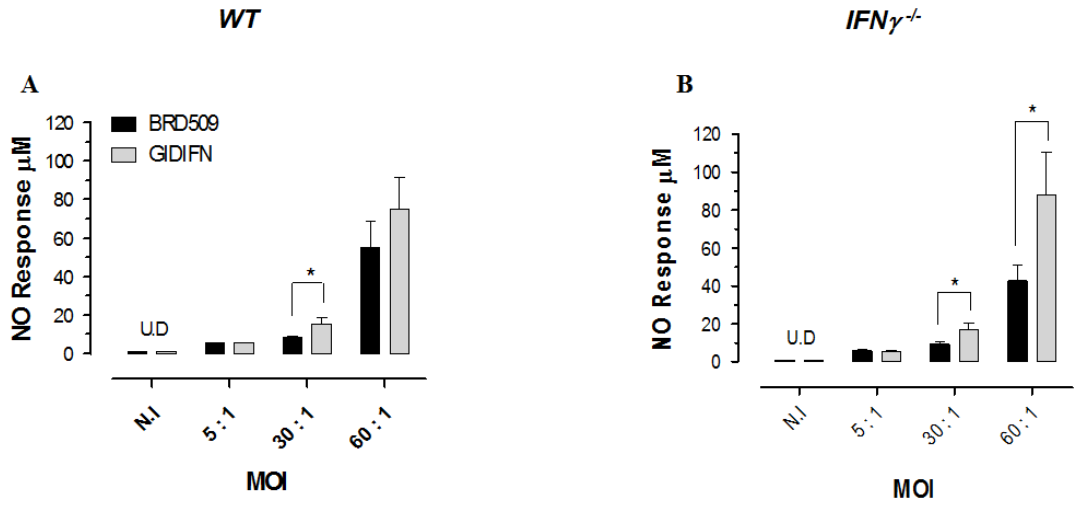

Figure 15: Enhanced production of NO from PECs infected ex-*vivo* by GIDIFN.

Thioglycolate elucidated PEC were harvested from WT (A) and IFNγ$^{-/-}$ (B) and infected for 1 hour with BRD509E or GIDIFN in antibiotic-free medium at different MOI. At the end of the infection period, non-internalized bacteria were killed with gentamicin, and cells were further incubated for 4 hours, after which cell-free culture supernatants were collected and analyzed for nitrite content using the Greiss method. Each data point represents the mean ± SEM of the supernatants of 5 ex *vivo* independent experiments. One way ANOVA was used to calculate statistical difference.

Finally, we compared the gene expression profiles of cells from the above ex-vivo experiment in the expression of NO gene regulator (iNOS) and S100A9 (an early inflammatory marker). Data of fold change and relative expression of WT and IFNγ$^{-/-}$ mice are shown in **Figure 16**. Starting with iNOS, BRD509E-infected WT cells, shows a minor increase in the expression of iNOS presented as 1.25-fold increase compared to saline-treated group **(Fig. 16*A*/*C*)**. On the other hand, GIDIFN-infected cells, shows superiority with 8.8-fold and 7-fold increase compared to uninfected and BRD509E-infected cells, respectively **(Fig. 16*A*/*C*)**. Remarkably, the expression of iNOS in IFNγ$^{-/-}$ mice is much higher than WT, with 15.7-fold and 73.7-fold higher gene expression in BRD509E and GIDIFN infected cells, respectively

(Fig. 16*E*/*G*). In addition, GIDIFN infected to IFNγ$^{-/-}$ mice, express iNOS 648.6-fold higher than control, and 33.1-fold higher than BRD509E-infected cells of the same mouse strain **(Fig. 16*E*/*G*)**.

Next, S100A9 gene expression of WT cells shows high level of expression in response to Salmonella infection, presented as 25.4-fold and 34.3-fold increase of BRD509E and GIDIFN, respectively as compared to control. Moreover, GIDIFN express the gene 1.3-fold higher than BRD509E **(Fig. 16*B*/*D*)**. By contrast, IFNγ$^{-/-}$ cells expressed the S100A9 gene in a similar trend as in the WT when compared to control, with the exception of GIDIFN expressing the gene 0.5-fold lower than BRD509E **(Fig. 16*F*/*H*)**. Interestingly, the expression of S100A9 by WT is far more than IFNγ$^{-/-}$, which could suggest that expression of this gene is impaired in the absence of IFN-γ **(Fig. 16*B*/*D; F/H*)**.

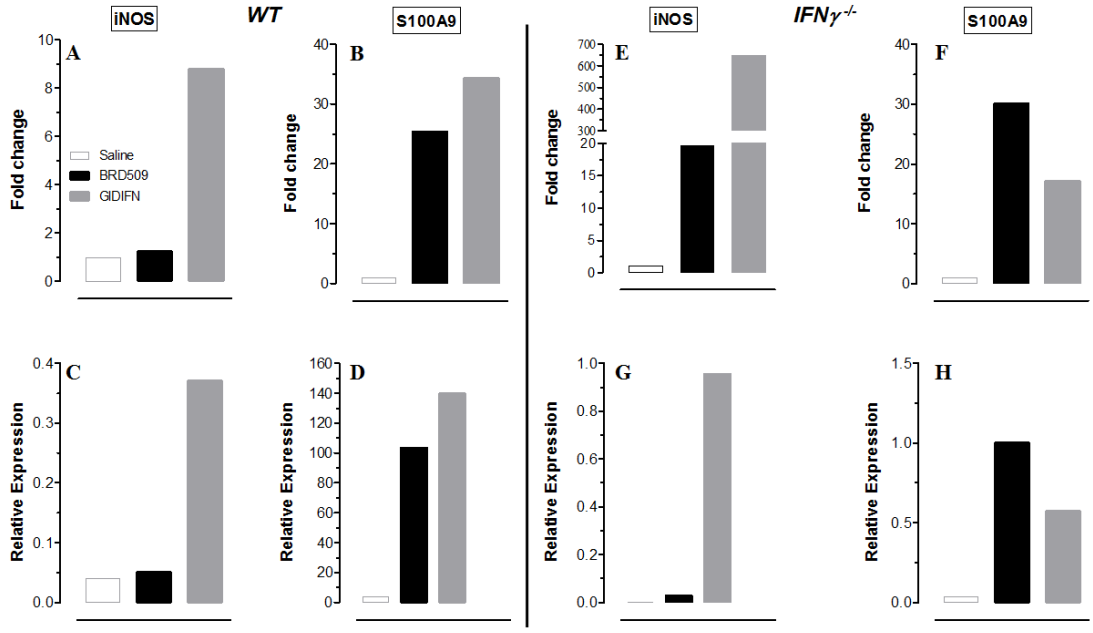

Figure 16: Enhanced antimicrobial gene expression of GIDIFN infected cells.

Thioglycolate elucidated PEC were harvested from WT (A-D) and IFNγ$^{-/-}$ (E-H) and infected for 1 hour with BRD509E or GIDIFN in antibiotic-free medium at 5:1 MOI. At the end of the infection period, non-internalized bacteria were killed with gentamicin, and cells were further incubated for 4 hours. Cells were analyzed for their gene expression by RT-PCR for iNOS and S100A9. Data is presented as fold change compared to the non-infected sample for the WT on the left panel (A-B) and IFNγ$^{-/-}$ on the right panel (E-F), or as relative expression of mRNA of WT (C-D) and IFNγ$^{-/-}$ (G-H). Each data represents the gene expression of one experiment.

Chapter 4: Discussion

Our lab has previously demonstrated the immune-potentiating properties of GIDIFN, a recombinant derivative of the attenuated *aroA/aroD* double auxotrophic mutant BRD509E, which has been engineered to express murine IFN-γ under the control of nirB promoter (al-Ramadi et al., 2001). Recently, GIDIFN was shown to be a more efficient vaccine candidate in two different immunodeficient mouse models, the C3H/HeJ ($TLR4^{-/-}$ $Nramp1^{R}$) and $CD154^{-/-}$ ($TLR4^{+/+}$ $Nramp1^{S}$) mice (Al-Ojali et al., 2013; Al-Ojali et al., 2012).

In the present study, IFN-γ expression by an attenuated Salmonella strain, GIDIFN, was able to modulate the immune response of two hypersusceptible mouse strains, MyD88-deficient ($MyD88^{-/-}$) and IFN-γ-deficient ($IFN\gamma^{-/-}$) mice. This provides support for the notion that cytokine-expressing, vaccine, strains of Salmonella may be more amenable for use in immunodeficient hosts in comparison with the parental, non-cytokine-expressing strain.

4.1. IFN-γ expression by GIDIFN enhances the survival of extremely susceptible $IFN\gamma^{-/-}$ mice

Salmonella is a facultative intracellular pathogen that causes systemic typhoid infection. Virulence of these organisms is linked to their capacity to penetrate the intestinal epithelium and target the phagocytic cells as its replication niche inside a compartment called Salmonella containing vacuole (SCV) (Chakravortty, 2002). Controlling bacterial replication at this stage is a decisive factor in determining the susceptibility of the host to Salmonella infection. This is in part controlled by the *Nramp1* gene, an innate resistance trait for Salmonella infection (Gruenheid & Gros,

2000; Lang et al., 1997). *Nramp1* is essential in resistance to salmonella infection through the enhancement of phagocytic cell recruitment and phagocytosis process of intracellular pathogen (Soo et al., 1998). Hosts with mutated *Nramp1* as, in the case of C57BL/6 mice, are considered susceptible to Salmonella due to the reduced macrophage killing efficacy (Govoni et al., 1999).

Immunity to Salmonella is accomplished through a robust Th1 response, that is mainly dependent on IFN-γ, that would regulate the other components of the immune system to clear the infection (Shtrichman & Samuel, 2001; al-Ramadi et al., 2006). The role of IFN-γ in susceptibility to Salmonella was first demonstrated in a study in which depletion of IFNγ by a specific mAb resulted in increased susceptibility of naturally resistant mice, with bacteria proliferating systemically and mice succumbing to infection after a week of infection (Nauciel & Espinasse-Maes, 1992). Furthermore, Bao and colleagues demonstrated the susceptibility of IFN-γ deficient mice in an oral infection model using 5 x 10^8 CFUs of BRD509E strain, which resulted in disseminated septicemia two weeks later (Bao et al., 2000).

In the current study, IFN-γ deficient mice were tested for their differential susceptibility to BRD509E and GIDIFN strain. Our results show that mice infected with a very low dose of 300 CFUs/mouse via the i.p. route succumb to the infection by day 28 post inoculation. These results emphasize the role of IFN-γ in immunity to attenuated Salmonella. Accordingly, we hypothesized that IFN-γ-expression by the recombinant GIDIFN strain could compensate for the lack of IFN-γ and induce a better immune response in IFN-γ deficient hosts.

Compared to BRD509E, GIDIFN strain significantly enhanced the survival of IFN-γ-deficient mice infected i.p with the same dose. Susceptibility of IFN-γ-

deficient mice to BRD509E is associated with a loss of bacterial control in target organs. As shown here, even at a dose of 300 CFUs/mouse, the bacterial burden in the liver of IFN-γ-deficient mice was 94-, 1900-and 78,500-fold higher than WT mice at 7, 10 and 14 days post infection, respectively. Although the expression of IFN-γ by GIDIFN was not enough to provide the same level of bacterial control as in WT mice, it nevertheless resulted in a significantly reduced bacterial burden in the liver compared to the BRD509E strain that was observed at early time points following infection. This is consistent with early identification of IFN-γ as a key regulator of innate immunity to Salmonella infection (Hess et al., 1996).

The effect of GIDIFN on susceptibility of MyD88-deficient mice was also investigated. Many studies have shown the susceptibility of these mice to bacterial and viral infections (Scanga et al., 2002; Lund et al., 2003). Furthermore, mice lacking MyD88 are highly susceptible to attenuated strains of *S. typhimurium* due to their inability to control systemic bacterial spread and growth in target organs, accompanied with delayed recruitment of phagocytic cells, and defective cytokine production (Issac et al., 2013; al-Ramadi et al., 2004).

As an adaptor molecule for the signaling pathways of most TLRs and other proinflammatory cytokines, such as IL-2 and IL-18, MyD88 has a great impact in the initiation of anti-Salmonella immune response through the production of these proinflammatory cytokines (Raupach et al., 2006). MyD88$^{-/-}$ mice exhibited increased susceptibility to attenuated Salmonella, with an overall survival rate of only 30% when given a low dose of 10^3 CFUs/mouse i.p. In sharp contrast, infection with GIDIFN resulted in a significant enhancement with ~90% of the infected animals surviving a similar dose infection. The enhancement in survival was associated with better control of bacterial replication in target organs.

A comparison of host susceptibility in the two immunodeficient mouse models clearly shows that IFN-$\gamma^{-/-}$ mice are far more susceptible than MyD88$^{-/-}$ counterparts. Infection of IFN-$\gamma^{-/-}$ mice with a dose that was 3 fold lower than the lowest dose used in MyD88$^{-/-}$ mice (300 vs. 1000 CFUs/mouse) still resulted in earlier and more pronounced mortality in the former mouse strain. The fact that GIDIFN could enhance the survival of these extremely susceptible animals is an indication of the potential significance of this strategy to boost anti-bacterial immunity in susceptible hosts.

4.2. GIDIFN enhances the recruitment of peritoneal myeloid cells

Early recognition of a pathogen is the first stage of the early innate immune response. In the case of Salmonella infection, phagocytes can sense the presence of Salmonella through the recognition by TLRs of Salmonella-associated PAMPs, including lipopolysaccharide (by TLR4), bacterial lipoproteins (TLR2), flagellin (TLR5) and CPG DNA (TLR9) (Knapp, 2010). Activation of TLRs leads to the production of proinflammatory cytokines such as TNF-α, IL-6 and IL-12 in a MyD88-dependent manner through the activation of transcription factor NF-κB (Akira & Takeda, 2004).

Phagocytic cells, including macrophages, neutrophils and dendritic cells, are crucial during the early stage of an infection for the control of bacterial growth and recruitment of other immune cells by production of cytokines and chemokines. Cytokines such as IL-12 mediates immunity to Salmonella through the induction of IFN-γ production by NK cells and facilitates the development of a Th1 response (Castro et al., 1995; Manetti et al., 1993).

Flow cytometric analysis of peritoneal cells collected at 4 hr or 48 hr after infection with BRD509E or GIDIFN Salmonella strain revealed the superior capacity of GIDIFN to recruit myeloid inflammatory cells. This was particularly observed by the sustained increase in these cells at 48 hr post inoculation. Previous studies have ascribed a role for IFN-γ in controlling neutrophil influx through regulating the chemokine secretion in response to microbial triggers as well as modulating the expression of chemokine receptors (Robson et al., 2001; Bonecchi et al., 1999). In our study, hypersusceptibility of IFN-$\gamma^{-/-}$ mice to Salmonella infection could be partly due to defective recruitment of inflammatory cells. This is consistent with a study in which a defect in IFN-γ impaired neutrophil recruitment, which was restored upon treatment with recombinant IFN-γ through normalization of PMN-activating CXC chemokine expression (McLoughlin et al., 2003).

A comparison of the phenotypic changes at later time points in a systemic organ (spleen) between BRD509E or GIDIFN-infected WT and IFN-$\gamma^{-/-}$ mice surprisingly shows no difference in the extent of myeloid cell recruitment induced by either bacterial strain, suggesting that the observed GIDIFN-mediated enhancement is most likely due to its effect at the early phase of infection.

4.3. GIDIFN enhances the anti-microbial effector functions of macrophages

Salmonella is an intra-macrophage pathogen and elimination of such a microbe requires the activation of bacteria-infected macrophages through IFN-γ. This activation induces the bactericidal process through the production of nitric oxide (NO) and reactive oxygen radicals resulting in increased access of the lytic effector molecules to the salmonella containing vacuole (Richter-Dahlfors et al., 1997). In

addition, IFN-γ enhances antigen presentation via MHC class II and stimulate NK cells to produce more IFN-γ and NO via IL-12 production (Liew, 1995). Nitric oxide has an important role in the killing process of different microbes. Liew and his group demonstrated the effect of IFN-γ activated macrophages in killing intracellular leishmania parasites by NO, which could be inhibited through the addition of a compound (L-NMMA) which is an antagonist for the L-arginine necessary for the NO production pathway (Liew et al., 1990). Another study have shown the effect of neutralizing IL-12 in the dissemination of Salmonella to target organs, due to reduced level of serum IFN-γ and downregulation of MHC class II and iNOS (Nitric oxide synthase), all of which reversed upon treatment with recombinant IFN-γ (Mastroeni et al., 1998).

In the present study, co-culture of GIDIFN with PECs induced an enhancement in the level of NO and upregulated iNOS gene expression. In fact, co-culture of WT PECs with BRD509E at MOI of 5:1 for 1 hr failed to increase iNOS gene expression to any significant level above control. However, when GIDIFN was used at the same culture conditions, there was a dramatic 7-fold increase in iNOS expression. Similar findings were observed when IFN$\gamma^{-/-}$ PECs were used, with GIDIFN strain enhancing iNOS expression by 30-fold over the level seen in cells cultured with BRD509E strain. Together with previous studies, these findings suggest that IFN-γ expression by GIDIFN enhances the ability to activate effector macrophages even in IFN$\gamma^{-/-}$ mice. These findings are consistent with previous data from our laboratory that reported the effect of GIDIFN on peritoneal cells harvested from thioglycolate-injected BALB/c mice. The capacity of GIDIFN to induce macrophage activation as evidenced by upregulated expression of macrophage

activation markers, strong induction of proinflammatory cytokines including, TNF-α, IL-6 and IL-12 and increased production of antimicrobial effector molecules, including NO (Fernandez-Cabezudo et al., 2009).

Another marker used to assess macrophage activation is S100A9, which is a protein expressed on phagocytes that first infiltrate inflammatory sites (Nacken et al., 2003). S100A9 has an important role in neutrophil recruitment in response to LPS stimulation (Vandal et al., 2003). S100A9 is an important recognition molecule of endogenous danger signals in phagocytes and regulates myeloid cell function by binding to TLR-4 (Vogl et al., 2007; Ehrchen et al., 2009). S100A9 dimerizes with S100A8 to form a complex (known as calprotectin), which is found predominantly in myeloid cells, and is actively secreted in response to stress in phagocytic cells (Markowitz et al., 2013). In one study, S100A9 was reported to act as an inducer of nitric oxide production by murine macrophages in iNOS-dependent manner (Pouliot et al., 2008). In addition, this study showed that S100A9 and IFN-γ have a synergistic effect on nitric oxide generation, suggesting an important role of S100A9 in cytokine-mediated activation of phagocytes during the innate immune response and its effect on modulating antimicrobial functions (Pouliot et al., 2008).

Analysis of S100A9 gene expression in BRD509E- vs. GIDIFN-treated PECs yielded a different picture. In this case, expression of S100A9 was predominantly induced by co-culture of PECs with BRD509E bacteria, resulting in 25- and 30-fold increase over control in WT and IFNγ$^{-/-}$ PECs, respectively. Similar cultures with the GIDIFN bacterial strain showed either a modest increase (~1.3-fold in WT) or decrease (~1.7-fold in IFNγ$^{-/-}$) compared to BRD509E. These data demonstrate the co-requirement for TLR-mediated signaling and IFN-γ in the optimal activation of

iNOS. In contrast, S100A9 induction appears to be dependent on TLR signals but mostly independent of IFN-γ.

In summary, this study demonstrated the capacity of a genetically-engineered, IFNγ-expressing Salmonella strain (GIDIFN) to significantly enhance the survival of two immunodeficient, Salmonella-hypersusceptible mouse strains, namely MyD88$^{-/-}$ and IFN-γ$^{-/-}$ mice. This enhancement was correlated with decreased bacterial loads in systemic organs, possibly due to the enhanced ability of GIDIFN to activate effector macrophages, as shown by increased synthesis of anti-microbial effector molecules, including NO. These findings suggest the potential use of attenuated bacterial strains expressing immunomodulatory genes as a therapeutic approach in immunodeficient hosts.

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