The Roles of Selectin Ligands and Innate Immune Responses in Modulating Resistance to Intracellular Bacterial Infections in Murine Hosts with Altered Immunity

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ABSTRACT

Listeria monocytogenes (LM) and *Salmonella enterica* serovar Typhimurium (ST) are intracellular bacterial pathogens that cause invasive disease in immune-altered individuals, including the immunocompromised and pregnant women. The mechanisms that modulate innate immunity to intracellular infection, particularly during pregnancy, are not well-understood. Functional selectin ligands play critical roles in leukocyte recruitment during inflammation. Increased control of LM infection in functional selectin ligand-deficient (FtDKO) mice is associated with increased levels of circulating innate immune cells, despite defective leukocyte migration compared to WT mice. Adoptive transfer of WT and FtDKO bone marrow (BM) cells to irradiated WT and FtDKO recipients demonstrates that BM reconstitution and the increased neutrophil phenotype of FtDKO mice is independent of functional selectin ligand expression within the host environment. Thus, functional selectin ligand deficiency enhances inherent innate immune resistance to intracellular infection. We then examined the impact of pregnancy-associated immunological changes on maternal susceptibility to intracellular infections. ST infection in pregnant mice results in profound systemic infection, increased fetal loss and enhanced serum and placental expression of pro-inflammatory cytokines. Pregnant mice showed decreased ratios of pro-inflammatory Th17 cells relative to anti-inflammatory regulatory T cells (Tregs) when compared to non-pregnant mice during infection. Functional inactivation of Tregs *in vivo* restored control of infection and normal Th17-to-Treg ratios, and reduced fetal loss. These indicate that modulation of Th17 and Treg responses impacts maternal and fetal protection from ST infection. Lastly, we examined the roles of type I interferons (IFNs) in modulating innate immunity to intracellular infections during pregnancy. Type I IFN receptor deficiency ($IFNAR^{-/-}$) enhances immunity

to LM and ST in the non-pregnant state by limiting pathogen-induced leukocyte death. We show that pregnant IFNAR$^{-/-}$ mice infected with LM retain increased protection from infection relative to WT controls. In contrast, protection conferred by IFNAR deficiency against ST infection in the non-pregnant state is abrogated during pregnancy. Distinctive maternal responses to LM and ST are associated with differential regulation of leukocyte distribution and cytokine expression in maternal systemic and/or placental compartments. Taken together, modulation of key mechanisms involved in leukocyte recruitment, immune-regulation and cytokine signaling impact host susceptibility to intracellular infections.

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LIST OF ABBREVIATIONS

APC	Antigen-Presenting Cell
B16-OVA	Murine Melanoma Cell Line Expressing Plasmid-Derived Ovalbumin
B6.SJL	B6.SJL-*Ptprc*^{a} *Pep3*^{b}/BoyJ mouse
BCR	B Cell Receptor
BHI	Bacto™ Brain-Heart Infusion Media
BM	Bone Marrow
BMDM	Bone Marrow-Derived Macrophage
bp	Base Pair
CBA	Cytometric Bead Array
CD	Cluster of Differentiation
CD62E	E-selectin
CD62L	L-selectin
CD62P	P-selectin
cDNA	Complementary DNA
CFU	Colony Forming Unit
CFU-G	Granulocyte Colony-Forming Unit
CFU-GM	Granulocyte-Macrophage Colony-Forming Unit
CFU-M	Macrophage Colony-Forming Unit
CLP	Common Lymphoid Progenitor
CMP	Common Myeloid Progenitor
DC	Dendritic Cell
EDTA	Ethylenediaminetetraacetic Acid

FtDKO	α-(1,3)-Fucosyltransferase-IV and -VII-Deficient Knock Out Mouse
FucT-IV	α-(1,3)-Fucosyltransferase-IV
FucT-VII	α-(1,3)-Fucosyltransferase-VII
GMP	Granulocyte-Macrophage Progenitor
HSC	Hematopoietic Stem Cell
IFNAR$^{-/-}$	Type I IFN-α Receptor-Deficient Mouse
IFN-γ	Interferon-Gamma
IL-6	Interleukin-6
IL-10	Interleukin-10
IL-12p70	Interleukin-12 (p35 and p40 Heterodimer)
IL-17	Interleukin-17
iNOS	Inducible Nitric Oxide Synthase
i.p.	Intraperitoneal
i.v.	Intravenous
KC	Keratinocyte Chemoattractant
LM	*Listeria monocytogenes* 10403s (wild-type)
LN	Lymph Node
LPS	Lipopolysaccharide
mAb	Monoclonal Antibody
MCP-1	Monocyte Chemoattractant Protein-1
MFI	Mean Fluorescence Intensity
µg	Microgram
MHC	Major Histocompatibility Complex
µL	Microliter

mL	Milliliter
MOI	Multiplicity of Infection
ng	Nanogram
NK	Natural Killer Cell
NPI	Non-Pregnant Infected
NPNI	Non-Pregnant Non-Infected
Nramp1	Natural Resistance-Associated Macrophage Protein-1
OD	Optical Density
PAMP	Pathogen-Associated Molecular Pattern
PBS	Phosphate-Buffered Saline
pg	Picogram
PI	Pregnant Infected
PNI	Pregnant Non-Infected
PRR	Pattern Recognition Receptor
R8	RPMI Medium With 8% Fetal Bovine Serum
RNS	Reactive Nitrogen Species
RORγ-t	Retinoic Acid Receptor-Related Orphan Receptor Gamma
ROS	Reactive Oxygen Species
s.c.	Subcutaneous
ST	*Salmonella enterica* serovar Typhimurium SL1344
TCR	T Cell Receptor
Th1	T Helper-1 Cell
Th2	T Helper-2 Cell
Th17	T Helper-17 Cell

TLR	Toll-Like Receptor
TNF	Tumor Necrosis Factor
Treg	Regulatory T Cell
WT	Wild-Type C57BL/6J Mouse

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1.0 INTRODUCTION

1.1 Immunity to Infection

Immune responses to infection are classically categorized into innate and adaptive immunity. Innate immunity provides the first line of defense against invading pathogens. Various components of innate immunity then promote antigen-specific responses of adaptive immunity, which mediate pathogen clearance and provide protective recall responses to secondary infections. This chapter discusses the major components of innate and adaptive immunity, with focus on host protection from systemic infection by intracellular bacterial pathogens. We also present mechanisms involved in modulating host resistance to infection in the context of an altered maternal immune response during pregnancy.

1.2 Innate Immunity

Host recognition of the invading pathogen by pattern recognition receptors (PRRs) triggers a cascade of immune activation mechanisms, including pro-inflammatory cytokine expression and leukocyte (immune cell) recruitment, tasked to control primary infection. Innate immunity is often characterized as 'non-specific' due to its ability to initiate host defenses without prior encounter with the pathogen.

1.2.1 Toll-Like Receptors

Toll-like receptors (TLRs) are a major class of PRRs that initiate pro-inflammatory responses upon recognition of highly-conserved pathogen-associated molecular patterns (PAMPs) expressed by microbial pathogens. These transmembrane proteins are expressed by epithelial cells, natural killer (NK) cells and phagocytic cells, including neutrophils, monocytes, macrophages and dendritic cells (DCs) (Chalifour et al., 2004). Currently, 10

human and 13 murine TLRs have been identified, each having a specific microbial ligand (Table 1) (Leone and Moreau, 2014). TLR-dependent microbial recognition occurs through two main pathways: MyD88-dependent and MyD88-Independent/TRIF-dependent. The former pathway signals through the myeloid differentiation primary-response protein 88 (MyD88), an adaptor molecule that interacts with all TLRs (except TLR3 in most cases) to initiate downstream effector functions (Akira and Takeda, 2004). In contrast, the latter pathway requires the recruitment of Toll/interleukin-1 receptor-domain-containing adaptor protein inducing IFN-β (TRIF) to TLR3 and TLR4 to induce type I IFN cytokine expression (Yamamoto et al., 2003; Kawasaki and Kawai, 2014). It also features delayed activation kinetics relative to the MyD88-dependent pathway (Yamamoto et al., 2003). The two pathways lead to the activation of nuclear factor-κB (NF-κB) and mitogen- activated protein (MAP) kinases, both of which induce pro-inflammatory cytokine expression (Kawasaki and Kawai, 2014).

1.2.2 Cytokines

Cytokines are small, soluble proteins that mediate interactions between cells. They bind specific cytokine receptors and are secreted by both immune and non-immune cells, such as endothelial cells and fibroblasts (Zhang and An, 2007). Cytokines often exhibit functional redundancy and pleiotropy, wherein a single cytokine can act on multiple cell types (Ozaki and Leonard, 2002). These characteristics enable cytokines to exert wide-ranging effects on various physiological processes, including blood cell formation (hematopoiesis), inflammation and homeostasis. Together with other growth factors, cytokines drive the differentiation of hematopoietic stem cells (HSCs) into granulocytic, monocytic and lymphocytic progenitors and the maturation of progenitors into leukocytes (Robb, 2007). Chemotactic cytokines or

Table 1. Toll-like Receptors and Their Microbial Ligands*

TLRs	Localization	Ligand	Origin of the Ligand
TLR1/TLR2	Plasma membrane	Triacyl lipopeptides	Bacteria, mycoplasma
TLR6/TLR2	Plasma membrane	Diacyl lipopeptides Lipoteichoic acid Zymosan	Bacteria, viruses, parasites
TLR3	Endolysosome	Double-stranded RNA	Virus
TLR4	Plasma membrane	Lipopolysaccharide	Bacteria, viruses
TLR5	Plasma membrane	Flagellin	Bacteria
TLR6	Plasma membrane	Diacyl lipoprotein	Bacteria, viruses
TLR7	Endolysosome	Single-stranded RNA	Virus, bacteria
TLR8	Endolysosome	Single-stranded RNA	Virus, bacteria
TLR9	Endolysosome	Unmethylated DNA with CpG motifs	Virus, bacteria, protozoa
TLR10	Endolysosome	Unknown	Unknown
TLR11†	Plasma membrane	Profilin-like molecule	Protozoa
TLR12†	Endolysosome	Profilin	Protozoa (*Toxoplasma gondii*)
TLR13†	Endolysosome	23S ribosomal RNA	Bacteria

*TLRs may also recognize endogenous (*i.e.*, host) molecules. †Expressed in mice but not humans.
TLR = Toll-like receptor.

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Marc Leone, Richard Moreau; Leukocyte Toll-like Receptor 2-mitochondria Axis in Sepsis: Unraveling Immune Response Sophistication. *Anesthesiology* 2014;121(6):1147-1149. doi: 10.1097/ALN.0000000000000471.

chemokines specialize in cell migration during embryonic development, tissue vascularization, and in homeostatic and inflammatory conditions (Sokol and Luster, 2015; Wang and Knaut, 2014). Pro-inflammatory cytokines, including IFN-gamma (IFN-γ), tumor necrosis factor (TNF) and interleukin-6 (IL-6), are produced by innate and adaptive immune cells during infection to augment non-specific immune responses and promote the development of antigen-specific responses by T and B cells (Turner et al., 2014). Anti-inflammatory cytokines such as IL-10 regulate the cytokine "storm" induced by infection to minimize host tissue injury and prevent chronic activation of cells after infection (Dinarello, 2007). Dysregulation of pro- and anti-inflammatory cytokine expression can lead to autoimmune and other chronic inflammatory diseases (Dinarello, 2007).

1.2.2.1 Type I Interferons

Type I Interferons (IFNs) comprise a large family of cytokines produced in response to infection. The IFN-α and IFN-β subtypes play critical roles in activating innate and adaptive immune mechanisms towards early pathogen control and development of antigen-specific and memory cell responses (Alsharifi et al., 2008). Type I IFNs are induced upon pathogen recognition via transmembrane TLRs and cytoplasmic PRRs and in response to cytokines (Ivashkiv and Donlin, 2014; Trinchieri, 2010). TLR4 signaling is well-characterized for robust type I IFN production during bacterial infection (McNab et al., 2015). Cytoplasmic PRRs such as nucleotide-binding and oligomerization domain (NOD)-like receptors (NLRs) promote type I IFN expression upon recognition of foreign nucleic acids and bacterial peptides (Boxx and Cheng, 2016; McNab et al., 2015). Type I IFNs are expressed by various immune and non-immune cells, and signal through the ubiquitous transmembrane IFN alpha receptor (IFNAR) (Ivashkiv and Donlin, 2014). They can both act directly on cells and cross-talk with other

cytokines to shape immune responses. IFN-α is a potent inducer of NK cell trafficking, proliferation, cytolytic activity and type II IFN (IFN-γ) production during the innate immune response (Brassard et al., 2002). It also stimulates IFN-γ production by $CD4^+$ and $CD8^+$ T cells, and antibody production by B cells of the adaptive immune response (Brassard et al., 2002). Cross-talk between type I IFN and IFN-γ exert immune activation and regulatory functions. Increased type I IFN expression restrict IFN-γ-induced macrophage activation through downregulation of IFN-γ receptor 1 (IFNGR1) expression (Trinchieri, 2010). In contrast, reduced constitutive levels of type I IFNs sensitizes cells to stimulation by other cytokines, such as IL-6 and types I and II IFNs (Trinchieri, 2010). Other cytokines induced directly or indirectly by type I IFNs include IL-12, IL-15 and TNF (Brassard et al., 2002).

Type I IFNs play prominent roles in host protection from viral and bacterial infections. IFNα/β expression generally confers host protection from viral infections through induction of IFN-stimulated genes (ISGs), which encode proteins that inhibit viral replication, and promote innate immune activation and host cell death (Boxx and Cheng, 2016). However, IFNAR signaling is associated with reduced host resistance to chronic infection. For example, *in vivo* blockade of IFNAR prior to lymphocytic choriomeningitis virus (LCMV) infection compromises immunity to acute infection, but reduces viral burden upon establishment of chronic infection (Wilson et al., 2013). Enhanced control of chronic infection is associated with decreased expression of IL-10 and programmed death-ligand 1 (PD-L1) in the serum and on myeloid cells, respectively (Wilson et al., 2013). In a similar study, control of chronic infection in the absence of IFNAR signaling correlates with increased numbers of cytokine-producing LCMV-specific $CD4^+$ T cells in the spleen (Teijaro et al., 2013). These results indicate that type I IFNs are essential in the resolution of acute viral infection. However, they

promote infection persistence through modulation of immune-suppressive mechanisms and protective $CD4^+$ T cell responses. IFNAR signaling also induces differential effects on host immunity to bacterial infections. Type I IFNs control intracellular growth of *Legionella pneumophila* in murine macrophages through polarization of pro-inflammatory responses by classically-activated M1 macrophages and induction of anti-microbial nitric oxide (NO) production (Plumlee et al., 2009). IFN-α also limits *Chlamydia trachomatis* proliferation in human epithelial cells by regulating intracellular availability of tryptophan and iron to the bacteria (Ishihara et al., 2005). Synergistic cross-talk by IFN-α with IFN-γ and TNF also augments cytokine responses against infection (Ishihara et al., 2005). In contrast, C57BL/6J wild-type (WT) mice infected with *Listeria monocytogenes* (LM) bacteria exhibit reduced resistance to systemic infection relative to mice lacking functional IFNAR ($IFNAR^{-/-}$) due to enhanced expression of IFN-inducible apoptosis-associated genes and lymphocyte death (O'Connell et al., 2004; Carrero et al., 2004). WT mice also exhibit decreased splenic proportions of TNF-producing macrophages relative to $IFNAR^{-/-}$ mice during LM infection (Auerbuch et al., 2004). Similarly, WT mice are susceptible to systemic *Salmonella enterica* serovar Typhimurium (ST) bacterial infection due to enhanced necroptotic death of macrophages relative to their $IFNAR^{-/-}$ counterparts (Robinson et al., 2012). Necroptosis is an inflammatory form of programmed cell death characterized by receptor-interacting serine/threonine-protein kinase 1 (RIP1)-mediated activation of downstream mediators RIP3 and mixed lineage kinase domain-like (MLKL) pseudokinase (Zhou and Yuan, 2014). Type I IFNs mediate necroptosis by promoting RIP1 and RIP3 activation, and RIP1 engagement of IFNAR (Robinson et al., 2012). Thus, the differential impact of IFNAR signaling on host immunity to viral and bacterial pathogens highlights the complex immune activation and regulatory networks mediated by type I IFNs.

1.2.3 Functional Selectin Ligands

Leukocyte recruitment involves tethering, rolling and adhesion of leukocytes on the endothelial wall of blood vessels prior to transmigration into infection sites. This process is initiated upon recognition of PAMPs by PRRs expressed on innate immune cells (Mogensen, 2009). The initial step of leukocyte recruitment is mediated in part by interactions between selectins and their corresponding ligands (Figure 1.1) (Rivera-Nieves et al., 2008). Selectins are a family of adhesion molecules comprised of L-, E- and P-selectins. L-selectin (CD62L) is constitutively expressed by leukocytes and P-selectin (CD62P) by platelets and endothelial cells (Patel et al., 2002). In contrast, E-selectin (CD62E) expression on the endothelial wall only occurs after cell activation by cytokines (Patel et al., 2002). P-selectin glycoprotein ligand 1 (PSGL-1) interacts with all three selectins and is the major ligand involved in P-selectin mediated neutrophil rolling (Yang et al., 1999). Selectins bind to the carbohydrate antigen sialyl Lewis X and related structures on their ligands following post-translational modifications, including terminal fucosylation by α(1,3) Fucosyltransferase-IV and -VII (FucT-IV and -VII) (Homeister et al., 2001). FucT-VII has been attributed the primary role in mediating E- and P-selectin-ligand activities in leukocytes, and L-selectin-mediated lymphocyte homing to lymphoid compartments via high endothelial venules (HEVs) (Homeister et al., 2001). In contrast, FucT-IV provides a supportive role to FucT-VII in functional selectin ligand interactions, as evidenced by residual HEV-derived L-selectin ligands lymphocyte homing activity in FucT-VII-deficient mice (Maly et al., 1996; Homeister et al., 2001). FucT-IV and -VII deficiency (FtDKO) is associated with defective leukocyte transmigration into extravascular sites, increased numbers of circulating neutrophils, lymph node (LN) atrophy and reduced lymphocyte homing to lymphoid compartments (Homeister et

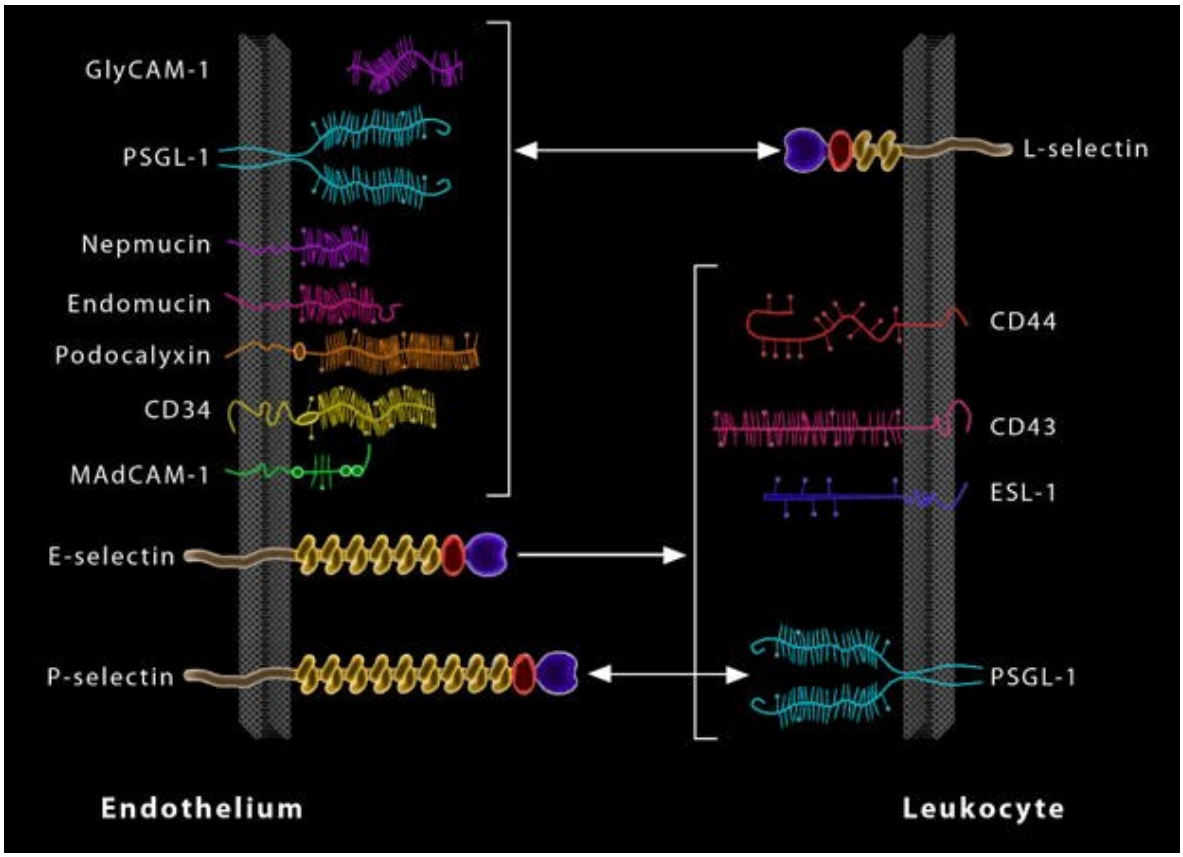
GlyCAM-1
PSGL-1
Nepmucin
Endomucin
Podocalyxin
CD34
MAdCAM-1
E-selectin
P-selectin
L-selectin
CD44
CD43
ESL-1
PSGL-1
Endothelium
Leukocyte

Figure 1.1 Selectins and their ligands. During the inflammatory response, leukocyte adhesion to endothelial cells is controlled by the binding of selectins to complementary carbohydrate ligands. All known selectin ligands relevant for lymphocyte trafficking are transmembrane glycoproteins, which present oligosaccharide structures to the selectins. Transient bond formations between the selectins and their ligands mediate the early steps of the adhesion cascade. All three selectins can recognize glycoproteins presenting the tetrasaccharide sialyl-LewisX (sialyl-CD15). This tetrasaccharide is found on all circulating myeloid cells and some activated T cells. It is composed of sialic acid, galactose, fucose, and N-acetyl-galactosamine. It is unclear how selectins achieve specific interactions with ligands, given this common carbohydrate recognition.

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Jesús Rivera-Nieves, Gezahegn Gorfu, Klaus Ley; Leukocyte adhesion molecules in animal models of inflammatory bowel disease, *Inflammatory Bowel Diseases*, Volume 14, Issue 12, 1 December 2008, Pages 1715–1735, https://doi.org/10.1002/ibd.20501.

al., 2001). Thus, FucT-IV and -VII play critical roles in modulating leukocyte migration and distribution in circulation and tissues.

1.2.4 Innate Immune Cells

1.2.4.1 Neutrophils

Neutrophils are among the first leukocytes that are recruited to sites of inflammation during infection. These polymorphonuclear cells comprise 50-70% of circulating leukocytes in humans and 10-25% in mice (Doeing et al., 2003; Mestas and Hughes, 2004). Human and murine neutrophils are distinguished by their cell surface expression of cluster of differentiation 177 (CD177) and lymphocyte antigen 6 complex, locus G (Ly-6G), respectively (Sachs et al., 2007; Daley et al., 2008). The primary site of neutrophil production is the BM, wherein granulocyte-macrophage progenitors (GMPs) differentiate into granulocytic and monocytic/macrophage populations (Nakorn et al., 2003; Seita and Weissman, 2010). Neutrophils are classified as granulocytes due to the presence of azurophilic, specific and gelatinase granules in the course of their maturation (Faurschou and Borregaard, 2003). These granules contain proteins that contribute to immune activation and microbial killing (Faurschou and Borregaard, 2003). Human and murine neutrophils were initially reported to have short half-lives of 8 and 1.5 hours (h), respectively, under *ex vivo* conditions (Kolaczkowska and Kubes, 2013). However, *in vivo* neutrophil labelling studies have reported longer half-lives of 5.4 days in humans and 8-10 h in mice (Pillay et al., 2010; Basu et al., 2002). Aged neutrophils are eliminated from the total blood granulocyte pool through apoptosis, a non-inflammatory mechanism of programmed cell death (Summers et al., 2010).

Neutrophils employ both phagocytic and non-phagocytic mechanisms in microbial killing. Phagocytosed pathogens are eliminated through generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Winterbourn et al., 2016). Extracellular release of ROS occurs upon direct sensing of pathogens via formyl-peptide receptors (Munafo et al., 2009). Phagocytosis also leads to degranulation, which involves the fusion of cytoplasmic granules with bacteria-containing phagosomes and the release of anti-microbial proteins, including lysozyme, elastase and bactericidal-permeability-increasing protein (Kobayashi and DeLeo, 2009). Extracellular pathogens are eliminated by neutrophils using neutrophil extracellular traps (NETs), which are web-like DNA structures that contain anti-microbial chromatin, histones and granules (Delgado-Rizo et al., 2017). Neutrophils perform indirect microbial killing through generation of pro-inflammatory cytokines, including IFN-γ, TNF and IL-6 (Witter et al., 2016). Differential modulation of neutrophil survival during infection and inflammation also impact disease progression and outcomes. Bacterial and viral infections by *Pseudomonas aeruginosa* and influenza A, respectively, impair innate immune responses by enhancing neutrophil apoptosis (Allen et al., 2005; Colamussi et al., 1999). In contrast, development of immune pathologies during sepsis is associated with delayed neutrophil apoptosis, which is attributed in part to downregulation of pro-apoptotic enzymes caspase-3 and caspase-9 (Sayeed, 2004; Taneja et al., 2004). Thus, achieving a balance in apoptotic and survival signals is critical in the resolution of infection and regulation of inflammation.

1.2.4.2 Dendritic Cells

DCs are professional antigen-presenting cells (APCs) that mediate critical interactions between innate and adaptive immunity. Professional APCs display endogenous (intracellular-derived) and exogenous (extracellular-derived) antigens via major histocompatibility complex

(MHC) class I and II molecules, respectively, to induce clonal expansion and differentiation of naïve T cells into effector T cells (Pozzi et al., 2005; Thery and Amigorena, 2001). In contrast, all other nucleated cells only present endogenous antigen via MHC class I molecules. Antigen presentation also expands antigen-specific memory T and B cell populations upon secondary antigen exposure (Knight and Stagg, 1993). APCs can be targeted for killing by T cells and NK cells as a mechanism of microbial control (Knight and Stagg, 1993). IL-12 generated by DCs augments pro-inflammatory responses by natural killer (NK) cells and promotes T cell activation during infection (Banchereau and Steinman, 1998). DCs also produce TNF, a key pro-inflammatory cytokine in the early control of intracellular infection (Serbina et al., 2003b; Zhao et al., 2006).

DCs are a highly-heterogeneous population of CD11c-expressing cells that are classified into two main subsets: classical/conventional DCs (cDCs) and non-classical DCs. cDCs originate from DC precursors in the blood and peripheral tissues (Belz and Nutt, 2012). These cells can be further classified according to CD11b, CD8α and CD103 expression. $CD11b^+$ cDCs comprise majority of cDCs in lymphoid organs other than the thymus (Mildner and Jung, 2014). They migrate from peripheral tissues to LNs to activate $CD4^+$ T cells via MHC class II (Dudziak et al., 2007). In contrast, $CD8\alpha^+$ DCs are lymphoid tissue-resident (non-migratory) cDCs that specialize in cross-presentation, which involves presentation of exogenous antigens via MHC class I to prime $CD8^+$ T cells (Rock, 2003; den Haan et al., 2000). Cross-presentation plays a critical role in the detection of intracellular pathogens that do not typically infect APCs and require $CD8^+$ T cell responses for the control of infection (Sigal et al., 1999). $CD8\alpha^+$ DCs also generate IL12p70, the bioactive form of IL-12, upon TLR stimulation. Furthermore, $CD103^+$ DCs are the non-lymphoid tissue (migratory) counterparts

of $CD8\alpha^+$ DCs that are similarly capable of cross-presentation and IL-12 production. $CD8\alpha^+$ and $CD103^+$ cDCs share transcription factors, such as IFN regulatory factor 8 (IRF8) and basic leucine zipper ATF-like 3 transcription factor (BATF3) (Mildner and Jung, 2014). On the other hand, non-classical DCs are comprised of monocyte-derived DCs (moDCs), plasmacytoid DCs (pDcs) and Langerhans cells. As the term implies, moDCs are derived from monocytes that have undergone differentiation during inflammation. Monocyte stimulation with granulocyte-macrophage colony-stimulating factor (GM-CSF) *in vitro* or TLR4 *in vivo* induces moDC differentiation (Belz and Nutt, 2012). moDCs exhibit similar features as the cDC subsets, including CD11b expression and cross-presentation (Qu et al., 2014). They can be distinguished from cDCs through CD64 expression (Plantinga et al., 2013; Mildner and Jung, 2014). In contrast, pDCs are derived from common DC progenitors in the BM, and are found in peripheral blood and tissues (Belz and Nutt, 2012). These cells are characterized by robust production of type I IFNs in response to viral infection (Gary-Gouy et al., 2002). However, pDCs exhibit weak antigen-presenting capabilities relative to other DC subsets (Reizis et al., 2011). Lastly, Langerhans cells are epidermal skin layer-resident DCs that exhibit migratory and antigen presenting-capabilities (Belz and Nutt, 2012). They are generated through on-site differentiation of myeloid precursor cells, independent of renewal by BM HSC-derived cells (Chorro et al., 2009). Taken together, DCs shape various aspects of innate and adaptive immunity according to their functional specialization and tissue localization.

1.2.4.3 Monocytes

Monocytes are mononuclear phagocytes that comprise 4% and 10% of nucleated cells in the blood of mice and humans, respectively. BM GMPs differentiate into the monocytic

lineage in response to macrophage colony-stimulating factor (M-CSF), also known as colony stimulating factor 1 (CSF1) (Ginhoux and Jung, 2014; Stanley et al., 1997). In mice, monocytes are mainly classified according to their cell surface expression of lymphocyte antigen 6 complex, locus C (Ly-6C). Ly-6C^{high} monocytes or classical monocytes are derived from common monocyte progenitors, and macrophage and DC precursors in the BM. These cells have a half-life of 20 h and express C-C chemokine receptor type 2 (CCR2) for emigration from the BM (Yona et al., 2013). In contrast, Ly-6C^{low} monocytes are derived from Ly-6C^{high} monocytes and have a longer half-life of 2 days (Yona et al., 2013). The equivalents of Ly-6C^{high} and Ly-6C^{low} monocytes in humans are CD14^{+} and CD14^{low}CD16^{+} subsets, respectively (Ingersoll et al., 2010).

Ly-6C^{low} monocyte function is associated with endothelial surveillance and repair, in coordination with neutrophils (Auffray et al., 2007; Carlin et al., 2013). In contrast, Ly-6C^{high} monocytes are critical in the host defense against intracellular pathogens. During infection, these cells migrate to inflammation sites and differentiate into tissue-resident macrophages and moDCs. Ly-6C^{high} monocytes also exhibit phagocytic activity and produce ROS and pro-inflammatory cytokines, such as TNF and type I IFNs as mechanisms of bacterial and viral killing (Serbina et al., 2008; Barbalat et al., 2009). However, monocytes can contribute to disease progression during infection by transporting bacteria that have escaped intracellular killing into host tissues (Drevets et al., 2004).

1.2.4.4 Macrophages

Macrophages are mononuclear phagocytes that perform diverse immune and homeostatic functions owing to their plasticity and tissue-specificity. As professional APCs,

macrophages prime naïve T cells towards the development of effector and memory T cell responses (Pozzi et al., 2005). Macrophages also provide early and critical defense against pathogens through phagocytosis, ROS and RNS generation, and cytokine production (Zhang and Wang, 2014). Homeostatic functions of macrophages include immune regulation, clearance of erythrocytes and other cellular debris, and tissue repair and remodeling (Biswas and Mantovani, 2010). Monocytes that migrate from the bloodstream into tissues are traditionally regarded as the main source of macrophages. However, local self-renewal from embryonic precursors has been shown to replenish the macrophage pool in tissues (Yona et al., 2013). The cell surface glycoprotein F4/80 is expressed by various murine tissue-resident macrophages, including splenic macrophages, skin Langerhans' cells, liver Kupffer cells and brain microglia (McKnight and Gordon, 1998). Similarly with monocytes, M-CSF is a key regulator of macrophage proliferation, differentiation and survival (Ginhoux and Jung, 2014; Stanley et al., 1997).

Macrophages are classified into two main subsets: classically-activated (M1) and alternatively-activated (M2). Induced by IFN-γ and/or Gram-negative bacteria-derived lipopolysaccharide (LPS), M1 cells play critical roles in immunity to intracellular infection. Their effector functions are characterized by robust phagocytic activity and production of ROS and pro-inflammatory cytokines, including IL-6, TNF and IL-1β (Kong and Gao, 2017). M1 cells also contribute to polarization of T helper-1 (Th1) lymphocyte responses through IL-12 production. Phenotypic M1 cell markers include inducible nitric oxide synthase (iNOS), and co-stimulatory receptors CD86 and PD-L1 (Kong and Gao, 2017). In contrast, M2 cells are subdivided into M2a, M2b and M2c cells. M2a cells are IL-4- or IL-13-induced macrophages that provide anti-parasitic responses and promote polarization of T helper-2

(Th2) cell responses. Phenotypic markers for M2a macrophages include arginase-1 and mannose receptor (CD206) (Kong and Gao, 2017). On the other hand, M2b cells are generated through stimulation with TLR, IL-1 receptor antagonist (IL-1ra) and immune (antigen-antibody) complexes. They are typically distinguished by CD64, CD86 and suppressor of cytokine signaling-3 (SOCS-3) expression (Kong and Gao, 2017). M2b cells are major sources of anti-inflammatory IL-10 and express low levels of pro-inflammatory cytokines. Lastly, M2c cells are induced by IL-10, TGF-β or glucocorticoids, and express CD163 and heme oxygenase-1 (HMOX-1). Their main functions include immune-regulation, debris clearance, and tissue repair and remodeling (Kong and Gao, 2017). Thus, phenotypic and functional heterogeneity enable macrophages to perform critical roles in both pathogen control and maintenance of homeostasis.

1.2.4.5 Natural Killer Cells

NK cells are innate lymphoid cells that provide cytotoxic and cytokine-mediated effector functions in immunity to pathogens. They originate from common lymphoid progenitors, which also give rise to B and T cells. Two major NK cell subsets have been identified in humans according to phenotype and functionality: $CD56^{bright}$ $CD16^{dim/-}$ and $CD56^{dim}$ $CD16^{+}$. Primarily located in lymphoid tissues, $CD56^{bright}$ cells are characterized by increased cytokine production and reduced cytotoxicity upon activation (Poli et al., 2009). The opposite is observed for $CD56^{dim}$ $CD16^{+}$ cells, which comprise majority of NK cells in the peripheral blood (Ferlazzo et al., 2004). In mice, CD49b (DX5) is considered a pan-NK cell marker, while NK1.1 is expressed by WT mice and select strains (Arase et al., 2001; Carlyle et al., 2006). Murine NK cell subsets can be further classified according to CD27 expression. $CD27^{high}$ cells feature robust cytokine expression and cytotoxicity upon stimulation

(Hayakawa and Smyth, 2006). Constitutive expression of chemokine receptor CXCR3 in $CD27^{high}$ cells also enable increased migratory capability in response to CXCR3 ligands (Hayakawa and Smyth, 2006). In contrast, the $CD27^{low}$ subset exhibits highly-regulated activation mechanisms due to increased expression of inhibitory receptors (Hayakawa et al., 2006). Signals from activating and inhibitory receptors regulate NK cell function. Activating signals prompt NK cells to destroy target cells through the release of perforin and granzyme, and induction of apoptosis via engagement of death receptors (Smyth et al., 2005). In contrast, inhibitory receptors regulate NK cell activation and promote tolerance of host cells through recognition of self-MHC class I molecules (Orr and Lanier, 2010). Ly49 is a major family of murine NK cell receptors that is primarily comprised of inhibitory receptors (Yokoyama and Seaman, 1993). Functional homologues of Ly49 receptors in humans are known as killer-cell immunoglobulin-like receptors (KIRs) (Orr and Lanier, 2010).

Upon TLR recognition of bacterial PAMPs, NK cells produce α-defensins and pro-inflammatory cytokines IFN-γ and TNF to promote microbial killing and activation of macrophages and T cells (Chalifour et al., 2004). Furthermore, cytokine production by B cells, epithelial cells and other innate immune cells promote NK cell activation, proliferation and cytotoxicity (Chalifour et al., 2004; Souza-Fonseca-Guimaraes et al., 2012). Thus, NK cells contribute to host immune responses to bacterial infection through production of cytokines and anti-microbial mediators, and cross-talk with accessory cells.

1.3 Adaptive Immunity

Induction of pathogen-specific responses by adaptive immunity is dependent on antigen recognition and activation signals provided by innate immunity. Adaptive immunity

is often characterized as acquired immunity for its critical role in the development of immunological memory, which provide rapid responses upon re-encounter of the pathogen. In contrast to innate immunity, adaptive immunity requires a longer period to develop, and is mediated primarily by B and T lymphocyte subsets.

1.3.1 B Cells

B cells are a lymphocyte subset that is classically associated with humoral or antibody-mediated responses. Antibodies, also known as immunoglobulins (Igs), recognize a specific antigen to initiate immune responses to pathogens. However, B cells also perform various functions associated with cellular immunity, including antigen presentation, co-stimulation and cytokine production. As APCs, they are highly capable of internalizing and processing antigens for presentation to T cells upon BCR engagement (Treanor, 2012). B cell stimulation through BCR and CD40 ligand (CD40L) induces expression of pro-inflammatory TNF-α, lymphotoxin, and IL-6, while stimulation through CD40L alone generates anti-inflammatory IL-10 (Duddy et al., 2004). Furthermore, B cells also promote the recruitment of Th2 cells through production of C-C motif chemokine ligand 17 (CCL17) and CCL22 (Vazquez et al., 2015). B cells originate from common lymphoid progenitors and undergo several stages of development. The pro-B cell stage involves functional rearrangement of Ig gene segments of the heavy chain (V_H, D_H, and J_H) and light chain (V_L-J_L). The pre-B cell stage then forms immature B cells through functional rearrangement of κ and λ chain gene segments (Pieper et al., 2013). The final stage of development is characterized by migration of immature B cells to the spleen prior to differentiation into naïve B cells. These developmental stages result in a repertoire of B cells that generate antibodies with diverse antigen specificities (~5×10^{13} antigens) (Pieper et al., 2013). Furthermore, B cells undergo positive and negative

selection in the course of their development to be able to distinguish reactivity between pathogens and the host. Positive selection promotes the survival of immature B cells that express low-affinity autoreactive BCRs (Melchers, 2015). In contrast, negative selection prompts the apoptosis of immature B cells upon binding self-antigen (Melchers, 2015). Cytokines that modulate B cell development and proliferation include IL-7, IL-4, IL-6, IFN-γ and type I IFNs (Vazquez et al., 2015). Human B cells express the cell surface antigen CD19, while murine B cells express both CD45R (B220) and CD19 (Lai et al., 1998).

Antibodies are glycoproteins that are typically comprised of two large heavy (H) chains and two smaller light (L) chains. The antigen-binding fragment (Fab) features one constant and one variable domain on the H and L chains. Binding to the epitope, which is the site of interaction on the antigen, occurs via the variable domain of Fab (Schroeder, Jr. and Cavacini, 2010). In contrast, the fragment crystallizable (Fc) region is the tail region of the antibody that binds Fc receptors expressed by various immune cells, including B cells, NK cells, neutrophils, monocytes and macrophages (Takai, 2005; Schroeder, Jr. and Cavacini, 2010). Fc receptor (FcR) engagement promotes various immune processes, including phagocytosis, cytolysis, Ig transport and internalization of antigens for presentation (Takai, 2005). Antibodies are classified into several isotypes according to differences in the constant region of their H chains: gamma-chain (IgG), alpha-chain (IgA), mu-chain (IgM), delta-chain (IgD) and epsilon-chain (IgE). IgG is the most abundant in circulation and exhibits the longest serum-half life among the isotypes. It is well-characterized for its roles in secondary responses, toxin neutralization and opsonization of pathogens (Forthal, 2014). Bacterial opsonization is the process by which specific epitopes on bacterial surface antigens are marked by antibodies and plasma proteins of the complement system for phagocytosis. IgG also plays crucial roles in fetal immunity with

its ability to cross the placental barrier (Palmeira et al., 2012). The second most abundant isotype is IgA, a critical component of mucosal immunity against bacteria and viruses. It exists as a monomer in serum and a dimer in the mucosa and secretions, including saliva and mucus (Woof and Kerr, 2006). IgA in the colostrum or the "first milk" also provides passive immunity to infants (Woof and Kerr, 2006). The largest and third most abundant isotype is IgM. Its pentameric structure enables efficient opsonization, while its monomeric form functions as the BCR in mature B cells (Bjornson and Detmers, 1995; Geisberger et al., 2006). In contrast to IgG, IgM is mainly involved in primary immune responses (Boes, 2000). The fourth most abundant isotype is IgD, a monomer that functions as a BCR. Although IgD is found in human serum but not in rodents, its role outside of the membrane-bound form remains poorly understood (Geisberger et al., 2006). Lastly, the IgE is the least abundant isotype in circulation. This monomer is generated in response to parasitic helminths, and during various allergic conditions (Fitzsimmons et al., 2014).

Antigen recognition by naïve B cells during a primary response generates plasma cells and memory B cells. Plasma cells are terminally-differentiated cells that generate antigen-specific antibodies. After the primary response, some plasma cells remain as long-lived plasma cells, which provide protective immunity by maintaining antibody production even after the clearance of initial immunizing antigen (Tangye and Tarlinton, 2009). In contrast, memory B cells are quiescent cells that rapidly undergo activation, differentiation and proliferation into plasma cells upon antigen re-exposure (Moens et al., 2016). Taken together, B cells mediate critical effector functions in host protection through antibody production, antigen presentation, co-stimulation and cytokine expression.

1.3.2 T cells

T cells are lymphocytes distinguished by their expression of cell surface T cell receptors (TCRs). They provide critical functions in host immunity through antigen recognition, cell-mediated cytotoxicity, immune-suppression and modulation of antibody production by B cells. In contrast to other lymphocyte subsets, T cell maturation occurs in the thymus. Committed lymphoid progenitors from the BM migrate to the thymus and develop into CD4-CD8- double-negative (DN) thymocytes (Germain, 2002). These cells first undergo beta selection to select cells that have expressed a functional TCR-β chain prior to differentiation into $CD4^+CD8^+$ double-positive (DP) thymocytes (Michie and Zuniga-Pflucker, 2002). Cells that fail to develop a functionally-rearranged TCR-β chain are prompted to undergo apoptosis (Michie and Zuniga-Pflucker, 2002). DP thymocytes then develop αβ-TCRs through TCR-α locus rearrangement (Germain, 2002). Similarly with B cells, T cells undergo a series of positive and negative selection steps during their development. DP thymocytes are positively-selected for survival upon TCR recognition of self-antigen presented by APCs via MHC class I or II. Cells then undergo lineage commitment into either $CD4^+$ (MHC class II-restricted) or $CD8^+$ (MHC class I-restricted) single positive (SP) thymocytes. Negative selection directs SP thymocytes that are strongly-reactive to self-antigen to undergo apoptosis. Taken together, T cell development generates mature $CD4^+$ and $CD8^+$ SP T cells expressing αβ-TCRs *(*αβ-T cells) that are MHC-restricted and tolerant to self-antigens. αβ-T cells comprise around 95% of the total T cell population in circulation (Kannan et al., 2012). The remaining T cells consist of γδ T cells, which express TCRs with one γ (gamma) chain and one δ (delta) chain (Hayday, 2000). In contrast to αβ-T cells, γδ T cells have a limited TCR diversity and exhibit antigen recognition that is not restricted by classical

MHC (Hayday, 2000). In the following sections, αβ-T cells will be referred to as T cells unless otherwise stated.

1.3.2.1 $CD4^+$ T cells

$CD4^+$ T cells are traditionally characterized as T helper (Th) cells due to their ability to promote effector immune responses, including cytokine-mediated stimulation of innate immune cells, activation of $CD8^+$ T cells and antibody production by B cells (den Haan and Bevan, 2000). Antigen recognition via MHC class II induces differentiation of naïve $CD4^+$ T cells into specific Th subsets (Luckheeram et al., 2012). Differentiated $CD4^+$ T cells were classically divided into Th1 and Th2 subsets. IL-12 and IL-18 promote Th1 development, while IL-4 drives Th2 differentiation (Nakanishi et al., 2001; Saito et al., 2010). Each Th subset can be identified by its cytokine profile, transcription factor and function. Th1 cells are components of cell-mediated immunity, which are crucial in immunity to intracellular pathogens. They generate IL-2 and the pro-inflammatory cytokines IFN-γ and lymphotoxin-alpha (TNF-β) (Saito et al., 2010; Szabo et al., 2000). Th1 cells also express the transcription factor T-bet, which induces and maintains IFN-γ production (Szabo et al., 2000). In contrast, Th2 cells are involved in the modulation of antibody-mediated (humoral) immunity and host defense against helminths and products of allergens. Major cytokines produced by Th2 cells include IL-4, IL-5, IL-9, IL-13 and IL-25 (Paul and Zhu, 2010). Generally, IL-4 promotes the differentiation of naïve $CD4^+$ T cells into Th2 cells. It also induces the expression of GATA-3, a Th2-specific transcription factor that activates IL-4 and IL-13 production by Th2 cells and maintains GATA-3 expression (Paul and Zhu, 2010).

The identification of Th17 and regulatory T (Treg) cell subsets prompted a paradigm shift in defining inflammatory and immune-suppressive mechanisms that modulate host immunity and disease progression. Th17 cells are pro-inflammatory T cells that produce IL-17 (IL-17A and IL-17F) cytokines, and express the transcription factor RORγT (Littman and Rudensky, 2010). Other cell types that express IL-17 include γδ T cells, neutrophils and macrophages (Ferretti et al., 2003; Martin et al., 2009). However, IL-17 production by Th17 cells is distinctive in that it is dependent not only on cytokine receptor stimulation and the presence of IL-23 and IL-1β, but also on TCR stimulation (Littman and Rudensky, 2010). IL-17 signals through the IL-17 receptor (IL-17R) expressed by immune and non-immune cells, including epithelial cells, endothelial cells and fibroblasts (Khader et al., 2009). Th17 cells also secrete IL-21 and IL-22, both of which are involved in neutrophilia or elevated neutrophil levels in the blood, anti-microbial protein production and tissue repair and remodeling (Littman and Rudensky, 2010). In contrast, Tregs promote immunologic tolerance through suppression of effector T cell and NK cell activities, DC maturation, and antibody production by B cells (Saito et al., 2010; Josefowicz et al., 2012; Chapoval et al., 2010). These cells are characterized by their transcription factor forkhead box P3 (FoxP3) and their surface protein CD25. FoxP3 expression is crucial to Treg development and maintenance of regulatory function (Josefowicz et al., 2012). Th17 cells and Tregs are closely-related in their developmental pathways. Naïve $CD4^+$ T cells can differentiate into Th17 cells in the presence of TGF-β and IL-6. IL-23 maintains but not induces the Th17 cell differentiation process, as it is not able to act directly on naïve $CD4^+$ T cells (Oukka, 2007). In contrast, TGF-β, together with IL-2, induces naïve $CD4^+$ T cell differentiation into Tregs. Th17 cells and Tregs also exhibit reciprocal regulation of immune function. Th17 cells promote inflammation in response to infection, while Tregs regulate T cell-mediated inflammation and maintain

homeostasis (Littman and Rudensky, 2010). CTLA-4 is a major component in the regulatory function of FoxP3$^+$ Tregs (Ngalamika et al., 2012; Friedline et al., 2009). Deficiency in CTLA-4 has been correlated with overproduction of lymphocytes and abnormal activation of T cells in mice despite normal T cell development, selection and TCR repertoire complexity (Friedline et al., 2009).

1.3.2.2 CD8$^+$ T cells

CD8$^+$ T cells are typically referred to as "killer" T cells due to their functional specialization of targeting and destroying infected cells. They play a crucial role in discriminating "self versus non-self" through recognition of antigens presented via MHC class I. Naïve CD8$^+$ T cells can differentiate into various subsets upon activation. Tc1 cells, also known as cytotoxic CD8$^+$ T cells, augment innate immune cell responses through production of interferon-γ (IFN-γ), TNF and lymphotoxin (Mosmann et al., 1997). Similar to Th1 cells, Tc1 cells express the transcription factor T-bet and are well-characterized for their antigen-specific responses to intracellular infections and tumors. In contrast, Tc2 express the transcription factor GATA3 and generate IL-4, IL-5 and IL-13, resembling the expression profile of Th2 cells. Increased frequencies of Tc2 cells relative to Th1 cells have been associated with disease progression in viral infections (Maggi et al., 1995). Both Tc1 and Tc2 subsets perform cytotoxic killing using perforin and granzyme, and Fas/Fas ligand interaction (Mosmann et al., 1997; Sad et al., 1997; Mittrucker et al., 2014). Furthermore, Tc17 cells resemble Th17 cells in their expression of the transcription factor RORγT and production of IL-17 and IL-21 (Mittrucker et al., 2014). Tc17 cells lack cytolytic activity and do not produce granzyme and perforin compared to Tc1 and Tc2 cells (El-Behi et al., 2014; Hamada et al., 2009). Lastly, CD8$^+$ suppressor T cells share similarities with CD4$^+$ Tregs in their expression

of the transcription factor FoxP3 and anti-inflammatory cytokine IL-10. The immune-regulatory function of $CD8^+$ suppressor T cells have been implicated in reduced immunity to chronic bacterial and viral infections, and tumors (Filaci et al., 2007; Joosten et al., 2007; Billerbeck et al., 2007; Garba et al., 2002). Majority (~95%) of antigen-specific $CD8^+$ T cells are programmed for cell death after primary infection, while a minor population remains as memory $CD8^+$ T cells (Obar and Lefrancois, 2010). These long-lived cells are able to proliferate and express effector molecules rapidly upon antigen re-exposure (Veiga-Fernandes et al., 2000).

1.4 Intracellular Bacterial Pathogens

Listeria monocytogenes (LM) and *Salmonella enterica* serovar Typhimurium (ST) are intracellular pathogens that are widely-used models of acute and chronic infections, respectively. Acute infection features the rapid onset of disease that is typically resolved with the development of adaptive immune responses. In contrast, chronic infection is characterized by pathogen persistence following acute infection, despite the activation of innate and adaptive immune responses. Infection persistence is primarily influenced by pathogen-specific interactions with the host, including bacterial localization within host cells, and regulation of host immune activation and cell death mechanisms (Robinson et al., 2012; Luu et al., 2006). The following sections will discuss the mechanisms of pathogenicity by LM and ST, and host responses that mediate clearance of acute and chronic intracellular infections.

1.4.1 *Listeria monocytogenes*

LM is a Gram-positive, facultative, anaerobic bacterium that causes food-borne illness known as listeriosis. Its ability to cross the intestinal barrier poses a high risk of systemic

disease, particularly in immunocompromised hosts and pregnant women. It is also able to cross the placental and blood-brain barriers to induce feto-maternal infections and meningoencephalitis, respectively (Vazquez-Boland et al., 2001). LM exhibits a predilection for infecting phagocytic cells, including neutrophils, macrophages and DCs to promote intracellular survival. Once inside the cell, LM escapes into the cytoplasm after being engulfed by the phagosome using listeriolysin O (LLO) and two phospholipases C, a phosphatidylinositol-specific phospholipase C (PI-PLC) and a broad-range phospholipase C (PC-PLC) (Smith et al., 1995; Cossart et al., 2003). However, LM is also able to adhere to and invade non-phagocytic cells, such as epithelial cells and hepatocytes using internalin surface proteins (Cossart et al., 2003). Bacterial cell-to-cell spreading is facilitated by PI-PLC, PC-PLC and actin assembly-inducing protein (ActA) (Smith et al., 1995; Cossart et al., 2003). ActA also promotes bacterial intracytoplasmic mobility (Portnoy et al., 2002).

During systemic infection, innate immune responses are initiated upon recognition of LM-derived peptidoglycan (TLR2), flagellin (TLR5), and DNA (TLR9) by phagocytic immune cells (Zenewicz and Shen, 2007). Cytoplasmic recognition of LM can also occur via two NLRs, NOD2 and cryopyrin (Mariathasan et al., 2006; Kim et al., 2008). NOD2 recognizes the muramyl dipeptide component of LM peptidoglycan cell layer (Girardin et al., 2003). Cryopyrin activates the inflammasome, an intracellular protein signaling complex that activates capase-1 and, subsequently, induces production of pro-inflammatory cytokines IL-1β and IL-18 (Mariathasan et al., 2006). Early control of infection is mediated by neutrophils and macrophages through phagocytosis of bacteria in the blood and infected organs (Zenewicz and Shen, 2007). Recognition of LM-derived formylated peptides through formyl peptide receptors expressed on neutrophils also induces ROS production (Liu et al., 2012; Southgate

et al., 2008). Macrophage-mediated bacterial killing is enhanced by ROS and RNS (Endres et al., 1997; MacMicking et al., 1995). Neutrophils and NK cells also promote macrophage activation through IFN-γ production (Dussurget et al., 2014; Yin and Ferguson, 2009). IL-12 generated by macrophages and cDCs induces naïve $CD4^+$ T cell differentiation into Th1 cells, which then augment innate immune responses through IFN-γ production (Dussurget et al., 2014; Hsieh et al., 1993; Zhan et al., 2010). Recruitment of inflammatory monocytes to inflammation sites generates Tip-DCs, which serve as primary sources of TNF and iNOS in the early phase of primary infection (Serbina et al., 2003b; Serbina et al., 2008). Taken together, robust innate immune responses are critical for rapid and early control of LM infection.

Despite the critical roles of innate immune responses in host protection, adaptive immune responses mediate bacterial clearance and confer protective immunity to LM (Pamer, 2004). Antigen presentation via MHC class I induces activation and differentiation of naïve $CD8^+$ T cells into Tc1 cells. In mice, antigen-specific $CD8^+$ T cell responses to low (0.8×10^5 colony-forming units or CFUs) and high (0.8×10^7 CFUs) doses of LM peak at day 7 and contract thereafter in the course of systemic infection (Badovinac et al., 2002). Long term host protection to LM is primarily mediated by memory $CD8^+$ T cells (Kaufmann et al., 1986; Czuprynski and Brown, 1990). $CD4^+$ T cells are essential in maintaining memory $CD8^+$ T cell number and function after acute infection (Sun et al., 2004). Deficiencies in MHC classes I and II have been shown to compromise control of infection, indicating crucial roles for $CD8^+$ and $CD4^+$ T cell-mediated responses, respectively (Ladel et al., 1994). However, $CD4^+$ T cell responses are not sufficient to clear LM infection in the absence of $CD8^+$ T cell responses (Serody et al., 1996). B cells also contribute to protective immunity to LM through regulation

of $CD8^+$ T cell contraction and, consequently, memory $CD8^+$ T cell development (Shen et al., 2003). Taken together, highly-coordinated interactions between innate and adaptive immunity are essential in host protection from LM infection.

LM infection in mice provides a valuable model in examining immune responses associated with acute systemic infection. Resistance to LM infection in WT mice is associated with the resistant allele at the *Hc* locus on chromosome 2 encoding the component 5 (C5) protein of the complement system (Gervais et al., 1984). C5a anaphylatoxin produced through cleavage of C5 enhances neutrophil and macrophage migration to inflammation sites in the early stages of primary infection (Garifulin and Boyartchuk, 2005).

1.4.2 *Salmonella enterica* serovar Typhimurium

Salmonella is a Gram-negative, facultative, anaerobic bacterium that causes food-borne disease in humans and mice. Salmonellosis poses a high risk of invasive illness in the immunocompromised and pregnant women. *Salmonella* is comprised of two main species, *S. bongori* and *S. enterica*. In addition to their genetic differences, *S. bongori* and *S. enterica* are distinguished by their ability to cause systemic disease in cold-blooded and warm-blooded animals, respectively (Chan et al., 2003). Furthermore, *S. enterica* is comprised of 6 subspecies with over 2,500 serovars or serotypes. *Salmonella* serotyping is based on somatic O and flagellar H antigen expression on the bacterial surface. The two major clinical syndromes of salmonellosis are enteric fever (typhoid/systemic infection) and gastroenteritis (non-typhoid). Most *S. enterica* serovars cause non-typhoidal disease in humans, except *S. enterica* serovar Typhi and *S. enterica* serovar Paratyphi A, B and C (Ohl and Miller, 2001).

ST is a natural mouse pathogen that causes systemic illness in susceptible strains. ST infection in mice is a widely-used animal model for typhoid fever in humans due to similarities

in bacterial distribution in tissues (Santos et al., 2001). Macrophages and DCs phagocytose ST upon recognition of PAMPs, such as lipoproteins (TLR1, 2, 6), LPS (TLR4) and flagellin (TLR5) during systemic dissemination (Broz et al., 2012). Despite their crucial roles in bacterial control, macrophages and DCs can be exploited by ST as intracellular niches for growth and immune evasion. The Type III Secretion System (T3SS) encoded by *Salmonella* Pathogenicity Island 2 (SPI-2) transports virulence effector proteins to the cytoplasm (Uchiya et al., 1999). It promotes bacterial survival by inhibiting fusion of *Salmonella*-containing vacuoles (SCVs) with lysosomes containing anti-microbial peptides and degradative enzymes (Haas, 2007). T3SS also regulates RNS (iNOS) and ROS (NADPH oxidase) localization to the SCV (Chakravortty et al., 2002; Vazquez-Torres et al., 2000). However, macrophages and DCs are not completely defenseless, as they are able to recognize PAMPs within the cytoplasm through NLRs. NOD1 and NOD2 are NLRS that interact with RIP2 to induce NF-κB activation and pro-inflammatory cytokine expression (Kobayashi et al., 2002). Macrophages can also eliminate the intracellular niche of *Salmonella* through pyroptosis, a cell death mechanism induced through inflammasome activation and caspase-1 formation (Broz et al., 2012; Bergsbaken et al., 2009). Pyroptosis is characterized by cell lysis and the release of cytokines and other cellular contents, leading to the induction of inflammatory responses that augment innate immunity to ST (Broz et al., 2012). Neutrophils contribute to control of infection through direct killing of ST released by pyroptotic macrophages (Miao et al., 2010). Both macrophages and neutrophils generate TNF and IFN-γ during the early phase of primary infection (Kirby et al., 2002). Other sources of IFN-γ include NK cells and Th1 cells (Kirby et al., 2002; Kupz et al., 2013). Furthermore, IFN-γ induces IL-12 production by monocytes, macrophages and DCs to further enhance IFN-γ production by NK cells (Verreck et al., 2004; van de Wetering et al., 2009; Hunter et al., 1997).

Adaptive immunity to ST infection is characterized by delayed expansion and contraction of $CD8^+$ T cell responses relative to LM infection (Luu et al., 2006). This is attributed to delayed antigen presentation resulting from antigen localization within the phagosome, as opposed to the cytoplasm (Tzelepis et al., 2012). ST has also been shown to inhibit T cell proliferation and cytokine production in a contact-dependent manner (van der Velden et al., 2005). Despite the immune-suppressive mechanisms of ST, adaptive immune responses are essential for host resistance to infection. Deficiencies in αβ T cells, MHC class-II and T-bet expression by $CD4^+$ T cells are associated with incomplete resolution of infection in mice, indicating a central role for $CD4^+$ T cells in immunity to ST (Hess et al., 1996; Ravindran et al., 2005; Weintraub et al., 1997). In contrast, immunized mice deficient in classical MHC class-Ia, perforin, or granzyme expression are still able to resolve secondary infection with virulent ST (Lee et al., 2012a). Furthermore, mice deficient in granzyme B and perforin/granzyme B also exhibit delayed clearance of attenuated ST (Lee et al., 2012a). These indicate that $CD4^+$ T cells primarily mediate bacterial clearance and protective immunity to ST, while $CD8^+$ T cells contribute to resolution of primary infection. B cells also promote the development of primary effector and memory Th1 cell populations through B cell-intrinsic MyD88 signaling and antigen-specific BCR-mediated antigen presentation, respectively (Barr et al., 2010).

Susceptibility to ST infection in WT mice is attributed in part to the lack of a functional natural resistance-associated macrophage protein 1 (Nramp1) gene (Vidal et al., 1995). Nramp1 is a membrane protein that regulates ion transport in phagocytic cells (Forbes and Gros, 2001). Nramp1-expressing DCs and macrophages respond to ST more rapidly compared

to Nramp1-deficient cells through increased production of inflammatory cytokines (Valdez et al., 2008).

1.5 Pregnancy

1.5.1 Immunological Paradox of Pregnancy

Pregnancy presents an immunological paradox, wherein the semi-allogeneic fetus evades immune rejection by the maternal host and continues to develop. In 1953, Medawar proposed three hypotheses to address maternal tolerance: (i) the mother and fetus are anatomically separated; (ii) the fetus lacks antigen expression; and (iii) maternal lymphocyte function is suppressed (Chen et al., 2012a). In regard to the first hypothesis, it was later determined that maternal-fetal interaction occurs as early as embryonic implantation (Red-Horse et al., 2004). During this process, the maternal endometrium undergoes tissue reorganization to enable invasion by embryonic trophoblast cells. Furthermore, bidirectional cell transfer occurs between the mother and fetus throughout pregnancy and leads to maternal microchimerism or the persistence of maternal cells in the offspring (Nelson et al., 2007). The second hypothesis was disproven by Hoskin and Murgita by showing that co-culture of syngeneic fetal thymus cells induce proliferation of splenic T cells derived from mice pregnant by syngeneic matings (Hoskin and Murgita, 1989). In regard to the third hypothesis, it was shown that maternal lymphocyte function is not suppressed during normal pregnancy; rather, it is associated with the predominance of Th2 over Th1 responses (Lin et al., 1993). Furthermore, pregnancy complications such as recurrent spontaneous abortion are mediated by Th1 responses (Raghupathy, 1997). In 1964, Billingham proposed a fourth hypothesis that the uterus is an “immune-privileged” site, wherein the fetal tissue is tolerated by maternal immune responses in a similar manner as the brain and anterior eye chamber (Billingham,

1964; Billington, 2003). However, Poppa *et al.* demonstrated the rejection of intrauterine parathyroid allografts in pseudopregnant, parathyroidectomized rats, indicating the presence of uterine immune responses (Poppa et al., 1964). Despite the disproval of their hypotheses, Medawar and Billingham laid the groundwork for research that would unravel the mechanism of maternal tolerance, revealing a central role for the placenta and immune regulation by T cells in the success of pregnancy.

1.5.2 Immune Regulation During Pregnancy

1.5.2.1 Placenta

The placenta is a transient organ that acts as the interface between the mother and the fetus. Its main physiological function during early gestation is to support embryonic implantation (Cross, 2006). In the course of fetal development, the placenta regulates the exchange of nutrients, gases and metabolic waste products between maternal and fetal blood (Erlebacher, 2013). It also functions as an endocrine organ through its production of hormones, which promote maternal blood cell production and modulate insulin production and resistance (Cross, 2006). Human and murine placentas are characterized as hemochorial, wherein maternal blood is in contact with the fetal trophoblast layer (Enders and Carter, 2012). The placenta is also an immune-rich environment that acts as a barrier against pathogens and modulates maternal tolerance. Uteroplacental immune cells that play crucial roles in the success of pregnancy include decidual macrophages, uterine NK (uNK) cells, decidual/uterine DCs and Tregs (Bulmer et al., 2010).

Macrophages in the maternal-fetal interface perform various homeostatic and immune functions in the course of pregnancy. Human macrophages in the decidua, which is the uterine

tissue on the maternal side of the placenta, express CD14, CD68 and MHC class II (Nagamatsu and Schust, 2010). In mice, placental macrophages express the F4/80 cell surface antigen and perform Fc receptor-mediated phagocytosis, but exhibit reduced antigen-presenting capabilities relative to splenic macrophages (Chang et al., 1993). Circulating monocytes that have extravasated into uterine tissues provide significant sources of decidual macrophages (Tagliani et al., 2011). During early pregnancy, macrophages eliminate apoptotic cells in the course of placental growth, and support vascularization and remodeling of the decidua (Faas and de, 2017). They also promote immune tolerance to fetal antigens through production of IL-10, indoleamine 2,3-dioxygenase (IDO) and prostaglandin E_2 (Nagamatsu and Schust, 2010). Decidual macrophages phagocytose pathogens, generate ROS and express pro-inflammatory cytokines, such as IL-1β, TNF, IL-6 and IL-12 during infection (Duriez et al., 2014; Singh et al., 2005). Dysregulation of TNF and IL-10 balance is associated with increased incidence of pregnancy complications, further highlighting the critical role of macrophages during pregnancy (Shynlova et al., 2013; Brogin et al., 2012).

uNKs are a specialized subset of NK cells involved in decidualization or the remodeling of endometrial stromal cells to support embryonic implantation (Ramathal et al., 2010). In humans, $CD56^{bright}CD16^{-}$ cells comprise 70-80% of decidual NK cells during early pregnancy (Saito et al., 2008). Reduced CD16 expression in this cell population is associated with decreased cytotoxic activity relative to peripheral NK cells and a minor subpopulation of $CD56^{dim}CD16^{+}$ uNK cells (Jokhi et al., 1994; Verma et al., 2000). Murine uNK cells exhibit increased Ly49 receptor expression but reduced cytotoxicity relative to peripheral NK cells (Gaynor and Colucci, 2017). Ly49 receptors enable recognition of placental trophoblast cells via MHC class I (Madeja et al., 2011; Kieckbusch et al., 2014). Murine uNK cells are classified

into two populations according to phenotype and functionality. Uterine conventional NK (cNK) cells are CD49a^{-} DX5^{+} cells that produce IFN-γ and express CD69 upon activation (Gaynor and Colucci, 2017). In contrast, uterine tissue-resident NK (trNK) cells are CD49a^{+} DX5$^{+/-}$ cells that generate both TNF and IFN-γ, and constitutively express CD69 (Peng and Tian, 2017; Sojka et al., 2014). Decreased proportions of uterine cNK cells relative to uterine trNK cells is associated with defective vascular remodeling in pregnant mice lacking nuclear factor interleukin-3 (NFIL3), a transcription factor involved in innate lymphoid cell development and functionality (Boulenouar et al., 2016). Murine uNK cells also exhibit functional differences according to their reactivity to *Dolichos biflorus* agglutinin (DBA) glycoprotein. DBA^{+} uNK cells exhibit increased IL-22 and angiogenic factors, but decreased IFN-γ mRNA levels relative to their DBA^{-} counterparts (Chen et al., 2012b).

Decidual DCs (dDCs) are migratory cells that play critical immune activation and regulatory roles. In humans, decidual DCs are mainly classified into DC-SIGN^{+} immature DCs (iDCs) and CD83^{+} mature DCs (mDCs). DC-SIGN^{+} iDCs generate IL-15 during decidualization and embryonic implantation to promote NK cell recruitment to the endometrium (Liu et al., 2017). They also contribute to maternal tolerance through induction of Tregs, which will be described in greater depth in the following section (Hsu et al., 2012). Decreased frequency and altered functionality of DC-SIGN^{+} dDCs are associated with spontaneous abortion and pre-eclampsia, respectively (Tirado-Gonzalez et al., 2010; Hsu et al., 2012). Pre-eclampsia a systemic syndrome characterized by increased protein levels in the urine (proteinuria) and hypertension at 20 weeks post-gestation (Toldi et al., 2008). Furthermore, iDCs differentiate into mDCs upon antigen or cytokine stimulation, indicating a role for dDCs in the initiation of immune responses (Kammerer et al., 2003). IL-15 production

by $CD83^+$ dDCs can also induce the proliferation of NK cells (Verma et al., 2000). In mice, uterine DCs (uDCs) are $CD11c^+$ MHC class I^+ cells that play critical roles in decidualization and embryonic implantation (Plaks et al., 2008). Although uDCs can be classified according to CD11b or CD103 expression, their functional specializations are not well-understood (Tagliani and Erlebacher, 2011; Collins et al., 2009). The distinct uterine localizations of $CD11b^+$ and $CD11b^-$ DCs, and expression of alpha4beta7-integrin (ITGA4/ITGB7) and alphaEbeta7-integrin (ITGAE/ITGB7), respectively, suggest differential roles for uDC subsets in various stages of pregnancy (Behrends et al., 2008). Phenotypic markers shared by murine uDCs and human dDCs include CD205, CD40, CD80 and CD86 (Tagliani and Erlebacher, 2011).

Taken together, immune cells at the maternal-fetal interface contribute to the success of pregnancy through their roles in embryonic implantation, induction of maternal tolerance and immunity to infection.

1.5.2.2 Th17/Treg Paradigm

Pregnancy is often characterized as a balancing act, wherein maternal immune alterations associated with fetal tolerance also modulates host susceptibility to pathogens. Maternal tolerance was initially attributed to the Th1/Th2 paradigm, which is characterized by the shift from a pro-inflammatory cell-mediated Th1 profile to an anti-inflammatory humoral and Th2 profile. However, the incidence of recurrent abortions despite the predominance of Th2 responses during pregnancy indicated that other immune-regulatory mechanisms are involved in maternal tolerance (Bates et al., 2002; Chaouat et al., 2003). The discovery of Th17 cells and Tregs led to a paradigm shift in defining inflammatory and immune-suppressive

mechanisms that modulate host immunity and disease progression during pregnancy. Normal pregnancy is characterized by increased frequencies of Tregs in both humans and mice (Heikkinen et al., 2004; Saito et al., 2005; Sasaki et al., 2004; Somerset et al., 2004; Aluvihare et al., 2004; Robertson et al., 2009; Zenclussen, 2005; Zenclussen et al., 2005; Zhu et al., 2005). Using mouse models of pregnancy, it was determined that Tregs are the primary immune cells that mediate fetal implantation and maintenance of early pregnancy (Shima et al., 2010). It is proposed that induction of Tregs during pregnancy is directly elicited by the fetal alloantigen and not by pregnancy-related hormones. Furthermore, maternal tolerance is primarily attributed to fetal alloantigen-specific Tregs generated in the periphery (Samstein et al., 2012).

The imbalance between Treg and Th17 cell proportions is associated with various pregnancy complications. Reduced Treg frequencies in the decidua and/or circulation were observed in pregnant women with pre-eclampsia and spontaneous abortions (Toldi et al., 2008; Sasaki et al., 2004). A similar trend was observed in a murine spontaneous abortion model, which involved mating of CBA/J female (H2k) mice with DBA/2J male (H2d) (Zenclussen et al., 2005). Fetal rejection was inhibited upon adoptive transfer of $CD4^+CD25^+$ T cells from normal pregnant CBA/J mice into abortion CBA/J mice. However, the same effect was not observed when $CD4^+CD25^+$ T cells from non-pregnant mice were adoptively-transferred into abortion CBA/J mice. This indicated potential functional differences between pregnancy-induced and non-pregnancy-induced Tregs. In contrast, increased Th17 cells frequencies were observed in the peripheral blood of patients with unexplained early recurrent miscarriage or the loss of three or more consecutive pregnancies (Wang et al., 2010). It is proposed that altered levels of IL-6 trans-signaling regulators such as serum IL-6 and soluble IL-6 receptor of

patients with recurrent spontaneous abortion impair the suppressive function of Tregs, potentially, favoring a Th17 response (Arruvito et al., 2009). IL-27 has also been implicated in modulating IL-17 and IL-10 expression by human decidual $CD4^+$ T cells (Wang et al., 2013). Taken together, these studies indicate that modulation of Th17/Treg frequencies during pregnancy is critical in determining pregnancy outcome.

In conclusion, pregnancy requires a balance between immune-suppressive mechanisms required for maternal tolerance, and inflammatory responses for host protection from infection. Evaluation of immune responses in the maternal systemic and placental compartments can aid the development of immune-modulation strategies against adverse maternal and fetal outcomes.

1.6 Rationale

Deciphering the complex mechanisms by which host-pathogen interactions modulate innate immunity to infection remains a challenge. Intracellular pathogens, such as LM and ST, have developed mechanisms to evade immune responses and cause persistent infections. Furthermore, the consequences of modulating innate immune responses on host resistance to intracellular bacterial infection, particularly in hosts with altered immunity, are not well-understood.

Our laboratory had previously showed the critical role of functional selectin-ligand interactions in tumor immunity. FtDKO mice challenged with B16-OVA (melanoma cells expressing ovalbumin protein) exhibited reduced long-term tumor protection and homing by $CD8^+$ T cells, even after generating potent OVA-specific T cell responses from previous LM-OVA (LM expressing ovalbumin) vaccination (Stark et al., 2012). These results indicated that

FtDKO mice were able to mount an adequate immune response against bacterial infection, despite compromised immunity against tumor cells. Thus, I surmised that examining the roles of functional selectin ligands during LM infection may elucidate the mechanisms of innate immune-modulation during intracellular bacterial infections. I focused on unravelling the specific innate immune cell types that were differentially modulated in FtDKO mice.

Immunological changes associated with pregnancy modulate susceptibility to various infections. Previous research from our laboratory showed that decreased maternal resistance and increased fetal loss during ST infection is associated with reduced splenic recruitment and/or expansion of innate immune cells, and enhanced placental expression of pro-inflammatory cytokines (Chattopadhyay et al., 2010). As Treg responses are also differentially modulated in the maternal host during pregnancy, it was unclear how this regulatory response interfaced with innate immunity in the context of ST infection. Adverse maternal and fetal outcomes resulting from infection by prenatal pathogens, including LM and *Toxoplasma gondii* have been correlated with altered Th17/Treg ratios (Rowe et al., 2011; Zhang et al., 2012). In contrast, Th17 cell responses and inflammation are important components of host immunity to combat the severity of bacterial infections, such as LM and ST (Curtis and Way, 2009). The interplay between Th17 cell and Treg responses during pregnancy may have major implications, not only on immunity to infection, but also on the development of immune pathologies. Therefore, I evaluated Th17 cell and Treg responses during ST infection and determined their roles in modulating maternal innate immunity during pregnancy.

Type I IFNs provide both inflammatory and regulatory functions in response to infection. The protective and detrimental roles of IFNAR signaling in bacterial infections is largely dictated by specific host-pathogen interactions (e.g. intracellular vs. extracellular

pathogen localization and recognition) that impact innate immune activation and cell death mechanisms (Kovarik et al., 2016). IFNAR deficiency has been shown to enhance immunity to LM and ST in the non-pregnant state by promoting pathogen-induced immune cell death (Auerbuch et al., 2004; Carrero et al., 2004; O'Connell et al., 2004; Robinson et al., 2012). However, the extent of immune-regulation mediated by type I IFNs in response to intracellular infection during pregnancy was not well-understood. Thus, I rationalized that evaluating immune cell distribution and cytokine responses in maternal and placental compartments may provide insight into compartment-specific modulation of innate immune responses and their roles in determining maternal and fetal outcomes.

Taken together, my thesis examines the roles of functional selectin ligands, Th17/Treg balance and IFNAR signaling in the modulation of innate immune responses to two different intracellular infections. Collectively, I undertook to elucidate the roles of gross cellular and cytokine mediators in modulating innate immunity to intracellular infection.

1.7 Hypothesis

Immunity to intracellular infections in murine maternal hosts can be differentially modulated by increased circulation, functionality and/or pregnancy-induced regulation of host innate immunity.

1.8 Objectives

Our main objective is to provide a mechanistic insight into host immune responses that modulate resistance to intracellular pathogens. The specific objectives are to:

1. Evaluate the role of selectin ligands in modulating innate immunity to intracellular pathogens
2. Examine the role of Th17/Treg balance in pregnant host in modulating innate immunity to intracellular infection
3. Determine the role of IFNAR deficiency in modulating leukocyte recruitment and cytokine expression in response to intracellular pathogens during pregnancy

2.0 MANUSCRIPT ONE

Lack of functional selectin-ligand interactions enhances innate immune resistance to systemic *Listeria monocytogenes* infection

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The specific contributions of each author in the research article are listed below:

Gerard Agbayani: Designed and performed the experiments, analyzed the data, wrote and edited the manuscript

Komal Gurnani: Assisted in performing the injections, irradiation experiments and blinded progenitor CFU scoring, and independently performed experiments for Figure **2.12**

Ahmed Zafer: Performed the i.p. injections for *in vivo* antibody depletion studies and the cell sorting

Subash Sad: Conceptualized the study, designed the experiments, and contributed reagents

Lakshmi Krishnan: Conceptualized the study, designed the experiments, wrote and edited the manuscript, and contributed reagents

2.1 Abstract

Selectin-ligand interactions are important for leukocyte homing and functionality. The roles of selectin-ligand interactions in modulating immunity to intracellular infections are not completely understood. Mice lacking the expression of fucosyltransferase-IV and -VII (FtDKO) exhibit deficient functionality of selectin-ligand interactions. We addressed the kinetics of infection and immunity to *Listeria monocytogenes* (LM), an intracellular pathogen, in FtDKO mice. These mice exhibited enhanced ability to clear infection and increased survival to a lethal dose of LM infection relative to wild-type (WT) C57BL/6J controls. This was associated with increased levels of neutrophils, monocytes and DCs in the blood and/or infected organs. Adoptive transfer of bone marrow (BM) cells from FtDKO mice to WT mice resulted in enhanced neutrophil numbers and improved clearance of LM bacteria in recipients. *In vivo* depletion of myeloid innate immune cells, particularly neutrophils, monocytes, macrophages and DCs, using anti-Ly-6G (RB6-8C5) mAb reduced the ability of FtDKO mice to curtail LM infection. Nevertheless, depletion using anti-Ly-6G (1A8) known to exclusively deplete neutrophils did not abrogate increased resistance of FtDKO mice to LM infection, suggesting a role for other myeloid innate immune cells in this model. Examination of BM hematopoietic progenitors through flow cytometry and cell culture colony-forming unit assay showed increased frequencies of granulocyte-macrophage progenitors in FtDKO relative to WT mice, Overall, our results indicate that functional selectin ligand deficiency enhances innate immune-mediated resistance to systemic LM infection despite defective leukocyte migration and lymphocyte homing.

2.2 Introduction

Leukocyte recruitment to sites of infection is a critical mechanism of host defense. It involves leukocyte tethering, rolling and adhesion on the vascular wall and transmigration into extravascular compartments due to highly-coordinated interactions between adhesion molecules expressed on both leukocytes and endothelium. The initial leukocyte tethering and rolling steps occur in part due to interactions between selectins and selectin ligands. Selectins expressed on leukocytes, endothelium and platelets transiently interact with their ligand counter-receptors through recognition of the carbohydrate antigen Sialyl Lewis^X (Patel et al., 2002). Functional selectin ligand synthesis occurs through several glycosylation reactions, including the terminal fucosylation step mediated by α(1,3) Fucosyltransferase-IV and -VII (FucT-IV and -VII) (Homeister et al., 2001). FucT-VII has been attributed the primary role in functional selectin ligand expression, coordinating with FucT-IV to facilitate leukocyte recruitment and lymphocyte homing. FucT-IV and -VII deficiency (FtDKO) results in impaired leukocyte tethering, rolling and transmigration into extravascular components, increased leukocyte levels in circulation, reduced lymph node cellularity and absence of contact hypersensitivity (Erdmann et al., 2002; Homeister et al., 2001).

Listeria monocytogenes (LM) is facultative, intracellular bacterium that poses a high risk of infection in immune-compromised hosts (Vazquez-Boland et al., 2001). Infection occurs through ingestion of contaminated food, which can lead to systemic infection upon bacterial penetration of the intestinal epithelial barrier (Pamer, 2004). Macrophages and dendritic cells (DCs) provide early control of infection through phagocytosis of bacteria in the bloodstream, spleen and liver (Zenewicz and Shen, 2007). Neutrophils also mediate non-phagocytic bacterial killing through reactive oxygen species (ROS) production (Witter et al.,

2016). Inflammatory monocytes recruited to infection sites give rise to tumor necrosis factor (TNF)- and inducible nitric oxide synthase-producing (TIP) DCs, contributing to innate anti-microbial defense (Serbina et al., 2003b). IFN-γ produced by natural killer (NK) cells, and T cells also induces activation of macrophages, further augmenting innate immune responses (Dussurget et al., 2014). However, secondary bacterial clearance and long-term protection are primarily mediated by antigen-specific $CD8^+$ T cells, with the support of $CD4^+$ T cells (Pamer, 2004; Sun et al., 2004). Thus, efficient host protection from LM infection requires highly-coordinated interactions between innate and adaptive immune mechanisms.

We previously showed that lack of functional selectin ligand interactions compromises long-term protection against murine melanoma solid tumors (Stark et al., 2012). Vaccination with *Listeria monocytogenes* expressing ovalbumin (LM-OVA) induced a robust OVA-specific $CD8^+$ T cell response in both wild-type and FtDKO mice. However, subsequent protection against B16-OVA tumor challenge was significantly compromised in FtDKO mice. This correlated to reduced inherent ability of FtDKO $CD8^+$ T cells to infiltrate tumors and draining lymph nodes even when transferred to wild-type (WT) mice. FucT-IV and/or -VII expression has also been linked to enhanced metastases in lung, colorectal and prostate cancer models (Ogawa et al., 1996; Li et al., 2010; Barthel et al., 2009). Thus, regulation of lymphocyte and tumor cell trafficking by functional selectin-ligand interactions modulates cancer progression.

The consequences of the lack of functional selectin ligand interactions on the innate immune response to intracellular infection are not well-understood. Early studies demonstrated extreme leukocytosis characterized by increased neutrophil production and intravascular half-life in FtDKO mice (Homeister et al., 2001). In this study we show that FtDKO mice are able

to more efficiently control LM infection than WT mice due to increased innate immune cells in infected organs. Furthermore, this effect could be adoptively transferred to WT mice through FtDKO bone marrow (BM) transplant. Overall, our study demonstrates an enhanced modulation of innate immunity in the absence of alpha-1,3-fucosylation events of leukocytes.

2.3 Materials and Methods

2.3.1 Mice

C57BL/6J wild-type (WT) and B6.SJL-*Ptprc*^{a} *Pep3*^{b}/BoyJ (B6.SJL) mice were purchased from Jackson Laboratories (Bar Harbor, Maine, USA). Fucosyltransferase-IV and -VII double knockout (FtDKO) mice bred on a C57BL/6J background were originally generated by Dr. John Lowe and obtained from the Consortium for Functional Glycomics (Homeister et al., 2001). FtDKO mice were bred in-house at the National Research Council Canada small animal facility in Ottawa. All mice used in the study were 8-12 weeks of age. The study was performed in accordance with the protocols and procedures approved by National Research Council Canada in Ottawa, Canada and guidelines of the Canadian Council on Animal Care.

2.3.2 Infections

Listeria monocytogenes 10403S (LM) and *Salmonella enterica* serovar Typhimurium SL1344 (ST) were grown to mid-log phase at OD_{600} = 1.0 and OD_{600} = 0.8, respectively, in Bacto™ brain-heart infusion (BHI) medium (BD Biosciences, Mississauga, ON, Canada) supplemented with 50 µg/mL streptomycin (Sigma-Aldrich, Oakville, ON, Canada). Bacteria were maintained as frozen stocks in 20% glycerol at -80ºC. Frozen bacterial stocks were thawed and diluted in 0.9% saline solution. Mice were infected with 10^4 or 10^5 colony-forming units (CFUs) of LM suspended in 200 µL of saline solution, injected intravenously (i.v.) via the lateral tail vein.

At specific time points post-infection, organs were collected in RPMI 1640 with 8% fetal-bovine serum (FBS) and homogenized. Bacterial CFUs in infected organs were

determined by plating serial dilutions of the homogenates onto BHI agar plates. Data were presented as CFUs per organ.

2.3.3 Flow Cytometry Analysis

Organs were collected, homogenized to obtain a single-cell suspension and passed through a 100 μM cell strainer (BD Biosciences, Mississauga, ON, Canada). Blood samples were treated with Red Blood Cell Lysis Buffer (Sigma-Aldrich, Oakville, ON, Canada). Hepatic leukocytes were isolated through Percoll® (GE Healthcare; Sigma-Aldrich, Oakville, ON, Canada) density gradient centrifugation. Briefly, each homogenized liver was resuspended in 5 mL 40% Percoll® and underlaid with 5 mL 70% Percoll®. Cells were then centrifuged at 2,000 rpm for 25 minutes without braking using the Heraeus Megafuge 40R (Thermo Fisher Scientific, Burlington, ON, Canada). Leukocytes at the interphase between the 40%/70% gradient were isolated using plastic Pasteur pipettes.

Cells were centrifuged at 500 g for 5 minutes at 4°C. Cell pellets were resuspended in 1x PBS containing 2% FBS and incubated with anti-CD16/32 (FcγRIII/II) antibody for 10 minutes. Staining for cell surface antigens was then performed by incubating the cells with the following fluorochrome-conjugated monoclonal antibodies (mAbs) for 30 minutes: CD45 (30-F11), CD11b (M1/70), Ly-6G (1A8), CD8a (53-6.7), CD45R/B220 (RA3-6B2), CD11c (HL3) and CD45.2 (104) from BD Biosciences, Mississauga, ON, Canada; TCR-ß (H57-597), CD4 (RM4-5), CD49b (DX5) and CD45.1 (A20) from eBioscience, Burlington, ON, Canada; F4/80 (BM8) and Ly-6C (HK1.4) from BioLegend, San Diego, CA, USA. Samples were acquired using BD LSR Fortessa™ (BD Biosciences, Mississauga, ON, Canada). Cells were then washed and resuspended in 1x PBS containing 2% FBS prior to acquisition. Flow cytometry

data was analyzed using FlowJo® 7.6.5 software (FlowJo, LLC, Ashland, OR, USA). Immune cell populations were identified according to cell surface marker expression (Figure 2.1).

2.3.4 Antibody Treatments

In vivo depletion of neutrophils, monocytes, macrophages and DC subsets was performed through intraperitoneal (i.p.) injection of 100µg/100µL/mouse anti-mouse Ly-6G (RB6-8C5) functional grade purified mAb (eBioscience, Burlington, ON, Canada). Specific *in vivo* depletion of neutrophils was performed through i.p. injection of 200µg/200µL/mouse anti-mouse Ly-6G (1A8) functional grade purified mAb (eBioscience, Burlington, ON, Canada). Six hours later, mice were infected i.v. with 10^4 concentration of LM. The same doses of anti-Ly-6G mAbs were administered at day 2 post-infection. *In vivo* blockade of E-, P-, and L-selectins was performed through i.p. injection of 100µg/100µL/mouse anti-mouse CD62E (10E9.6), CD62P (RB40.34) and CD62L (MEL-14) purified no azide/low endotoxin (BD Biosciences, Mississauga, ON, Canada) 4 hours before infection and at day 1 post-infection. Bacteria were enumerated in the spleen and liver through CFU assay at day 3 post-infection. Blood and/or spleen were processed for flow cytometry analysis of immune cell frequencies.

2.3.5 Serum Cytokine Analysis

Serum levels of IL-6, IL-10, MCP-1, IFN-γ, TNF, and IL-12p70 cytokines in WT and FtDKO mice were quantified using the Cytometric Bead Array (CBA) Mouse Inflammation Kit (BD Biosciences, Mississauga, ON, Canada). Briefly, serum samples were incubated with cytokine capture beads and detection reagent for 2 hours. Samples were then washed and acquired using the BD LSRFortessa™ (BD Biosciences, Mississauga, ON, Canada). Cytokine expression levels were analyzed using the FCAP Array v3.0 software (Soft Flow Hungary

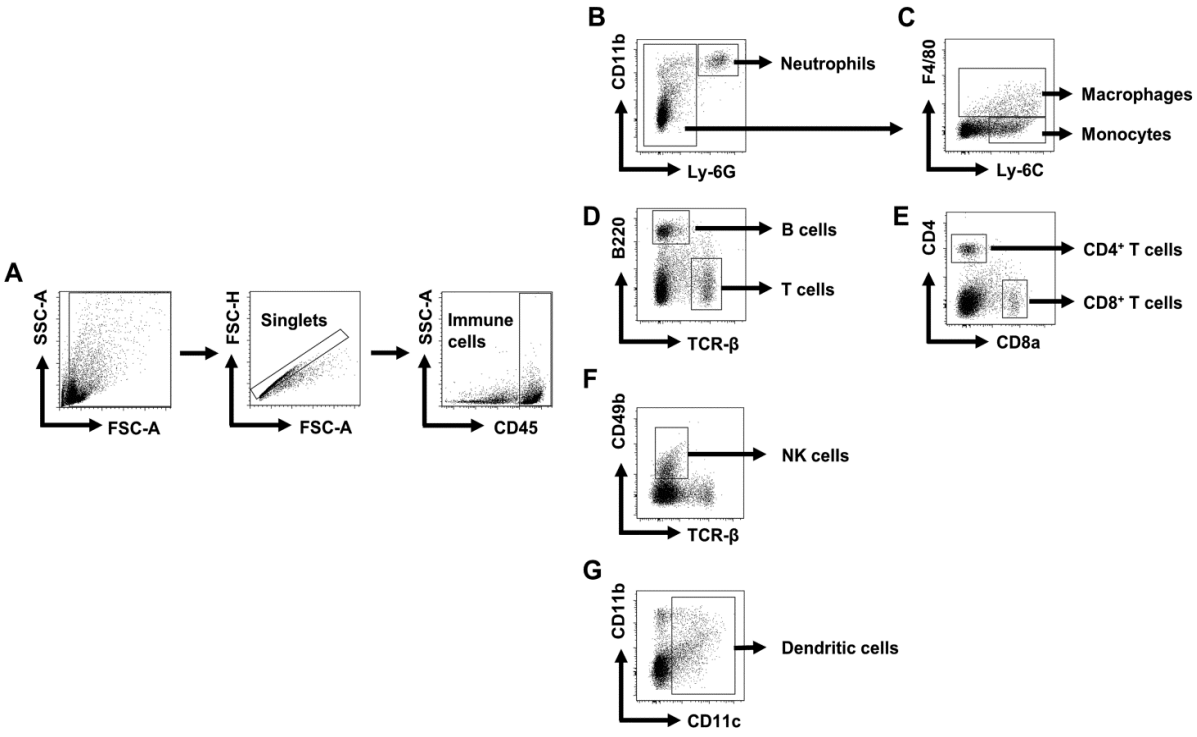
A
SSC-A
FSC-A
FSC-H
Singlets
FSC-A
SSC-A
Immune cells
CD45
B
CD11b
Ly-6G
Neutrophils
C
F4/80
Ly-6C
Macrophages
Monocytes
D
B220
TCR-β
B cells
T cells
E
CD4
CD8a
CD4+ T cells
CD8+ T cells
F
CD49b
TCR-β
NK cells
G
CD11b
CD11c
Dendritic cells

Figure 2.1. Flow cytometry gating strategy of major immune cell populations. (A) Immune cells were identified according to their forward scatter (FSC) and side scatter (SSC) properties, and CD45 expression. (B-G) Neutrophil ($CD11b^+$, $Ly\text{-}6G^+$), monocyte ($Ly\text{-}6C^+$, $Ly\text{-}6G^-$, $F4/80^-$, $CD11c^-$), macrophage ($F4/80^+$, $Ly\text{-}6G^-$, $CD11c^-$), B cell ($B220^+$, $CD11c^-$), T cell ($TCR\beta^+$, $CD11c^-$), $CD4^+$ T cell ($TCR\beta^+$ $CD4^+$, $CD11c^-$) $CD8^+$ T cell ($TCR\beta^+$ $CD8^+$, $CD11c^-$) and dendritic cell (DC) ($CD11c^+$) populations.

Ltd., Pécs, Hungary).

2.3.6 BM Chimeras

WT mice were irradiated with 4.8 Gy of cobalt-60 γ-rays and grouped according to immune cell phenotype of donor bone marrow (BM) cells to be adoptively transferred. Group A received 10^7 BM cells from B6.SJL mice ($CD45.1^+$ WT cell phenotype). Group B received 10^7 BM cells from FtDKO mice ($CD45.2^+$ cell phenotype). Group C received $5x10^6$ B6.SJL and $5x10^6$ FtDKO BM cells (50:50 ratio). Lastly, mice from Group D did not receive any BM cells to confirm that lethal irradiation was successful (100% death by day 13 post-irradiation). Similarly, irradiated FtDKO mice were grouped into Group A (CD45.1 B6.SJL cells) and Group B (CD45.2 FtDKO cells).

For preparation of donor cells, tibias and femurs from B6.SJL and FtDKO mice were harvested, washed once with 70% ethanol and twice with 1x PBS prior to flushing of BM cells with RPMI 1640 with 8% FBS. BM cells were then resuspended in 1x PBS and injected to recipient mice through the retro-orbital route 4 hours post-irradiation.

2.3.7 GMP Analysis

BM cells were harvested from WT and FtDKO mice as described previously. GMP frequencies were determined through flow cytometry using the following antibodies: mouse hematopoietic lineage/Lin (17A2, RA3-6B2, M1/70, TER-119, RB6-8C5), CD127 (A7R34), CD117 (2B8), Ly-6A/E (D7), CD34 (RAM34) and CD16/32 (93) from eBioscience, Burlington, ON, Canada. GMPs were identified as Lin^- IL-7rα$^-$ c-Kit^+ Sca-1^- $CD34^+$ $CD16/32^+$ cells (Challen et al., 2009; Akashi et al., 2000).

Colony-forming cell assay for the detection of GMPs was performed using MethoCult™ GF M3534 methylcellulose-based medium (StemCell Technologies, Vancouver, BC, Canada). Briefly, BM cells from WT and FtDKO mice were plated at $2x10^4$ cells per dish (in duplicate) on 35-mm dishes containing MethoCult™ GF M3534. Duplicate cultures were placed in a 100-mm Petri dish containing a 35-mm dish of sterile water and incubated at 37°C and 5% CO_2 in a water-jacketed incubator. At 12 days post-culture, colonies were scored single-blinded as CFU-GM (granulocyte-macrophage), CFU-G (granulocyte) and CFU-M (macrophage) based on morphological guidelines provided by the manufacturer. Clonal expansion of GMPs was evaluated through cell sorting (MoFlo™ Astrios - Beckman Coulter, Inc., Mississauga, ON, Canada). Briefly, BM cells were processed and stained with GMP markers, as described previously. A total of 5-10 GMP cells per well were seeded onto a 96-well plate containing MethoCult™ GF M3534 methylcellulose-based media (Stemcell Technologies, Vancouver, BC, Canada). Cells were incubated in a water-jacketed CO_2 incubator at 37°C and 5% CO_2. Colonies were scored single-blinded at day 12 of culture.

2.3.8 Statistical Analysis

All statistical analyses were performed using GraphPad® Prism 6 software. The results were presented as mean ± SEM or individual data points on a scatter plot. Statistical significance of CFU, flow cytometry and progenitor CFU data was determined through Mann-Whitney U test. Survival curves were analyzed using Gehan-Breslow-Wilcoxon test. P values of ≤ 0.05 were considered statistically significant. *$P \leq 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

2.4 Results

2.4.1 FtDKO mice control systemic infection more efficiently compared to WT mice

We assessed the survival capability of WT and FtDKO mice against a lethal dose of LM 10^5 CFUs i.v. (Figure 2.2A). WT mice succumbed to infection within 3 days post-infection. In contrast, FtDKO mice exhibited enhanced protection from infection, indicated by 80% survival rate until the study endpoint at day 120 post-infection. We then examined the bacterial load in the spleen and liver of WT and FtDKO mice infected with a sub-lethal dose of LM 10^4 CFUs i.v. (Figure 2.2B and 2.2C). At day 1 post-infection, WT and FtDKO mice had similar CFUs of approximately 10^4 in the spleen and liver. However, FtDKO mice showed rapid clearance of bacteria with a significantly decreased bacterial load relative to WT mice by day 3 post-infection. Bacterial load in FtDKO mice was cleared by day 5 post-infection, whereas it was cleared only by day 7 or beyond in WT mice. Enumeration of splenocyte numbers indicated increased cellularity in FtDKO mice in the steady state in comparison to WT mice (Figure 2.2D). Infection linearly increased splenic cellularity in both mouse strains, however this increase was more pronounced in FtDKO mice. Overall, FtDKO mice showed significantly higher spleen cell numbers at all time points compared to WT mice. These data suggest that an influx of cells in the spleen occurs in FtDKO mice in response to infection, despite defective leukocyte trafficking and lymphocyte homing. We also performed enumeration of bacteria in pooled inguinal lymph nodes (LNs) of FtDKO mice to assess the role of LN atrophy in the control of infection. FtDKO mice exhibited reduced bacterial numbers compared to WT mice in the LN compartment at day 3 post-infection (Figure 2.3A and 2.3B). Thus, LN atrophy does not appear to abrogate systemic resistance to LM infection in FtDKO mice.

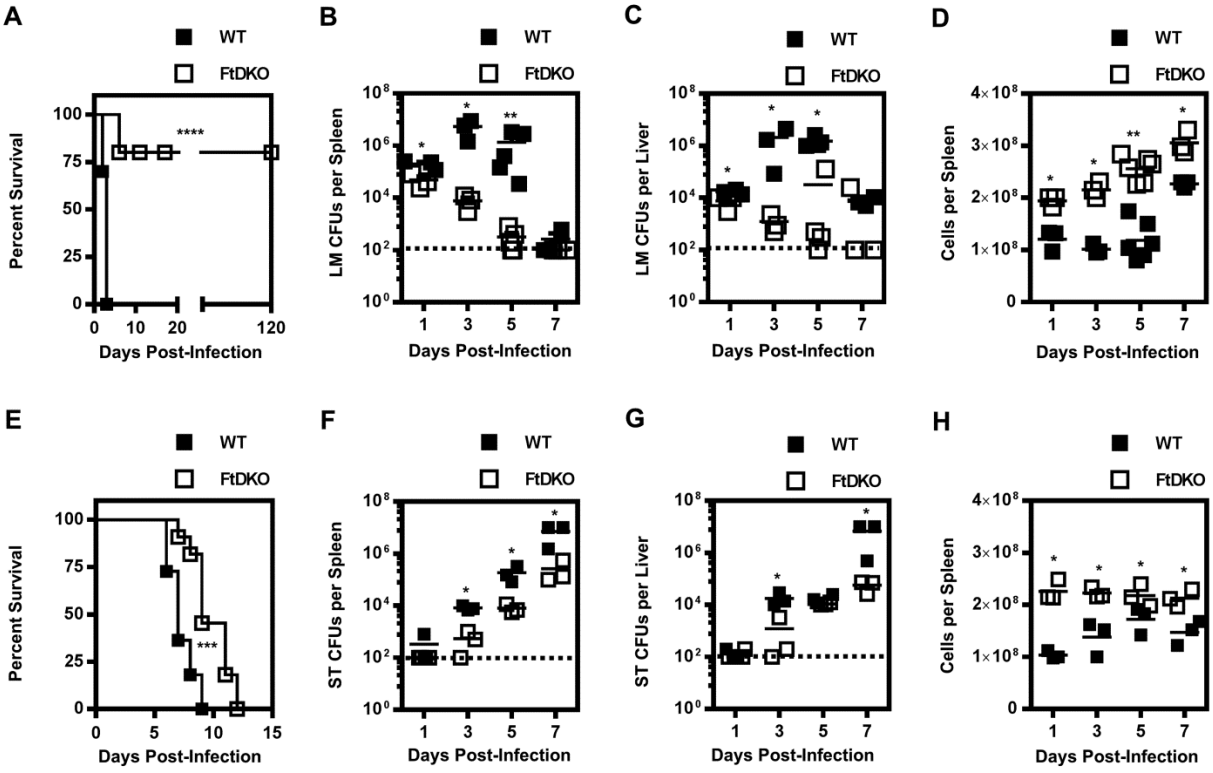
A
WT
FtDKO
Percent Survival
Days Post-Infection
****
B
LM CFUs per Spleen
Days Post-Infection
C
LM CFUs per Liver
Days Post-Infection
D
Cells per Spleen
Days Post-Infection
E
Percent Survival
Days Post-Infection
***
F
ST CFUs per Spleen
Days Post-Infection
G
ST CFUs per Liver
Days Post-Infection
H
Cells per Spleen
Days Post-Infection

Figure 2.2. FtDKO mice control intracellular infection more efficiently compared to WT mice. (A) WT and FtDKO mice were infected with LM 10^5 i.v. and monitored every day following infection. Mouse deaths were recorded upon observing >20% loss in body weight and/or 2 or more severe signs of clinical illness (morbidity, piloerection, lack of grooming). N = 10 mice per group. (B and C) WT and FtDKO mice were infected with LM 10^4 i.v. Spleen and liver bacterial burden was enumerated. Bacterial burden in organs of individual mice is shown, mean ± SEM (D) Splenic cell numbers were enumerated. (E) WT and FtDKO mice were infected with ST $5x10^2$ CFUs i.v. and monitored every day following infection. Mouse deaths were recorded upon observing >20% loss in body weight and/or 2 or more severe signs of clinical illness (morbidity, piloerection, lack of grooming). N = 11 mice per group. (F and G), WT and FtDKO mice were infected with ST 10^2 CFUs i.v. and bacterial burden as enumerated at specific time points post infection. Bacterial burden in organs of individual mice is indicated, mean ± SEM. (H) Splenic cell numbers were enumerated. Survival curves were analyzed using Gehan-Breslow-Wilcoxon test (A, D); and bacterial burden and cell numbers were analyzed by Mann Whitney U test. *: $P \leq 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

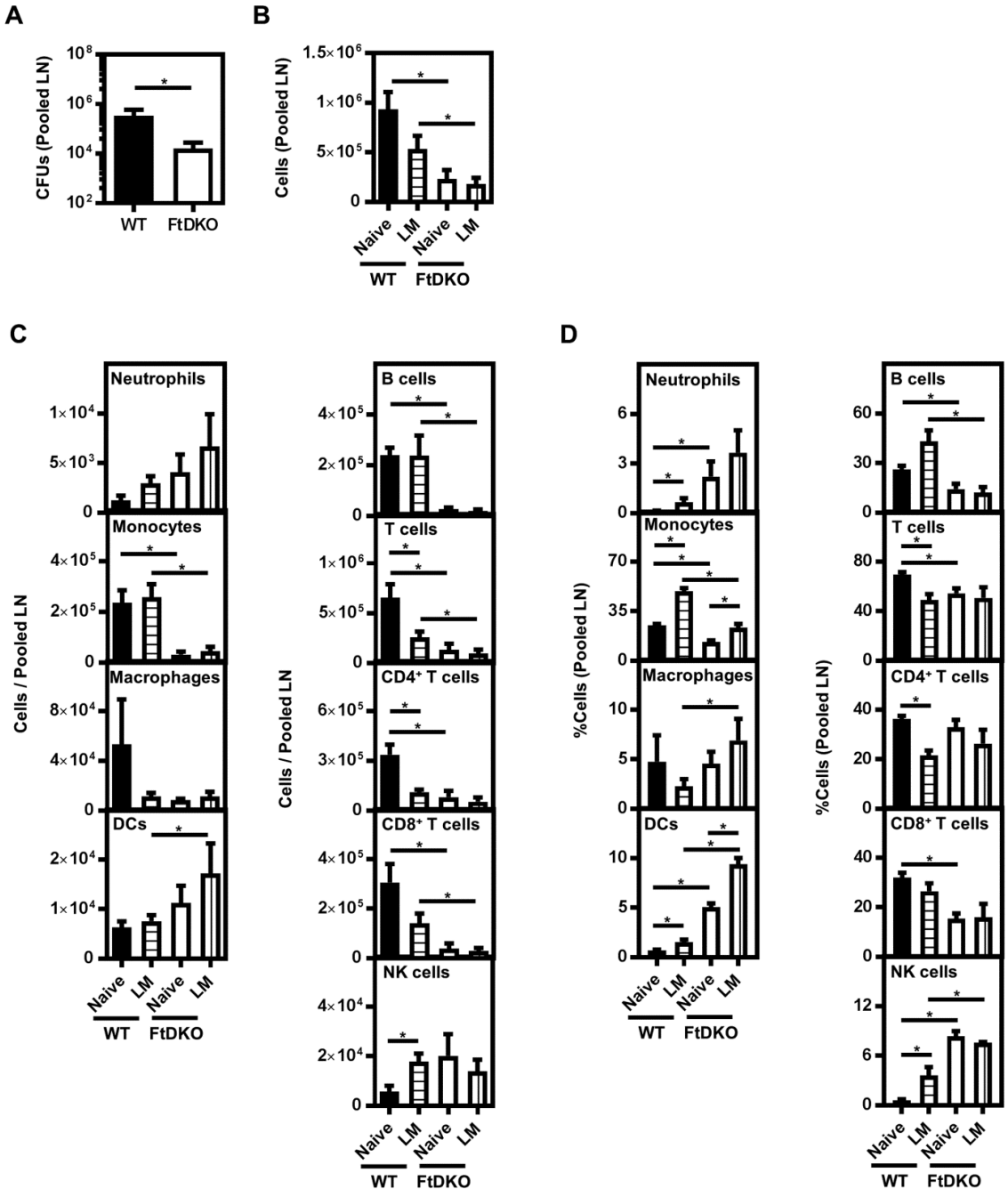
A
CFUs (Pooled LN)
WT
FtDKO
B
Cells (Pooled LN)
Naive
LM
WT
FtDKO
C
Neutrophils
Monocytes
Macrophages
DCs
Cells / Pooled LN
B cells
T cells
CD4+ T cells
CD8+ T cells
NK cells
D
%Cells (Pooled LN)

Figure 2.3. Decreased lymph node cellularity correlates with reduced bacterial numbers in FtDKO mice. WT and FtDKO mice were infected with LM 10^4 CFUs i.v. (A) Bacteria were enumerated in pooled inguinal lymph nodes (LNs) at day 3 post-infection. (B) LN cell numbers were determined using trypan blue and a hemayctometer. Immune cell populations were quantified through flow cytometry at day 3 post-infection. (C) Total cell numbers of immune cell types in pooled inguinal LNs. (D) Percentages of immune cell types in pooled inguinal LNs. Data are presented as mean ± SEM. N = 3 mice per group. Statistical analysis was performed by Mann Whitney U test. $*P \leq 0.05$.

We then infected FtDKO mice with another intracellular pathogen, *Salmonella enterica* serovar Typhimurium (ST), to determine whether the increased protection from infection is limited to LM. C57BL/6J WT mice are highly-susceptible to ST infection partly due to the lack of a functional natural resistance-associated macrophage protein-1 gene (Vidal et al., 1995). As expected, WT and FtDKO mice bred on the C57BL/6J background succumbed to ST ($5x10^2$ CFUs i.v.) infection (Figure 2.2E). However, FtDKO mice showed delayed susceptibility to infection (median survival: 9 days) compared to WT controls (median survival: 7 days). Moreover, bacterial numbers in the spleen were significantly lower (Figure 2.2F) in FtDKO mice at days 3, 5 and 7 post-infection with ST (10^2 CFUs i.v). A similar trend was observed in the liver post-infection (Figure 2.2G). Furthermore, increased splenic cellularity was observed at all time points post-infection (Figure 2.2H), similar to the response against systemic LM infection (Figure 2.2C). Thus, FtDKO mice exhibited general increased resistance to intracellular infections.

2.4.2 Control of LM infection is independent of E-, P-, and L-selectin function

We next performed *in vivo* blockade of E-, P-, and L-selectins to determine whether control of LM infection is directly dependent on selectin action. WT mice treated with a cocktail of anti-mouse CD62E, CD62P and CD62L monoclonal antibody cocktail showed similar bacterial numbers in the spleen and liver as non-treated controls (Figure 2.4A and 2.4B). Furthermore, no significant differences in splenic cell numbers were observed between the two groups (Figure 2.4C). Thus, the increased resistance to infection observed in FtDKO mice could not be recapitulated in WT mice, with blockade of selectins, suggesting involvement of downstream cellular function in the former group.

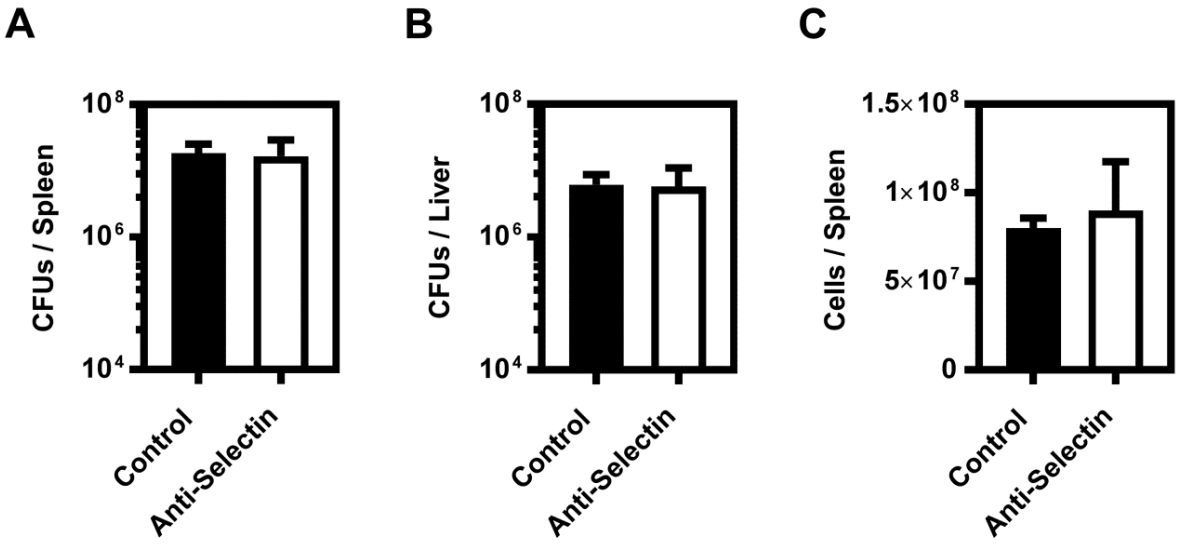
A
B
C
CFUs / Spleen
CFUs / Liver
Cells / Spleen
10^8
10^6
10^4
1.5×10^8
1×10^8
5×10^7
0
Control
Anti-Selectin

Figure 2.4. E-, P-, and L-selectins are not required in the control of LM infection. WT mice were treated with monoclonal antibodies against E-, P-, and L-selectins (anti-selectin) 4 hours pre-infection and 1 day post-infection with LM 10^4 CFUs i.v. (A and B) Bacteria were enumerated in the spleen and liver at day 3 post-infection. (C) Splenic counts were determined at day 3 post-infection. Data are presented as mean ± SEM. N = 3 mice per group. Statistical analysis was performed by Mann Whitney U test.

2.4.3 Increased immune cell numbers in FtDKO mice correlates with enhanced control of systemic LM infection

Functional selectin ligand deficiency results in elevated leukocyte levels in circulation, attributed in part to reduced neutrophil turnover (Homeister et al., 2001). Consistent with our previous study, our data presented below show that enlarged splenic sizes in naïve FtDKO mice is associated with increased cell numbers compared to their WT counterparts (Figure 2.5A and 2.5B) (Stark et al., 2012). A similar trend was observed between WT and FtDKO mice at day 3 post-infection with LM 10^4 CFU i.v. In contrast, no significant differences in liver cellularity were observed between both mouse strains in the naïve and infected states (Figure 2.5C). We then performed flow cytometry analysis of immune cell populations in the blood, spleen and liver at day 3 post-infection. Figure 2.5D and 2.5E indicate the gating strategies used for the identification of innate immune cell types (neutrophils, monocytes, macrophages) and DCs. WT mice exhibited lower numbers of splenic neutrophils, monocytes and DCs compared to FtDKO mice in the naïve state (Figure 2.5F). A similar trend was observed in neutrophil percentages in the spleen, liver and blood relative to FtDKO mice (Figure 2.5G-I). Upon infection, FtDKO mice retained increased neutrophil, monocyte and DC numbers in the spleen, and neutrophil percentages in the spleen and blood compared to WT mice. These results suggest that the increased levels of neutrophils, monocytes and DCs in tissues and/or blood may contribute to the rapid clearance of LM in FtDKO mice.

We also observed skewed distributions of other immune cell populations between both mouse strains. Figure 2.1 includes gating strategies used for lymphocyte subsets. Splenic T cell numbers were increased in naïve FtDKO mice relative to their WT counterparts (Figure 2.6A), consistent with a previous report (Harp and Onami, 2010). A similar trend was observed

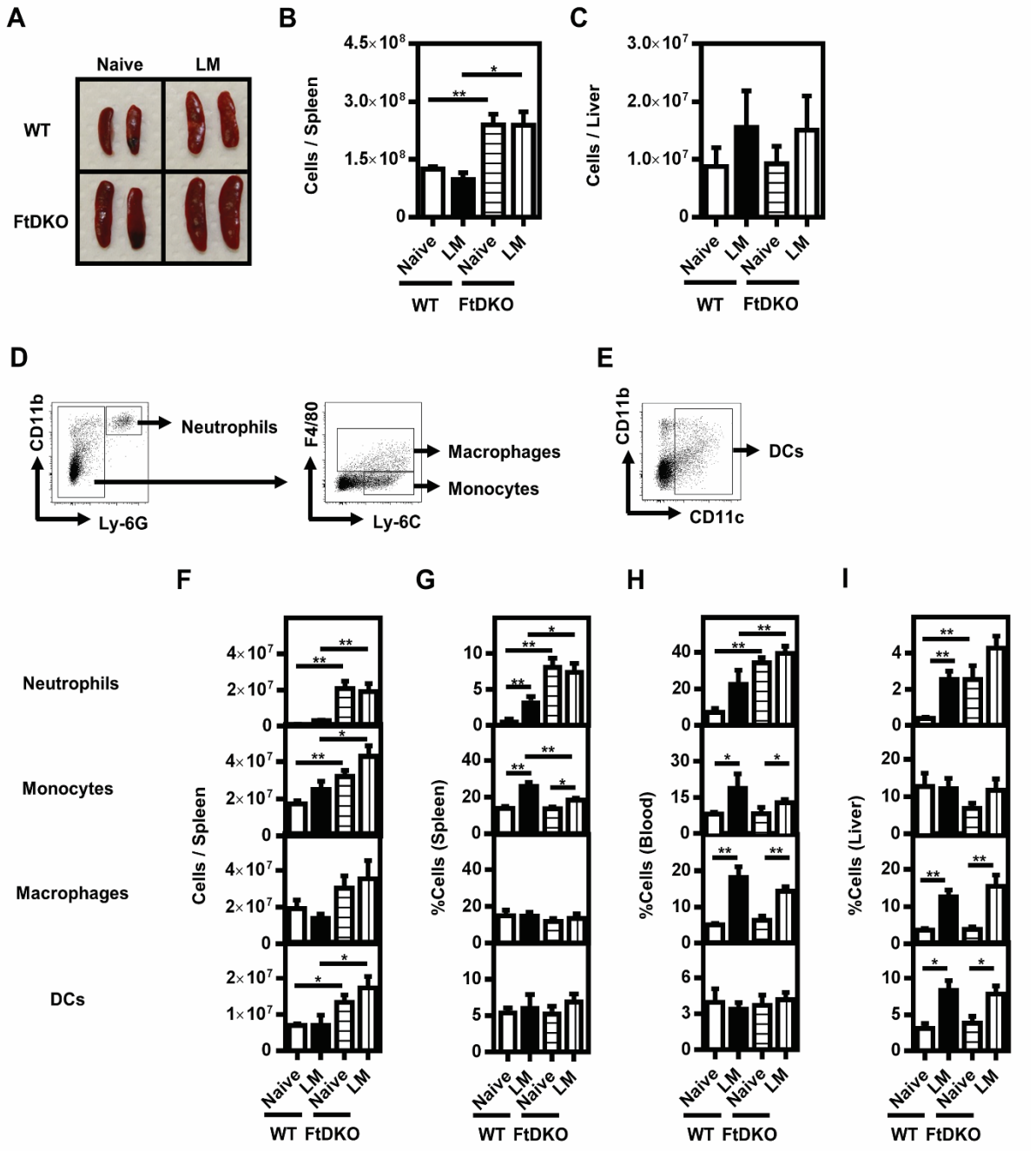
A
Naive
LM
WT
FtDKO
B
Cells / Spleen
4.5×10^8
3.0×10^8
1.5×10^8
0
Naive
LM
Naive
LM
WT
FtDKO
C
Cells / Liver
3.0×10^7
2.0×10^7
1.0×10^7
0
Naive
LM
Naive
LM
WT
FtDKO
D
CD11b
Ly-6G
Neutrophils
F4/80
Ly-6C
Macrophages
Monocytes
E
CD11b
CD11c
DCs
F
G
H
I
Neutrophils
Monocytes
Macrophages
DCs
Cells / Spleen
%Cells (Spleen)
%Cells (Blood)
%Cells (Liver)
Naive
LM
Naive
LM
WT
FtDKO

Figure 2.5. Increased levels of neutrophils, monocytes and DCs correlate with efficient clearance of systemic LM infection in FtDKO mice. WT and FtDKO mice were infected with LM 10^4 CFUs i.v. At day 3 post-infection, spleen, liver and blood immune cell populations were determined by flow cytometry (A) Representative images of spleens from naïve and infected WT and FtDKO mice. (B and C) Splenic and liver cell numbers. (D) Neutrophils were identified as $CD11b^+$ $Ly\text{-}6G^+$ cells. Monocytes and macrophages were defined as $Ly6\text{-}C^+$ $Ly\text{-}6G^-F4/80^-$ and $Ly6\text{-}G^-$ $F4/80^+$ cells, respectively. (E) $CD11c^+$ cells were classified as DCs. (F-I) Numbers and/or percentages of neutrophils, monocytes, macrophages and DCs were evaluated in the spleen, blood and liver of naïve and infected WT and FtDKO mice. Data are presented as mean ± SEM. N = 5-6 per group. Statistical analysis was performed by Mann Whitney U test. $*P \leq 0.05$, $**P < 0.01$.

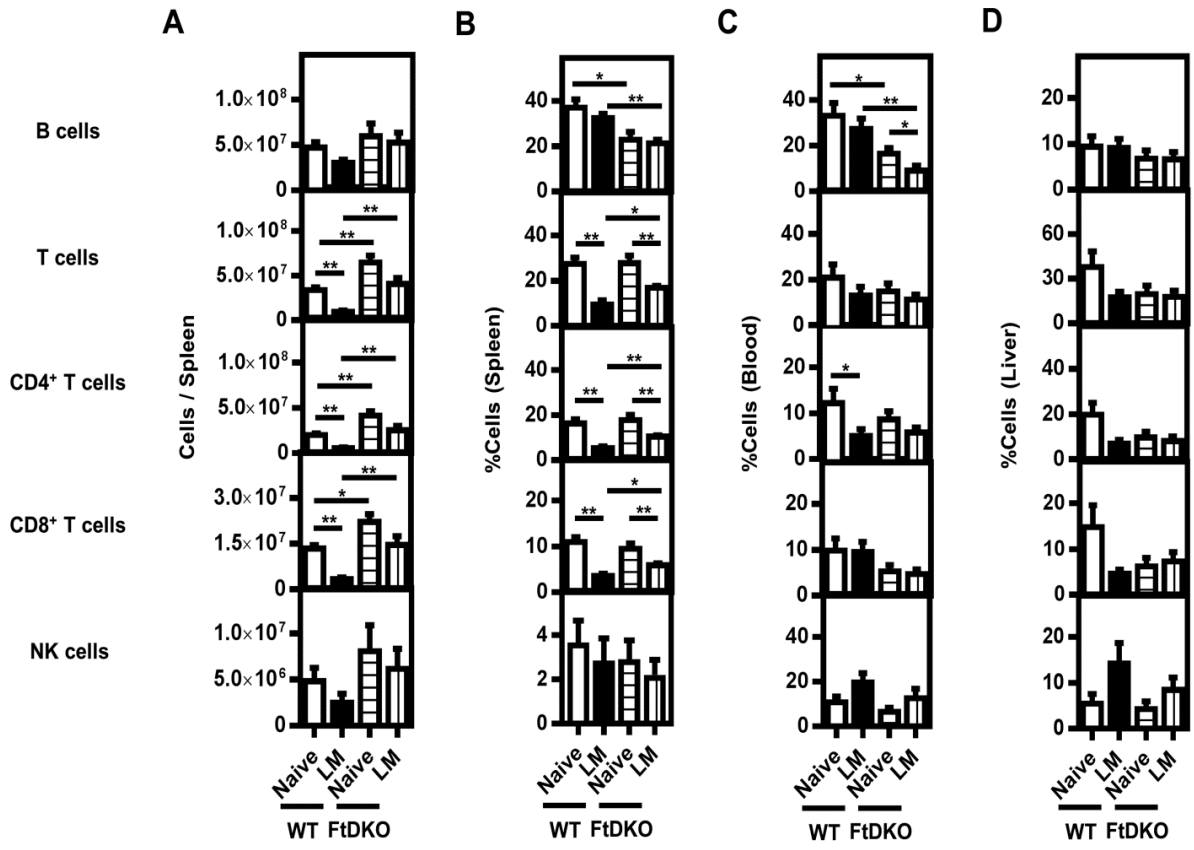
A
B
C
D
B cells
T cells
CD4+ T cells
CD8+ T cells
NK cells
Cells / Spleen
%Cells (Spleen)
%Cells (Blood)
%Cells (Liver)
1.0×10^8
5.0×10^7
3.0×10^7
1.5×10^7
1.0×10^7
5.0×10^6
Naive
LM
WT
FtDKO

Figure 2.6. FtDKO mice exhibit an altered distribution of immune cell types pre- and post-LM infection. WT and FtDKO mice were infected with LM 10^4 i.v. Blood and organs were collected and processed at day 3 post-infection. Cells were then stained with fluorochrome-conjugated antibodies for flow cytometry analysis of immune cell populations. (A and B) Total numbers and percentages of splenic immune cell populations in non-infected and infected WT and FtDKO mice. Immune cell frequencies in the blood (C) and liver (D) of WT and FtDKO mice pre- and post-infection. Data are presented as mean ± SEM. N = 5-6 mice per group. Statistical analysis was performed by Mann Whitney U test. $*P \leq 0.05$, $**P < 0.01$.

between WT and FtDKO mice at day 3 post-infection. Splenic B cell percentages were increased in FtDKO mice relative to WT mice in the naïve state. During infection, reduced B cell and increased T cell percentages were also observed in FtDKO mice. Furthermore, FtDKO mice exhibited decreased B cell proportions in the blood compared to WT mice before and after infection (Figure 2.6C). No significant differences were observed in the total liver cell numbers (Figure 2.5C) although neutrophil numbers in non-infected FtDKO mice liver were higher. Percentages of neutrophils and macrophages in the liver increased in response to infection even in WT mice, and this trend was paralleled in FtDKO mice (Figure 2.5I and 2.6D).

As expected, FtDKO mice also exhibited reduced overall LN cell numbers in the naïve state due to LN atrophy (Figure 2.3B). This was associated with decreased numbers of monocytes, B cells and T cells (Figure 2.3C). At day 3 post-infection, monocyte, B cell and T cell numbers remained reduced, while DC numbers increased relative to WT mice. Differences were also noted in the percentages of neutrophils, monocytes, macrophages, DCs, B cells, T cells and NK cells between both mouse strains in the naïve and/or infected states (Figure 2.3D). Thus, FtDKO mice retain increased protection from systemic LM infection despite defective leukocyte trafficking to LNs and LN atrophy.

2.4.4 FtDKO mice lack LM-induced production of pro-inflammatory cytokines

We assessed the levels of pro-inflammatory cytokines in the sera of WT and FtDKO mice at days 1 and 3 post-infection (Figure 2.7). Upon infection, WT mice exhibited a steady increase in the serum levels of inflammatory cytokines, IL-6, MCP-1, IFN-γ and TNF relative to FtDKO mice. Thus, the efficient control of LM infection in FtDKO mice was not associated

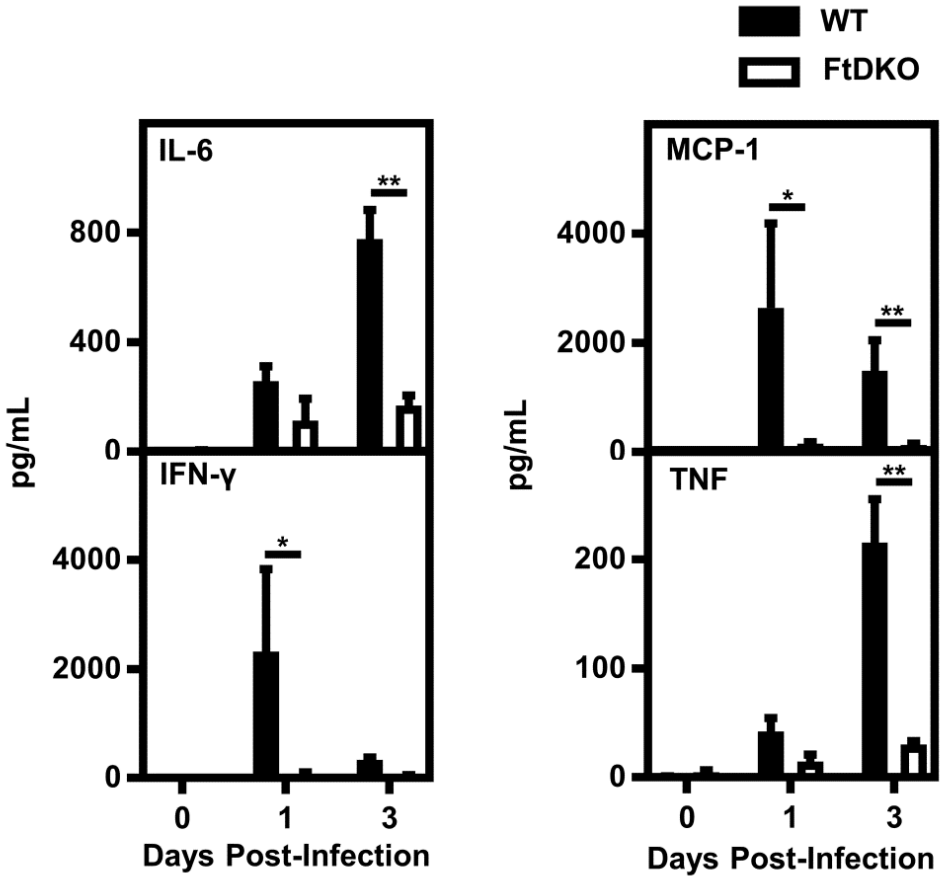
WT
FtDKO
IL-6
**
800
400
0
pg/mL
IFN-γ
*
4000
2000
0
0
1
3
Days Post-Infection
MCP-1
*
**
4000
2000
0
pg/mL
TNF
**
200
100
0
0
1
3
Days Post-Infection

Figure 2.7. Efficient control of infection in FtDKO mice is not associated with increased serum levels of pro-inflammatory cytokines. (A) Serum levels of IL-6, MCP-1, IFN-γ and TNF were measured before infection and at days 1 and 3 post-infection with LM 10^4 i.v. Data are presented as mean ± SEM. N = 3-6 mice per group per time point. Data were analyzed for statistical significance using the Mann Whitney U test. $*P \leq 0.05$, $**P < 0.01$.

with enhanced serum inflammatory cytokine expression. Our results suggest that the cytokine responses in WT mice correlated to increased bacterial burden.

2.4.5 FtDKO mice retain increased protection from LM infection despite neutrophil-specific depletion

We performed *in vivo* depletion of neutrophils to determine their role in the early control of systemic LM infection in FtDKO mice. RB6-8C5 mAb primarily recognizes Ly-6G expressed by neutrophils, but also cross-reacts with Ly-6C expressed by monocytes, macrophages and DCs (Daley et al., 2008; Fleming et al., 1993). In contrast, 1A8 mAb specifically binds to Ly-6G (Daley et al., 2008). Figure 2.8A and 2.8D demonstrate that two injections of either RB6-8C5 or 1A8 mAb depleted neutrophils in the blood in both WT and FtDKO mice by day 3 post-infection. In WT mice, RB6-8C5 mAb treatment resulted in dramatic exacerbation of infection, with death in ~50 % of the mice (CFUs plotted as 10^{10}), and/or significantly increased bacterial burden (Figure 2.8B and 2.8C). A similar increase in splenic and liver bacterial numbers was observed in WT mice treated with 1A8 mAb (Figure 2.8E and 2.8F). RB6-8C5 mAb treatment in FtDKO mice also had a profound effect of increasing splenic bacterial burden approximately 10,000-fold relative to non-treated FtDKO mice. In contrast, treatment with 1A8-mAb increased splenic and liver bacterial burden by ~100-fold in WT mice. However, no significant differences in splenic or liver bacterial numbers were observed between non-treated and 1A8 mAb-treated FtDKO mice (Figure 2.8E).

Next, we examined immune cell numbers in the spleens of neutrophil-depleted FtDKO mice during systemic LM infection (Figure 2.8H and 2.9). RB6-8C5 mAb-treated FtDKO mice

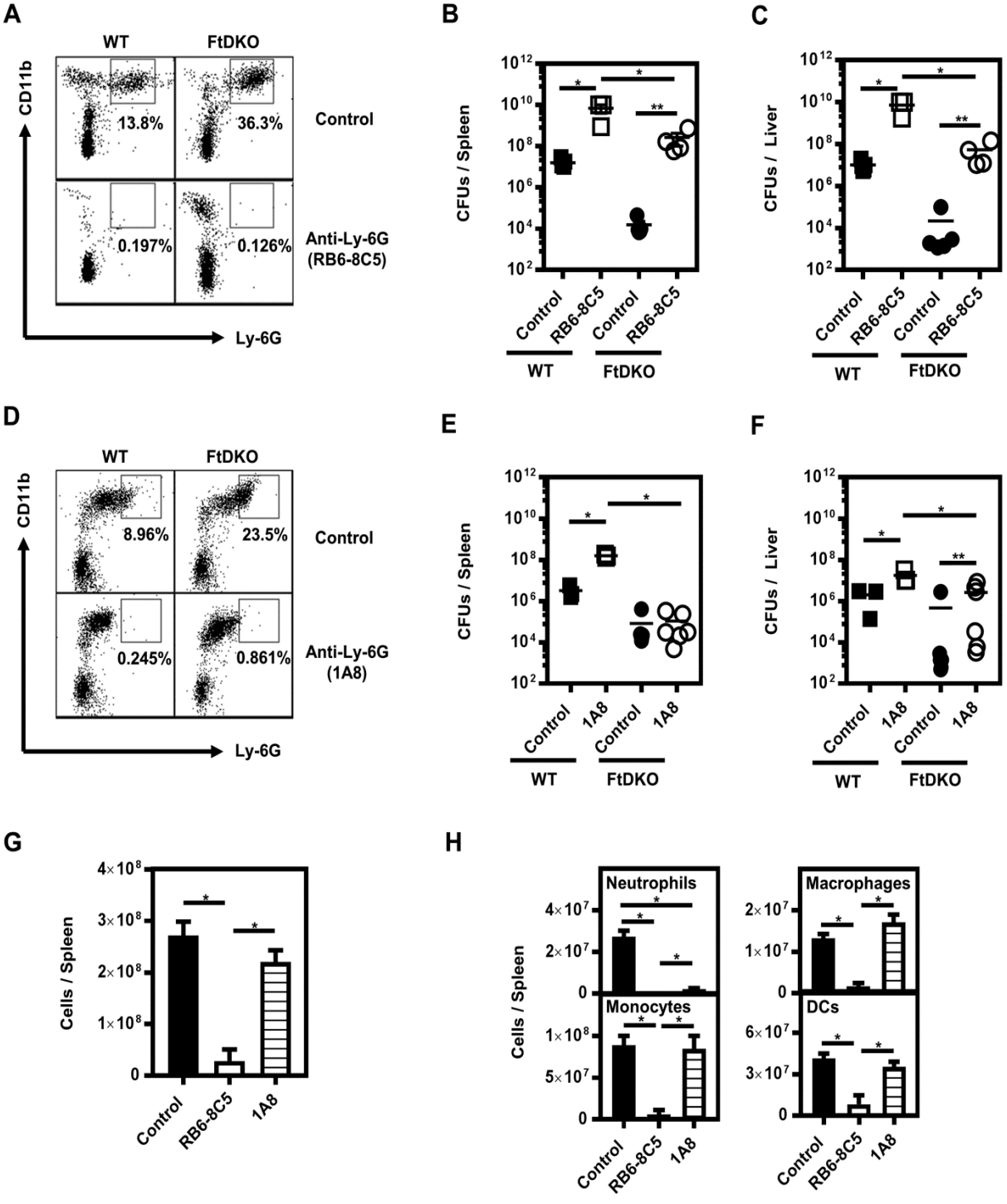
A
WT
FtDKO
CD11b
13.8%
36.3%
Control
0.197%
0.126%
Anti-Ly-6G
(RB6-8C5)
Ly-6G
B
CFUs / Spleen
Control
RB6-8C5
Control
RB6-8C5
WT
FtDKO
C
CFUs / Liver
Control
RB6-8C5
Control
RB6-8C5
WT
FtDKO
D
WT
FtDKO
CD11b
8.96%
23.5%
Control
0.245%
0.861%
Anti-Ly-6G
(1A8)
Ly-6G
E
CFUs / Spleen
Control
1A8
Control
1A8
WT
FtDKO
F
CFUs / Liver
Control
1A8
Control
1A8
WT
FtDKO
G
Cells / Spleen
Control
RB6-8C5
1A8
H
Cells / Spleen
Neutrophils
Monocytes
Macrophages
DCs
Control
RB6-8C5
1A8
Control
RB6-8C5
1A8

Figure 2.8. FtDKO mice exhibit enhanced protection against LM infection despite neutrophil-specific depletion. RB6-8C5 or 1A8 mAb was administered i.p. to WT and FtDKO mice, one day prior to LM 10^4 CFUs i.v. infection, and at day 2 post-infection. Blood from control and mAb-treated mice were collected at day 3 post-infection for bacterial enumeration and flow cytometry analysis of immune cell populations. (A and D) Representative flow cytometry plots of blood neutrophils from WT and FtDKO mice pre- and post-treatment with RB6-8C5 and 1A8 mAbs. (B, C, E, and F) Splenic and liver bacterial burden in organs of individual mice at day 3 post-infection. (G) Total splenocyte counts on day 3 post-infection (n=3-5 mice per group). (H and I) Numbers of immune cell subsets in the spleen at day 3 post-infection. Data are presented as mean ± SEM. N = 3-6 mice per group. Statistical significance between groups was determined by Mann Whitney U test. $*P \leq 0.05$, $**P < 0.01$.

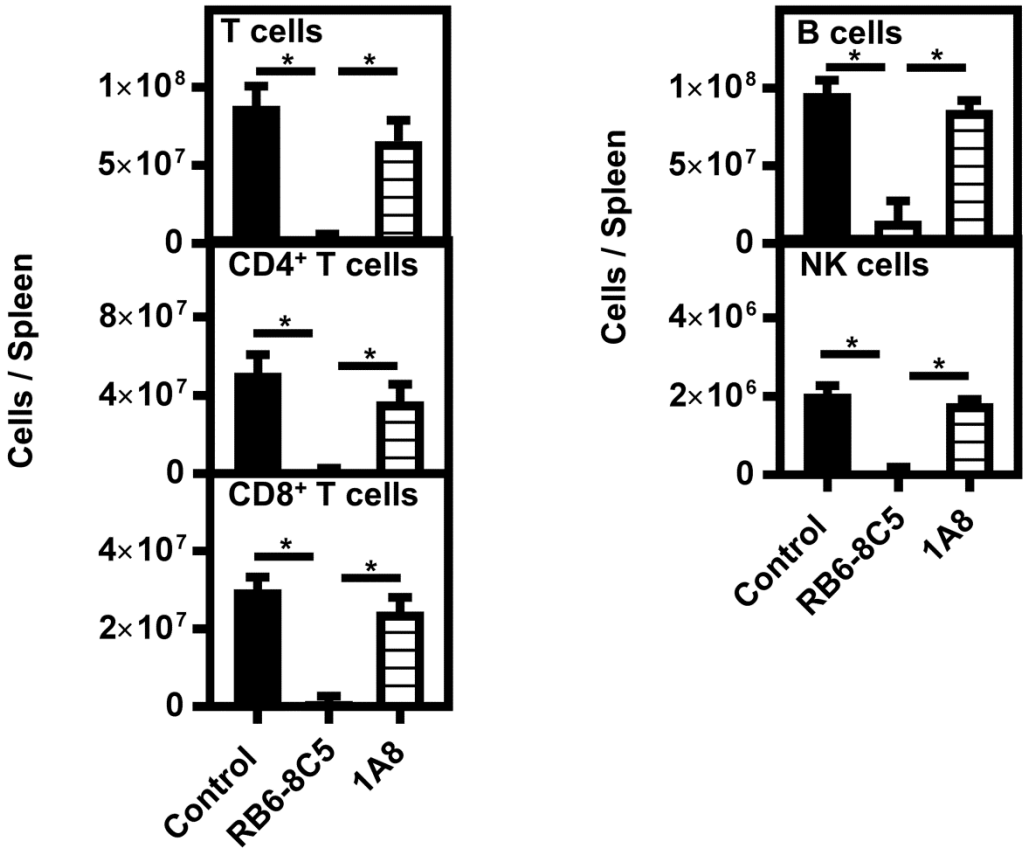
T cells
CD4+ T cells
CD8+ T cells
B cells
NK cells
Cells / Spleen
1×10^8
5×10^7
0
8×10^7
4×10^7
4×10^7
2×10^7
4×10^6
2×10^6
*
Control
RB6-8C5
1A8

Figure 2.9. Anti-Ly-6G (RB6-8C5) treatment leads to reduced splenic lymphocyte numbers in FtDKO mice during infection. FtDKO mice were treated with either RB6-8C5 or 1A8 anti-Ly-6G 6 hours before infection and at day 2 post-infection with LM 10^4 CFUs i.v. Spleens were collected at day 3 post-infection for analysis of lymphocyte populations. Data are presented as mean ± SEM. N = 3 mice per group. Statistical analysis was performed by Mann Whitney U test. *$P \leq 0.05$.

exhibited significantly decreased overall splenic cell numbers relative to non-treated controls and 1A8 mAb-treated FtDKO mice (Figure 2.8G). This correlated with an overall decrease in the numbers of not only splenic neutrophils but also macrophages, DCs and monocytes (Figure 2.8H). In contrast, 1A8 mAb-treated mice showed similar splenic cell numbers in comparison to untreated mice, and specific depletion of splenic neutrophil subset alone compared to non-treated controls (Figure 2.8G and 2.8H). This collective data suggest that eliminating neutrophils alone may be insufficient to abrogate the innate immune resistance to LM in FtDKO mice, as other innate immune cell subsets such as monocytes, macrophages and DCs may also contribute towards the control infection. Furthermore, efficient control of infection in FtDKO mice may be attributed to elevated monocyte and DC numbers in the spleen relative to WT mice (Figure 2.5F).

2.4.6 Hematopoietic compartment of FtDKO mice does not require functional selectin ligand interactions for efficient control of systemic LM infection

We evaluated whether the efficient innate immune response of FtDKO mice against systemic LM infection is dependent on cellular interactions within the functional selectin ligand-deficient environment. BM chimera studies were performed, wherein WT or FtDKO cells were adoptively transferred into lethally-irradiated WT mice, which exhibit normal functional selectin ligand expression. Figure 2.10A schematically outlines the groups of mice and the donor cells that were adoptively-transferred. Recipient mice were allowed to recover and return to normal weight gain following adoptive transfer. $CD45.1^+$ B6.SJL WT and $CD45.2^+$ FtDKO donor cells were successfully engrafted in groups A and B recipient mice, respectively, as observed in the blood at 4 (Figure 2.11B) and 15 (Figure 2.11C) weeks post-adoptive transfer. Furthermore, we achieved similar proportions of $CD45.1^+$ B6.SJL WT and

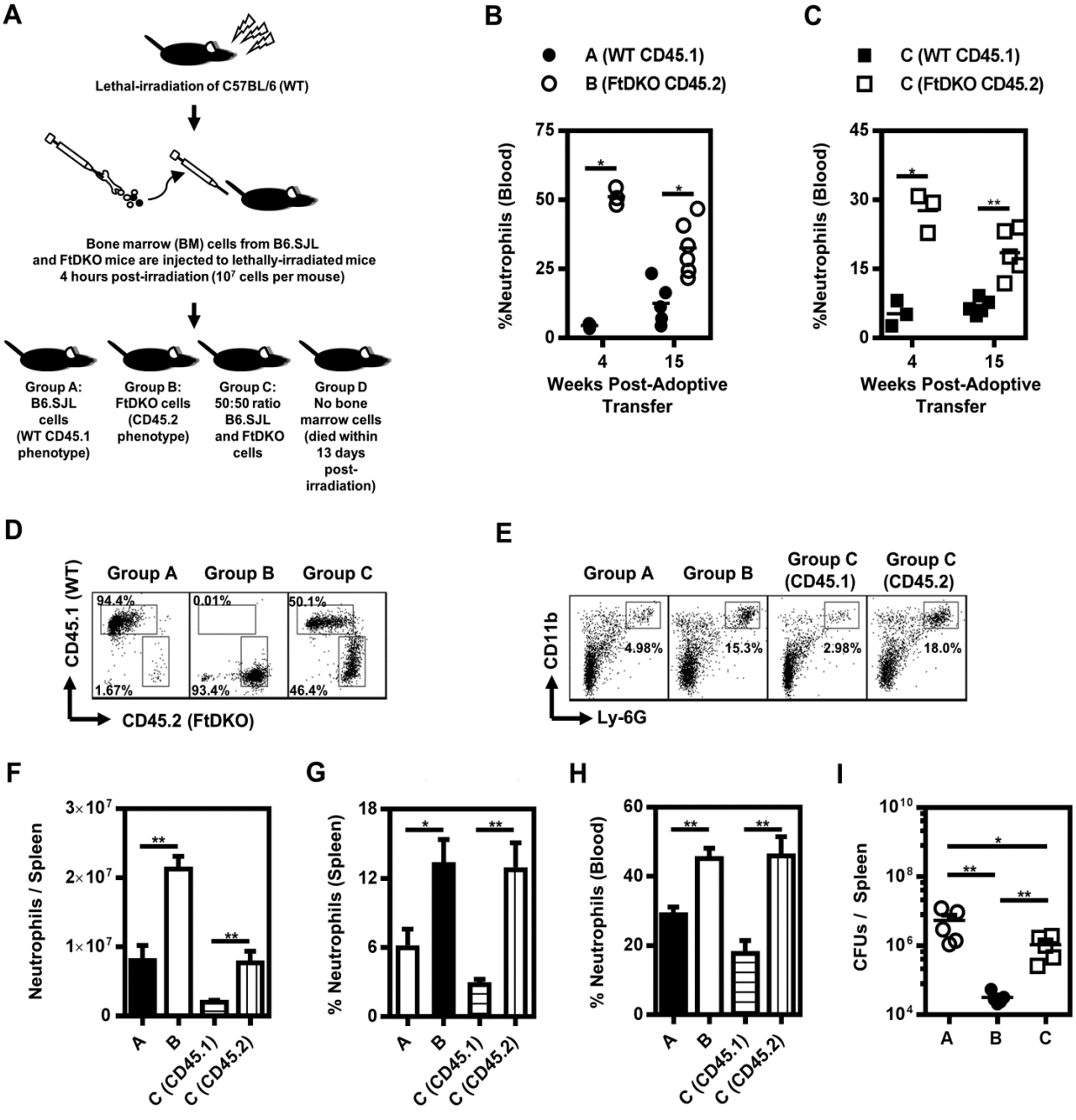
A
Lethal-irradiation of C57BL/6 (WT)
Bone marrow (BM) cells from B6.SJL
and FtDKO mice are injected to lethally-irradiated mice
4 hours post-irradiation (10^7 cells per mouse)
Group A: B6.SJL cells (WT CD45.1 phenotype)
Group B: FtDKO cells (CD45.2 phenotype)
Group C: 50:50 ratio B6.SJL and FtDKO cells
Group D No bone marrow cells (died within 13 days post-irradiation)
B
A (WT CD45.1)
B (FtDKO CD45.2)
%Neutrophils (Blood)
Weeks Post-Adoptive Transfer
C
C (WT CD45.1)
C (FtDKO CD45.2)
%Neutrophils (Blood)
Weeks Post-Adoptive Transfer
D
Group A
Group B
Group C
CD45.1 (WT)
CD45.2 (FtDKO)
94.4%
1.67%
0.01%
93.4%
50.1%
46.4%
E
Group A
Group B
Group C (CD45.1)
Group C (CD45.2)
CD11b
Ly-6G
4.98%
15.3%
2.98%
18.0%
F
Neutrophils / Spleen
A
B
C (CD45.1)
C (CD45.2)
G
% Neutrophils (Spleen)
H
% Neutrophils (Blood)
I
CFUs / Spleen

Figure 2.10. Resistance to systemic LM infection is mediated by factors specific to the hematopoietic compartment of FtDKO mice. (A) Schematic diagram of BM chimera mouse generation. (B and C) Neutrophil percentages in the blood were measured in naïve mice at weeks 4 and 15 post-adoptive transfer through flow cytometry. Mice from all BM chimera groups were then infected with LM 10^4 CFUs i.v. 15 weeks after irradiation and BM transfer. (D and E) Representative flow cytometry plots of total leukocyte ($CD45.1^+$ and $CD45.2^+$ cells) and neutrophil percentages in BM chimera mice at day 3 post-infection. (F) Number of splenic neutrophils on day 3 post-infection (G-H) Neutrophil percentages in the spleen and blood in BM chimera mice at day 3 post-infection. (I) Splenic bacterial burden on day 3 post-infection. Data are presented as mean ± SEM. N = 5-6 mice per group. Data were analyzed by Mann Whitney U test, $*P \leq 0.05$, $**P < 0.01$.

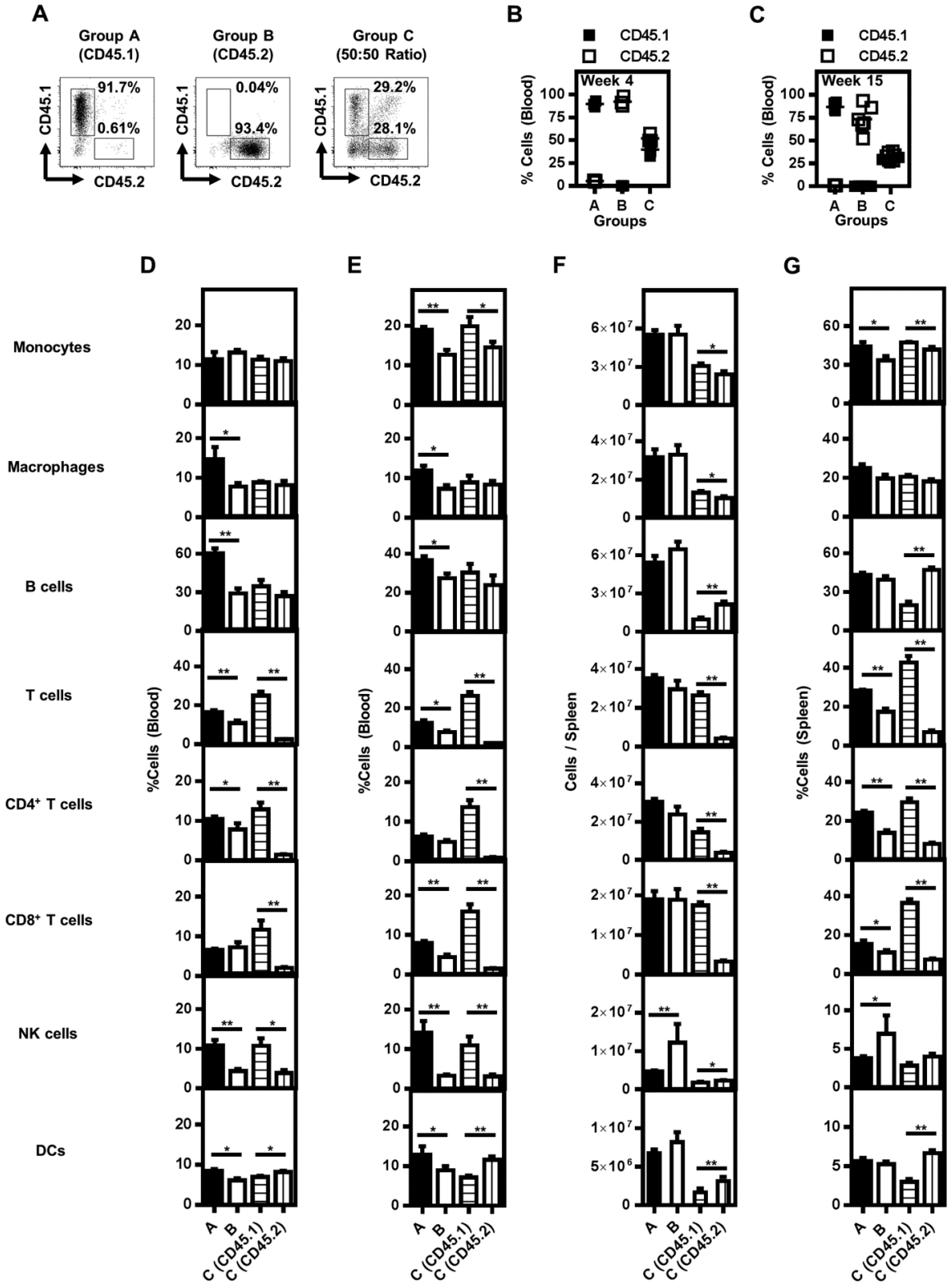
A
Group A (CD45.1)
Group B (CD45.2)
Group C (50:50 Ratio)
CD45.1
CD45.2
91.7%
0.61%
0.04%
93.4%
29.2%
28.1%
B
C
CD45.1
CD45.2
Week 4
Week 15
% Cells (Blood)
Groups
A B C
D
E
F
G
Monocytes
Macrophages
B cells
T cells
CD4+ T cells
CD8+ T cells
NK cells
DCs
%Cells (Blood)
Cells / Spleen
%Cells (Spleen)
A
B
C (CD45.1)
C (CD45.2)

Figure 2.11. Immune cells from FtDKO mice retain a skewed distribution in circulation despite adoptive transfer to a normal WT host environment. (A) Gating strategy for CD45.1$^+$ WT and CD45.2$^+$ FtDKO populations in BM chimera mice. CD45.1$^+$ WT and CD45.2$^+$ FtDKO cell proportions in the blood of groups A, B and C mice were measured at weeks 4 (B) and 15 (C) post-adoptive transfer. (D) Immune cell percentages in the blood of non-infected BM chimera mice at week 15 post-adoptive transfer. (E-G) Blood and spleen was collected for analysis of immune cell populations at day 3 post-infection with LM 10^4 i.v. Data are presented as mean ± SEM. N = 5-6 mice per group. Statistical analysis was performed by Mann Whitney U test. *$P \leq 0.05$, **$P < 0.01$.

CD45.2$^+$ FtDKO donor cells in the blood of Group C mice at both time points (Figure 2.11B and 2.11C). However, mice from Group B exhibited higher neutrophil frequencies in the blood at both 4 and 15 weeks post-adoptive cell transfer, resembling the immune profile of FtDKO mice when compared to mice from Group A in the steady state (Figure 2.10B). Similarly, CD45.2$^+$ neutrophil percentages were increased relative to their CD45.1$^+$ counterparts in Group C mice (Figure 2.10C), despite similar proportions of CD45.1$^+$ and CD45.2$^+$ immune cells in the blood (Figure 2.11B and 2.11C).

Mice were then infected with LM (10^4 CFUs i.v.) at week 15 post-adoptive transfer and immune cell phenotypes were examined in the spleen and blood at day 3 post-infection. Group B mice exhibited significantly higher neutrophil numbers and percentages in the spleen relative to Group A mice (Figure 2.10E-G). In Group C mice, CD45.2$^+$ FtDKO neutrophils retained increased numbers and percentages compared to their CD45.1$^+$ WT counterparts despite having similar overall CD45.1$^+$ and CD45.2$^+$ leukocyte proportions. A similar trend was observed in the blood (Figure 2.10H). Correspondingly, Group A had substantially higher splenic LM burden 3 days post-infection relative to Group B mice (Figure 2.10I). Group C mice had intermediate bacterial burdens. These results suggest that neutrophils and other factors specific to the hematopoietic compartment of FtDKO mice led to increased resistance to LM infection and this effect could be inherently transferred to a WT host by adoptive cell therapy.

We also phenotyped other immune cell types following BM chimera repopulations in the naive state in the blood (Figure 2.11D) and at day 3 post-infection in the blood and spleen (Figure 2.11E-G). Macrophages, B cells, T cells (CD4$^+$), NK cells and DCs were significantly decreased in the blood of Group B mice relative to Group A mice prior to infection. Group C

mice also exhibited reduced proportions of CD45.2$^+$ T cells (CD4$^+$ and CD8$^+$), NK cells and DCs compared to CD45.1$^+$ immune cell counterparts. During infection, blood levels of monocytes, macrophages, B cells, T cells (CD8$^+$), NK cells and DCs were decreased in Group B mice compared to Group A mice. A similar trend was observed in the proportions of CD45.1$^+$ and CD45.2$^+$ monocytes, T cells (CD4$^+$ and CD8$^+$) and NK cells in Group C mice. In the spleen, NK cell numbers were increased in Group B mice relative to Group A mice during infection. Group C mice also showed increased levels of CD45.2$^+$ B cells, NK cells and DCs and decreased numbers of monocytes, macrophages and T cells (CD4$^+$ and CD8$^+$) compared to their CD45.1$^+$ counterparts. Differences were also noted in the percentages of monocytes, T cells and NK cells between Groups A and B mice. Proportions of CD45.1$^+$ and CD45.2$^+$ monocytes, B cells, T cells and NK cells were also significantly different in Group C mice. Thus, BM cells from FtDKO mice promote a skewed innate immune leukocyte distribution despite adoptive transfer to a host environment with normal functional selectin ligand expression.

2.4.7 Altered leukocyte distribution in FtDKO mice is associated with cell-intrinsic functional selectin ligand deficiency

We determined whether the skewed leukocyte distribution in FtDKO mice is also attributed to functional selectin ligand deficiency of the host environment. The reverse BM chimera experiment was performed: FtDKO mice were lethally-irradiated and adoptively transferred with CD45.1$^+$ B6.SJL WT or CD45.2$^+$ FtDKO. Immune cell frequencies were monitored in the blood at week 4 post-adoptive transfer (Figure 2.12). CD45.1$^+$ B6.SJL WT (Group A) or CD45.2$^+$ FtDKO (Group B) donor cells showed distinct immune cell distributions as early as week 4 post-irradiation. Neutrophil levels were increased in Group B

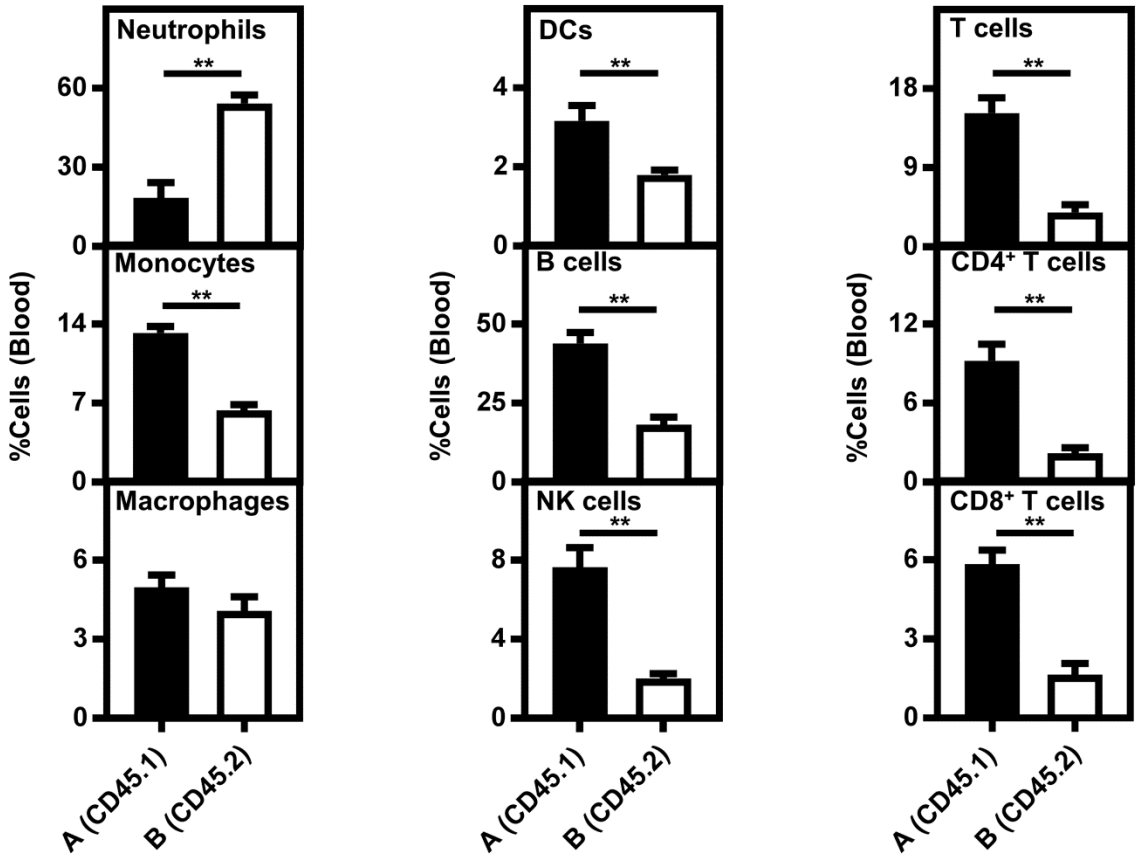
Neutrophils
**
60
30
0
Monocytes
**
14
7
0
Macrophages
6
3
0
%Cells (Blood)
A (CD45.1)
B (CD45.2)
DCs
**
4
2
0
B cells
**
50
25
0
NK cells
**
8
4
0
%Cells (Blood)
A (CD45.1)
B (CD45.2)
T cells
**
18
9
0
CD4+ T cells
**
12
6
0
CD8+ T cells
**
6
3
0
%Cells (Blood)
A (CD45.1)
B (CD45.2)

Figure 2.12. WT and FtDKO donor cells retain distinct cell distributions in circulation despite adoptive transfer to a functional selectin-ligand-deficient host. $CD45.1^+$ WT or $CD45.2^+$ FtDKO BM cells were adoptively-transferred to lethally-irradiated FtDKO mice. (A) At week 4 post-adoptive transfer, blood was collected from recipient mice for flow cytometry analysis of immune cell subsets among $CD45.1^+$ and $CD45.2^+$ immune cell populations. Data are presented as mean ± SEM of immune cell subsets. N = 5 mice per group per time point. Data were analyzed by Mann Whitney U test, $^{**}P < 0.01$.

mice, as seen previously in irradiated WT mice that received CD45.2^{+} FtDKO donor cells at the same time point (Figure 2.10B in comparison to Figure 2.12). Furthermore, monocyte, DC, B cell, NK cell and T cell frequencies were decreased in Group B mice relative to Group A mice. Thus, reconstitution of BM and skewing toward increased circulating neutrophil population by adoptively-transferred cells occurs in either WT or FucT-IV and -VII host environment. These results suggest that FtDKO mice exhibit cell-intrinsic changes that may contribute to the altered leukocyte distribution associated with functional selectin ligand deficiency.

2.4.8 FtDKO mice exhibit increased BM GMP frequencies

GMP, common myeloid progenitor (CMP) and common lymphoid progenitor (CLP) frequencies in the BM of WT and FtDKO mice were first determined through flow cytometry (Figure 2.13A-C). No significant differences in CMP and CLP frequencies were observed between the two mouse strains in the steady state (Figure 2.13C). However, FtDKO mice exhibited increased levels of GMPs relative to WT mice. This result correlated with significantly higher numbers of neutrophils and monocytes, and lower levels of macrophages in the BM of FtDKO mice compared to WT mice. Differences were also noted in B and T cell populations (Figure 2.13D).

We performed progenitor CFU assays to assess the proportions of CFU-GM, CFU-G, and CFU-M progenitors in the BM. Figure 2.13E shows the morphological characteristics of progenitor CFU types at day 12 of culture. Our results correlated with the flow cytometry analysis, showing FtDKO mice to have significantly higher proportions of CFU-GM compared to WT mice (Figure 2.13F). Furthermore, FtDKO mice had higher total progenitor CFUs at

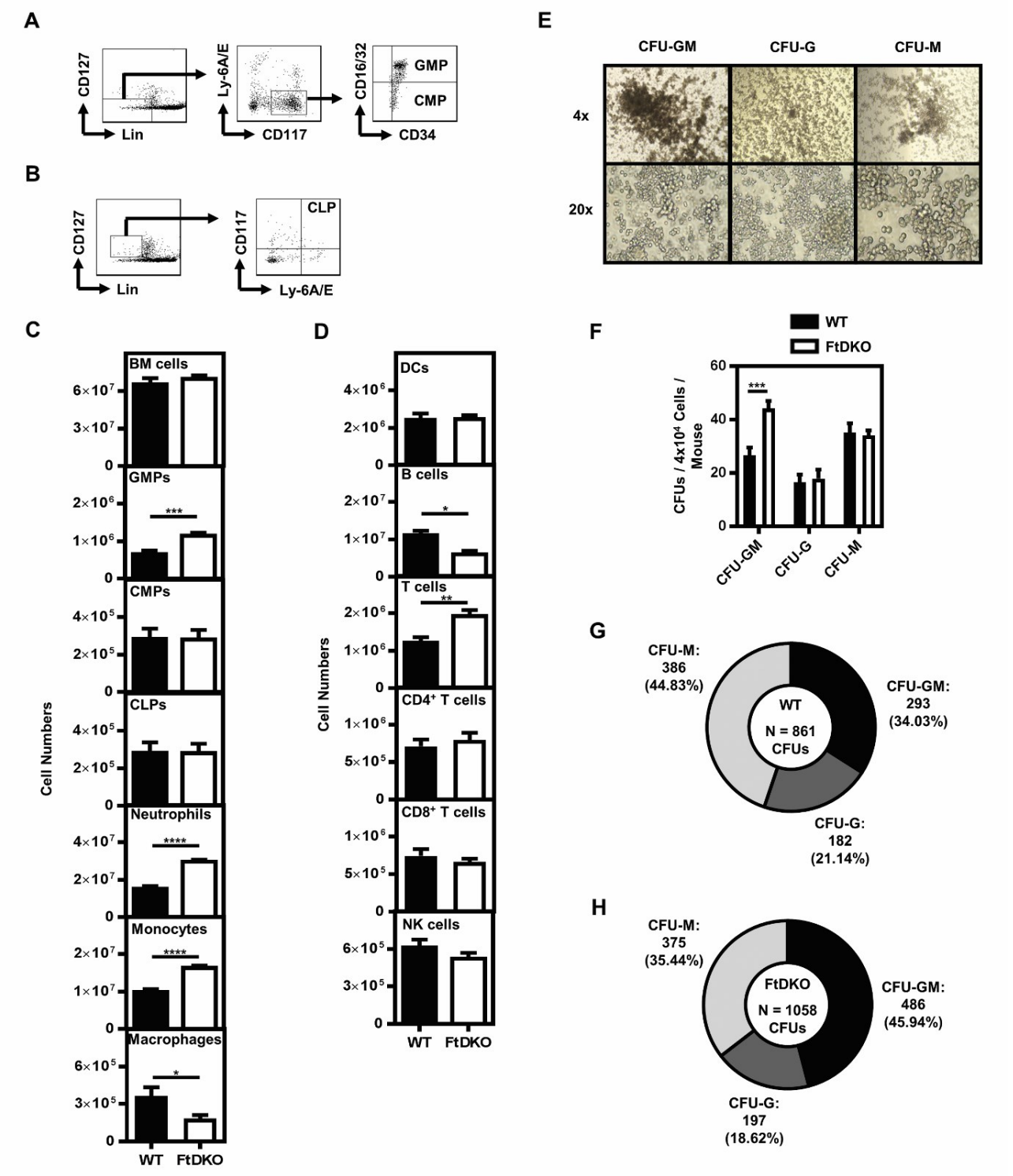
A
CD127
Lin
Ly-6A/E
CD117
CD16/32
CD34
GMP
CMP
B
CD127
Lin
CD117
Ly-6A/E
CLP
C
Cell Numbers
BM cells
GMPs
CMPs
CLPs
Neutrophils
Monocytes
Macrophages
WT
FtDKO
D
Cell Numbers
DCs
B cells
T cells
CD4+ T cells
CD8+ T cells
NK cells
WT
FtDKO
E
CFU-GM
CFU-G
CFU-M
4x
20x
F
WT
FtDKO
CFUs / 4x10^4 Cells / Mouse
CFU-GM
CFU-G
CFU-M
G
CFU-M: 386 (44.83%)
CFU-GM: 293 (34.03%)
WT
N = 861 CFUs
CFU-G: 182 (21.14%)
H
CFU-M: 375 (35.44%)
CFU-GM: 486 (45.94%)
FtDKO
N = 1058 CFUs
CFU-G: 197 (18.62%)

Figure 2.13. FtDKO mice exhibit increased GMP numbers in the BM. In the steady state, BM cells were harvested from WT and FtDKO mice for flow cytometry analysis. (A and B) Gating strategy for analysis of GMP, common myeloid progenitor (CMP) and common lymphoid progenitor (CLP) populations in the BM. (C and D) Total cell numbers of whole BM, progenitors, myeloid and lymphoid immune cell populations in the BM of WT and FtDKO mice were quantified. BM cells were plated in duplicate on MethoCult™ GF M3534 methylcellulose-based media at 2 x 10^4 cells per culture dish. Colonies were scored single-blinded under a light microscope as CFU-GM (granulocyte-macrophage), CFU-G (granulocyte), CFU-M (macrophage), according to morphological characteristics at day 12 of culture. (E) Representative light microscope images show GMP CFUs at 4x and 20x magnifications. (F) Percentages of GMP CFUs from duplicate culture dishes per mouse (4 x 10^4 cells) at day 12 of culture. (G and H) Total GMP CFUs from all mice examined at day 12 of culture. Data are presented as mean ± SEM. N = 8-11 mice per group. Data were analyzed by Mann Whitney U test, $*P \leq 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$.

day 12 of culture despite culturing the same quantity of BM cells from each mouse strain (Figure 2.13G and 2.13H). Thus, elevated levels of neutrophils and monocytes in the BM of FtDKO mice are associated with increased GMP numbers.

In order to examine GMP differentiation capacity on a per-cell basis, we sorted GMPs from the BM and performed the CFU progenitor differentiation assay. WT and FtDKO GMPs exhibited similar total CFU numbers and proportions of CFU-GM, CFU-G and CFU-M at day 12 of culture (Figure 2.14A and 2.14B). Taken together, our results suggest that increased neutrophil and monocyte numbers in FtDKO mice is attributed to higher numbers of GMPs relative to WT mice and not due to enhanced GMP expansion on a per-cell basis.

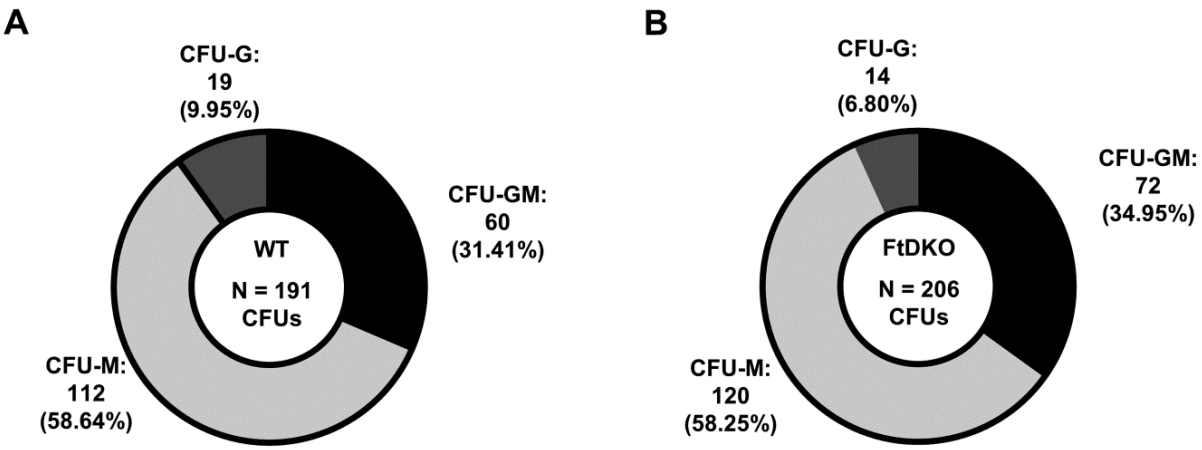
A
CFU-G:
19
(9.95%)
CFU-GM:
60
(31.41%)
WT
N = 191
CFUs
CFU-M:
112
(58.64%)
B
CFU-G:
14
(6.80%)
CFU-GM:
72
(34.95%)
FtDKO
N = 206
CFUs
CFU-M:
120
(58.25%)

Figure 2.14. WT and FtDKO GMPs exhibit similar expansion capabilities on a per-cell basis. Sorted GMPs from WT (A) and FtDKO (B) mice were seeded onto 96-well plates containing MethoCult® GF M3534 methylcellulose-based media at 5-10 cells per well. At day 12 of culture, progenitor CFUs were scored single-blinded according to morphological characteristics under a light microscope. N = 191-206 CFUs compiled from 3 independent experiments.

2.5 Discussion

Functional selectin-ligand interactions mediated by FucT-IV and -VII modulate leukocyte trafficking and homeostasis and may shape innate and adaptive immune responses during inflammation (Maly et al., 1996; Stark et al., 2012; Erdmann et al., 2002; Homeister et al., 2001; Smith et al., 1996; Smithson et al., 2001). We have previously shown the negative impact of functional selectin ligand deficiency on host protection against B16-OVA (murine melanoma expressing ovalbumin), as evidenced by impaired $CD8^+$ T cell infiltration into tumor sites (Stark et al., 2012). In this study, we addressed the implications of functional selectin ligand deficiency on LM infection using FucT-IV and -VII double-knockout (FtDKO) mice. We show that FtDKO mice are able to mount a robust and rapid innate immune response to systemic LM infection despite the expected lack of lymphocyte homing. This efficient control of infection was primarily attributed to increased levels of neutrophils, monocytes and DCs in the blood and/or infected organs. Furthermore, the FtDKO neutrophil-mediated control of infection could be adoptively transferred to a WT host environment, suggesting an inherent change in the neutrophil population in the absence of selectin-ligand interactions. We were able to correlate this to increased GMP frequencies in the hematopoietic compartment in FtDKO mice. Overall, our study demonstrates that lack of functional selectin-ligand interactions may compensate for defective leukocyte trafficking by augmenting innate immune function amongst leukocyte subsets.

Genetic resistance of C57BL/6J (WT) mice against LM infection has been attributed to the resistant allele at the *Hc* locus on chromosome 2, which encodes the component 5 (C5) protein of the complement system (Gervais et al., 1984). C5 is cleaved to generate C5a anaphylatoxin, which promotes chemotaxis of neutrophils and macrophages towards infection

sites during the early phase of primary infection (Garifulin and Boyartchuk, 2005). We found that efficient control of systemic LM infection in FtDKO mice at day 3 post-infection correlated with increased spleen cellularity and neutrophil, monocyte and DC numbers relative to WT mice. Although splenic cell numbers did not increase in FtDKO mice from the naïve to infected state at day 3 post-infection, we showed linear increase in both WT and FtDKO mice in the course of infection (Figure 2.2D). Furthermore, FtDKO mice consistently exhibited higher splenic cell numbers at all time points post-infection compared to WT mice. We also show delayed susceptibility of FtDKO mice to systemic infection by ST, another intracellular infection. It may therefore be surmised that innate immune cells are able to efficiently infiltrate primary lymphoid tissues in response to infection in the absence of functional selectin ligand interactions, unlike T and B lymphocytes. However, the possibility of infection-induced hematopoiesis in extramedullary tissues, such as the spleen, cannot be ruled out (Kim, 2010). FucT-IV and -VII expression has also been implicated in modulating host immunity to bacterial infections induced by *Mycobacterium tuberculosis* and *Anaplasma phagocytophilum* infections (Carlyon et al., 2003; Ehlers et al., 2009; Schreiber et al., 2006). Acute infection of FtDKO mice with low dose *M. tuberculosis* (~100 CFUs per lung) leads to control of bacterial proliferation. This is attributed to recruitment of activated T cells into the lungs similar to WT mice but delayed T cell and macrophage effector response (Schreiber et al., 2006). However, FtDKO mice with chronic *M. tuberculosis* infection exhibit decreased survival capability compared to WT controls despite similar bacterial growth and tissue pathology in infected organs (Ehlers et al., 2009). In contrast, the lack of FucT-IV and -VII expression has been associated with decreased neutrophil binding of *A. phagocytophilum,* a neutrophil-tropic bacterium (Carlyon et al., 2003). This leads to decreased infection of neutrophils and increased neutrophil respiratory burst relative to WT mice. These studies suggest that while regulating

leukocyte migration, FucT-IV and -VII potentially modulate host-pathogen interactions and, consequently, disease progression through their crucial roles in selectin ligand synthesis (Carlyon et al., 2003).

Neutrophil and monocyte recruitment to infection sites are hallmark features of the innate immune response to LM infection (Pamer, 2004; Rogers and Unanue, 1993). However, it was previously suggested that inflammatory monocytes and not neutrophils are required in controlling LM infection (Shi et al., 2011). Furthermore, IFN-γ generated by non-antigen-specific T cells early in the infection may enhance bactericidal activity of macrophages (Berg et al., 2003; Portnoy et al., 1989). Our results with RB6-8C5 or 1A8 antibodies that differentially deplete either many innate immune cell subsets (neutrophils, monocytes, macrophages and DCs) (Fleming et al., 1993) or specifically neutrophils respectively (Daley et al., 2008) indicate that besides neutrophils, other innate immune cells may also contribute towards protection against LM infection in FtDKO hosts. Indeed, the magnitude of bacterial load increase was ~10,000-fold in RB6-8C5-treated FtDKO mice indicating the profound protection afforded by the various innate immune cell subsets in this model. Specific depletion of neutrophils in FtDKO mice resulted in compromised but less pronounced control of infection. Consistent with the previous report of Shi *et al*., efficient control of splenic infection in FtDKO mice may be attributed to increased monocyte numbers during LM infection, despite the absence of neutrophils (Figure 2.8H) (Shi et al., 2011). Taken together, we show that innate immune responses sufficiently controlled bacterial numbers despite impaired lymphocyte homing in our acute infection model. However, it is important to note that adaptive immune responses mediated by lymphocytes are not dispensable during LM infection (Stark et al., 2012; Pamer, 2004; Sun et al., 2004). We previously showed that sterilizing immunity to LM

was mediated by antigen-specific $CD8^+$ T cells as well upon infection re-challenge (Stark et al., 2012). Thus, enhanced innate immune responses and robust recall $CD8^+$ T cell responses mediate bacterial clearance and long-term protection against LM infection in FtDKO mice.

The increased leukocyte phenotype in FtDKO mice is similarly found in individuals with leukocyte adhesion deficiency II (LAD II), which is attributed to a defective GDP-fucose transporter (SLC35C1) (Yakubenia et al., 2008; Harris et al., 2013). Mice lacking SLC35C1 expression ($SLC35C1^{-/-}$) also exhibit deficient selectin ligand fucosylation, abrogation of E-, L-, and P-selectin-dependent leukocyte rolling, impaired lymphocyte homing to lymph nodes (LNs) and reduced LN cellularity (Yakubenia et al., 2008; Hellbusch et al., 2007). However, lymphocytes are able to home to the spleen, indicating a SLC35C1-independent mechanism of lymphocyte trafficking. It was also suggested that lymphocyte homing to the spleen does not require fucosylation by FucT-IV and -VII (Homeister et al., 2001). In the naïve state, we show an association among LN atrophy, increased splenic accumulation of T cells and elevated levels of B cells in the blood of FtDKO mice compared to WT controls. Upon infection, splenic T cell numbers and blood B cell levels remained higher, while LN cellularity remained lower in FtDKO mice relative to WT mice. Splenic T cell numbers also decreased in WT mice from the naïve to infected state which may suggest efflux or death of T cells in response to infection. However, no significant changes were observed in FtDKO mice. Thus, it is possible that the increased presence of T cells in the spleen rather than LN compartment of FtDKO mice attributable to LN atrophy may also contribute to protection against LM. Nevertheless, as in this study we addressed primary infection, and differences in bacterial numbers between WT and FtDKO mice 3 days post-infection, it is likely that the resistance to infection was restricted to effects of innate immune cells.

During LM infection, IL-6 upregulation is associated with neutrophilia within the peripheral blood and consequent defense against infection (Dalrymple et al., 1995). Furthermore, increased expression of IFN-γ, TNF and MCP-1 correlates with enhanced bacterial clearance in infected organs (Havell, 1989; Buchmeier and Schreiber, 1985; Serbina et al., 2003a). We show that FtDKO mice have decreased levels of IL-6, TNF, IFN-γ and MCP-1 in the serum compared to WT mice in the naive state and at day 3 post-infection with LM. Our results suggest that increased numbers of neutrophils potentially results in rapid curtailment of bacterial numbers, preventing a sequel of inflammatory cytokine production that normally ensues following infection.

The BM contains a reserve pool of mature neutrophils that are promptly recruited into circulation following infection (Furze and Rankin, 2008). Control of LM infection requires a balance between bacterial load and BM neutrophil supply, wherein lethal infection dose may lead to terminal BM exhaustion and decreased control of infection (Navarini et al., 2009). Neutrophil percentages in the BM of FtDKO mice are significantly higher compared to WT mice in the naive state (Figure 2.13C). It is conceivable that the increased neutrophil reserve in FtDKO mice provides a large pool of cells that can readily respond to LM infection relative to WT mice. Elevated leukocyte levels in FtDKO mice have been previously attributed to increased neutrophil production and reduced neutrophil turnover in circulation relative to WT mice (Homeister et al., 2001). We also showed that donor FtDKO cells successfully reconstituted the BM of irradiated WT recipient mice. Given the normal expression of FucT-IV and -VII in the host environment of recipient mice, selectin ligands such as P-selectin glycoprotein ligand-1 (PSGL-1) expressed on FtDKO donor cells most likely were functional resulting in homing of donor cells to the BM. The precise mechanisms behind the decreased

disappearance rate of FtDKO neutrophils from circulation in WT hosts remain to be elucidated (Homeister et al., 2001). Nevertheless, infection induced in BM chimera recipient mice indicated that the ability of functional selectin ligand-deficient immune cells to confer resistance to LM infection could be transferred to a normal WT host environment. Thus, our data suggest that absence of selectin ligand expression inherently confers survival advantage even in a normal host environment. We also showed that donor WT cells successfully reconstituted the BM of irradiated FtDKO mice despite being transferred to a functional selectin ligand-deficient host environment. This suggests that homing of transplanted BM cells is not dependent on functional selectin ligand activity mediated by FucT-IV and -VII.

The BM also serves as the major niche for the maintenance and differentiation of hematopoietic stem cells (HSCs) in humans and mice (Mikkola and Orkin, 2006; Vazquez-Boland et al., 2001). HSCs are self-renewing cells that differentiate into progenitors, leading to the expansion of various mature effector cell types (Seita and Weissman, 2010; Mazo et al., 2011). Common myeloid progenitors (CMPs) generate granulocyte-macrophage progenitors (GMPs), which are non-self-renewing cells that develop into granulocytic and monocytic/macrophage populations (Nakorn et al., 2003; Seita and Weissman, 2010). In contrast, common lymphoid progenitors (CLPs) give rise to lymphocyte populations and some DC subsets (Nakorn et al., 2003; Seita and Weissman, 2010). We show that high neutrophil numbers in FtDKO mice correlate with increased GMP frequencies in the naive state. In humans, P-selectin interaction with PSGL-1 was shown to downregulate BM progenitor cell proliferation (Levesque et al., 1999). It was proposed that a similar mechanism occurs in mice, given that PSGL-1 requires fucosylation for functional interaction with P-selectin (Homeister et al., 2001). We also show increased CFU-GM numbers in FtDKO BM cells relative to their

WT counterparts at day 12 of progenitor culture, despite plating the same quantity of BM cells. However, no significant differences in progenitor CFU proportions were observed between both mouse strains upon culturing sorted GMPs. Thus, our *in vitro* results suggest that the preponderance of FtDKO neutrophils in lethally-irradiated WT recipients, as shown by our BM chimera studies, may be attributed to increased numbers of adoptively-transferred GMPs in FtDKO BM cells. It remains to be elucidated whether the lack of PSGL-1 fucosylation *in vivo* leads to enhanced GMP expansion on a per-cell basis and increased leukocyte levels in FtDKO mice.

In summary, functional selectin ligand deficiency contributes to the control of systemic intracellular infection through enhanced neutrophil responses complemented with increase in other innate immune cell types, despite defective leukocyte trafficking and lymphocyte homing. Transplantation of donor HLA-matched neutrophils may provide supportive treatment to antibiotics during severe infections or as prophylactic treatment, notably in patients with neutrophil deficiency (neutropenia) (Navarini et al., 2009; Fischer et al., 1994; Drewniak and Kuijpers, 2009). Cytokine- and/or cell-based immune therapies used in the treatment of cancer and inflammatory diseases may also provide therapeutic potential against infections in individuals with monocyte and/or macrophage dysfunction (Green et al., 2016; Possamai et al., 2014). Further studies are required in the development and validation of immune therapies and to decrease the risk of pathologies related to excessive inflammation.

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3.0 MANUSCRIPT TWO

Modulation of Th17 and regulatory T-cell responses during murine pregnancy contributes to increased maternal susceptibility to *Salmonella* Typhimurium infection

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The specific contributions of each author in the research article are listed below:

Gerard Agbayani: Designed and performed the experiments for Figures 3.1, 3.2, 3.3, 3.6, 3.7 and 3.8, analyzed the data, and wrote and edited the manuscript

Kristina Wachholz: Designed and assisted in performing the experiments for Figures 3.1, 3.2, 3.3, 3.6, 3.7 and 3.8, and analyzed the data

Anindita Chattopadhyay: Performed the experiments for Figure 3.5

Komal Gurnani: Performed the experiments for Figure 3.4

Shawn P. Murphy: Contributed to the discussion and ideas, and edited the manuscript

Lakshmi Krishnan: Conceptualized the study, designed the experiments, wrote and edited the manuscript, and contributed reagents

3.1 Abstract

Problem*: Salmonella* Typhimurium (ST) infection in pregnant mice results in massive placental infection and fetal loss but also exacerbated systemic infection and maternal death. The inflammatory Th17 host response is considered important for control of ST infection, whereas successful pregnancy correlates to a dampened inflammatory and enhanced regulatory T cell (Treg) response. It is unclear how Treg responses in pregnancy may modulate Th17 immunity against ST infection in pregnant mice.

Method of Study: Mice were infected systemically with ST and 24-72 h later, splenic bacterial burden was enumerated. Splenic Th17 and Treg cell numbers were determined through flow cytometry. Serum cytokine expression was analyzed using cytokine bead array. Additionally, splenic and/or placental mRNA expression of IL-17A, RORγ-t, IL-10 and TNF was determined through qRT-PCR. The effects of *in vivo* $CD25^+$ cell depletion and TLR4 blockade on the course of ST infection and Th17 response in non-pregnant and pregnant mice were determined.

Results: Enhanced ST burden in pregnant mice was associated with increased serum inflammatory cytokine signature. *In vivo*, TLR4 blockade reduced splenic ST bacterial burden, suggesting detrimental TLR4-mediated inflammation. However, the Th17 response was reduced both in the spleen and placentas in ST-infected pregnant mice relative to non-pregnant controls. In contrast, there was an increase in splenic Treg frequency in pregnant mice compared to the non-pregnant infected mice and depletion of this subset reduced bacterial burden while also increasing the systemic Th17 response in pregnant infected mice.

Conclusion: Down-regulation of systemic Th17 cell responses by Treg cells during pregnancy potentially contributes to exacerbation of ST infection in pregnant mice.

3.2 Introduction

Maternal tolerance to the semi-allogeneic fetus in mammalian pregnancy necessitates distinct immunological changes in both systemic and local utero-placental compartments. The classical Th1/Th2 paradigm, defined by the down-regulation of cell-mediated T-helper 1 (Th1) cell responses in favor of humoral and T-helper 2 (Th2) cell responses, was initially proposed as a simplistic explanation for promoting tolerance during pregnancy (Wegmann et al., 1993). However, a more complex network of immune regulatory mechanisms that comprises a balance between inflammatory and anti-inflammatory immune responses has been associated with successful pregnancy (Saito et al., 2010; Figueiredo and Schumacher, 2016). T-helper 17 (Th17) cells are pro-inflammatory $CD4^+$ T cells that express the retinoic acid-related orphan receptor gamma-t (RORγ-t) transcription factor and secrete interleukin-17A (IL-17A) cytokine (Moseley et al., 2003). In contrast, regulatory T cells (Tregs) are typically defined as immune-suppressive $CD4^+CD25^+$ T cells that express the forkhead box p3 (Foxp3) transcription factor, and secrete anti-inflammatory cytokines such as IL-10 and transforming growth factor-beta (TGF-β) (Josefowicz et al., 2012). Increased systemic frequencies of $CD4^+CD25^+$ Tregs are seen in both human and mouse pregnancies and these are considered a critical regulatory pathway for promoting fetal tolerance (Shima et al., 2010; Somerset et al., 2004). Furthermore, decreased Treg frequency and/or increased Th17 responses have been associated with pregnancy complications, including spontaneous abortions and pre-eclampsia (Sasaki et al., 2004; Odegard et al., 2000; Toldi et al., 2008).

Maternal immune alterations in pregnancy can exacerbate infection against certain intracellular pathogens such as *Salmonella* Typhimurium (ST). Systemic ST infection during pregnancy in normally resistant 129X1/SvJ mice, with a functional natural resistance-

associated macrophage protein-1 (Nramp1), leads to severe placental infection, rapid fetal loss and maternal death (Pejcic-Karapetrovic et al., 2007). This was associated with enhanced expression of pro-inflammatory cytokines such as IL-6, tumor necrosis factor (TNF) and interferon-gamma (IFN-γ) in the placental compartment (Pejcic-Karapetrovic et al., 2007). In contrast, there was decreased recruitment of innate immune cells such as neutrophils, dendritic cells (DCs) and natural killer (NK) cells to the splenic compartment of infected pregnant mice relative to resistant, infected non-pregnant mice (Pejcic-Karapetrovic et al., 2007). Thus, ST infection during murine pregnancy exhibited a dichotomous response; increased placental inflammation and decreased splenic innate immunity (Chattopadhyay et al., 2010; Pejcic-Karapetrovic et al., 2007).

The increase in Tregs during murine allogeneic pregnancy was correlated to reduced resistance to intracellular bacterial infections by pathogens such as *Listeria monocytogenes* (LM) and ST (Rowe et al., 2011). In contrast, Th17 cells are effector lineage cells that contribute to effective immunity against microbial pathogens such as ST. In the current study, we evaluated the link between Th17 and Treg responses after murine ST infection and their correlation to systemic maternal immunity during pregnancy.

3.3 Materials and Methods

3.3.1 Mice and Matings

129X1/SvJ and 129/Sv-E mice (8-10 weeks old) were purchased from Jackson Laboratories (Bar Harbor, ME, USA) and Charles River Laboratories (St. Constant, QC, Canada), respectively. One male and one to two females were housed overnight and the presence of a copulatory plug the following morning was designated as day 0 of pregnancy. The study was performed in accordance with the protocols (2011.04 and 2013.03) approved by National Research Council Canada in accordance with the guidelines of the Canadian Council on Animal Care (CCAC).

3.3.2 Infections and Bacterial Enumeration

Salmonella enterica serovar Typhimurium (ST) was grown to mid-log phase (OD_{600} = 0.8) in brain-heart infusion (BHI) broth (BD Biosciences, Mississauga, ON, Canada) supplemented with 50 μg/mL streptomycin (Sigma-Aldrich, Oakville, ON, Canada). Bacterial stocks were maintained frozen in 20% glycerol at -80ºC. Frozen bacterial stocks were thawed and diluted in 0.9% sodium chloride (saline) solution. Mice were infected intravenously (i.v.) via the lateral tail vein with 10^3 CFU of ST SL1344 suspended in 200 μL of saline solution.

Pregnant mice were infected at day 11 or 12 of gestation (mid-pregnancy). Mice were euthanized 24-72 hours (h) after infection. Spleens and placentas were harvested and placed in RPMI 1640 medium containing 8% fetal bovine serum (FBS). Single cell suspension was obtained following tissue homogenization. Bacterial numbers in infected organs were quantified as colony-forming units (CFUs) by plating serial dilutions of tissue homogenates suspended in 0.9% saline solution onto BHI agar plates.

3.3.3 Antibody Treatments

In vivo blockade of TLR4 was performed through intraperitoneal (i.p.) administration of 100 µg anti-mouse TLR4/MD-2 antibody (clone MTS510; eBioscience, Burlington, ON, Canada) on the day of ST infection. Spleens were collected 72 hours post-infection for bacterial enumeration. Anti-CD25 treatment was performed through i.p. administration of 100 µg anti-mouse CD25 antibody (clone PC61.5; eBioscience) per mouse 4 days prior to infection with ST.

3.3.4 Serum Cytokine Analysis

Blood was collected in Microtainer® serum separation tubes (BD) via submandibular bleeding of mice under anesthesia. Serum expression of IL-6, IL-10, MCP-1, IFN-γ, TNF, IL-1β, IL-12p70 and KC (CXCL1) cytokines was quantified using the Cytometric Bead Array (CBA) Mouse Inflammation Kit (BD Biosciences) and Mouse Flex Sets (BD Biosciences). Cytokine expression levels were analyzed using FCAP Array™ software (Soft Flow Hungary Ltd., Pécs, Hungary).

3.3.5 Quantitative Real-Time PCR (qRT-PCR)

Total RNA was isolated from spleens and placentas using the RNeasy Mini Kit (Qiagen, Toronto, ON, Canada). Complementary DNA (cDNA) was synthesized from 1,000 ng of RNA in a 40-µL reaction volume containing: 2 µL dNTP (10 mM; New England Biolabs, Whitby, ON, Canada), 2 µL anchored oligo$(dT)_{20}$ (5 µmol/L; Sigma-Aldrich, Oakville, ON, Canada), 4 µL 5x First Strand (FS) buffer (Invitrogen, Burlington, ON, Canada), 1 µL RNaseOUT (Invitrogen), 2 µL M-MLV Reverse Transcriptase (Invitrogen, Burlington, ON, Canada) and RNase-free water (Qiagen). IL-17A and RORγ-t mRNA expression levels

were measured based on primers and qRT-PCR conditions described by Zhang *et al*. (Zhang et al., 2012). The following primer sequences were used: IL-17A, 5'-TCTCTGATGCTGTTGCTGCT-3' (forward) and 5'-CGTGGAACGGTTGAGGTAGT-3' (reverse); RORγ-t, 5'-TGCAAGACTCATCGACAAGG-3' (forward) and 5'-AGGGGATTCAACATCAGTGC-3' (reverse); β-actin: 5'-CAGCCTTCCTTCTTGGGTAT-3' (forward) and 5'-TGGCATAGAGGTCTTTACGG-3' (reverse). Relative IL-17A and RORγ-t mRNA expression levels were normalized to β-actin. Four-fold dilutions (1, 1/4, 1/16, 1/64) of cDNA were used as templates to generate the standard curve for each primer pair. Samples were run in triplicates using 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) with the following conditions: 95ºC for 1 min followed by 40 cycles of 95ºC for 15 s, 61ºC for 15 s (64ºC for RORγ-t), 72ºC for 45 s. Each 20-µL qRT-PCR reaction volume contained 10 µL Fast SYBR® Green Master Mix (Applied Biosystems), 1 µL forward primer (5 µmol/L), 1 µl reverse primer (5 µmol/L), 2 µL cDNA template and 6 µL RNase-free water (Qiagen). IL-10 and TNF mRNA expression levels were quantified using primers and qRT-PCR conditions described previously (Pejcic-Karapetrovic et al., 2007).

3.3.6 Flow Cytometric Analysis

Single-cell suspensions were pelleted at 500 g for 5 min at 4ºC prior to incubation with anti-CD16/32 (Fc block) antibody in 1x phosphate-buffered saline (PBS) containing 2% fetal bovine serum (FBS) for 10 min. Cells were then stained with fluorochrome-conjugated monoclonal antibodies for cell surface antigens for 30 min: CD45 (30-F11; BD Biosciences) and CD4 (RM4-5; eBioscience). For intracellular staining, cells were fixed and permeabilized using the Mouse Foxp3 Buffer Set (BD Biosciences) prior to incubation with IL-17A (eBio17B7; eBioscience) and Foxp3 (FJK-16s; eBioscience). Stained cells were washed with

1x PBS containing 2% FBS before acquisition on the BD LSR Fortessa® flow cytometer using BD FACSDiva™ software (BD Biosciences). Data were analyzed using FlowJo 7.6.5 software (FlowJo, LLC, Ashland, OR, USA).

3.3.7 Statistical Analysis

Data are presented as the mean ± SEM or individual data points on a scatter plot. Statistical significance was determined through the Mann-Whitney test or one-way ANOVA with Tukey's multiple comparisons test as indicated. All statistical analyses were performed using GraphPad® Prism 6 software (GraphPad Software, Inc., La Jolla, CA, USA). *P* values of ≤ 0.05 were considered statistically significant. *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$, ****$P \leq 0.0001$.

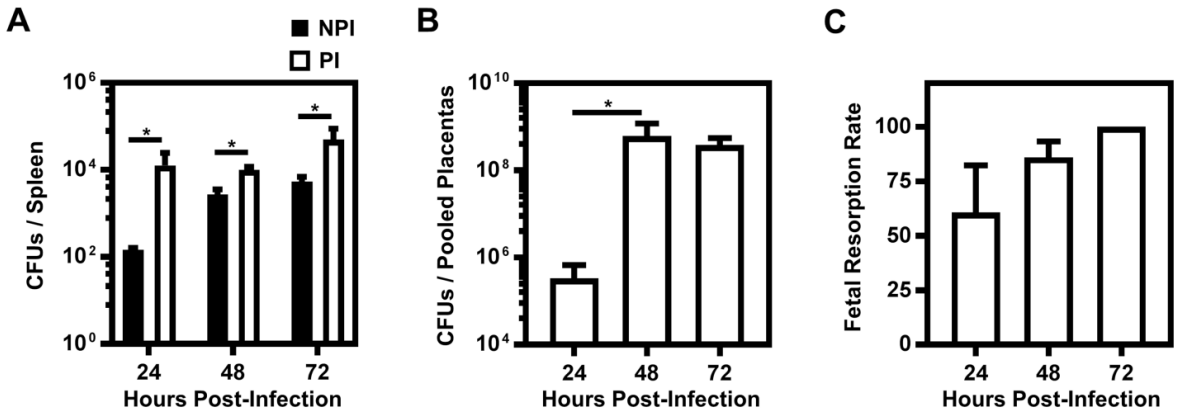
3.4 Results

3.4.1 Pregnancy promotes rapid productive infection of systemic and placental compartments by ST

We examined the bacterial numbers in the spleen and placentas of non-pregnant and pregnant mice kinetically at various times post-ST infection (with 10^3 CFUs dose given i.v.). Pregnant infected (PI) mice showed increased splenic bacterial numbers at 24 h post-infection compared to non-pregnant infected (NPI) mice (Figure 3.1A). Bacterial burden kinetically increased at 48 and 72 h post-infection, and pregnant mice consistently harbored significantly higher numbers of splenic bacteria relative to non-pregnant mice. In the placentas, bacterial numbers significantly increased from 24 to 48 h post-infection and remained at high levels at 72 h post-infection (Figure 3.1B). We also measured resorption rates, which are characterized as uterine necrotic scars or as necrotic and/or hemorrhagic fetuses and placentas. High resorption rates were observed even as early as 24 h post-infection and were consistent with the dramatic resorption rates seen at 48 to 72 h post-infection (Figure 3.1C). Thus, ST infection is significantly exacerbated in pregnant mice and correlates to the increased fetal loss.

3.4.2 Pregnant mice exhibit increased levels of serum pro-inflammatory cytokines during ST infection

We measured serum levels of cytokines at 4, 24, 48 and 72 h post-ST infection (Figure 3.2A and 3.3). Non-pregnant and pregnant mice showed a kinetic increase in TNF, IFN-γ, MCP-1, IL-12p70, IL-6, IL-1β and KC expression, correlating to the increase in bacterial burden over time. Serum cytokine levels peaked at 48 h post-infection. Moreover, the increase in cytokine expression from the non-infected to infected state was more pronounced in


A
NPI
PI
CFUs / Spleen
10^6
10^4
10^2
10^0
*
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*
24
48
72
Hours Post-Infection
B
CFUs / Pooled Placentas
10^{10}
10^8
10^6
10^4
*
24
48
72
Hours Post-Infection
C
Fetal Resorption Rate
100
75
50
25
0
24
48
72
Hours Post-Infection

Figure 3.1. Pregnancy enhances maternal susceptibilities to systemic and placental ST infection. Non-pregnant and pregnant 129/Sv-E mice (day 11 and 12 of gestation) were infected with ST 10^3 CFUs i.v. (A and B) Spleens and placentas were collected for bacterial enumeration at various time points post-infection. Placental bacterial numbers in one mouse at 24 h post-infection was beyond the limit of detection and was plotted as 10^6 CFUs. (C) Fetal resorption rates were determined using the formula $R/(R + V) \times 100$, where R is the number of resorbing fetuses and V is the number of viable fetuses per mouse. Data are presented as mean ± SEM and statistical significance was analyzed by Mann-Whitney test. *$P \leq 0.05$. N = 3-13 mice per group. NPI, non-pregnant infected; PI, pregnant infected.

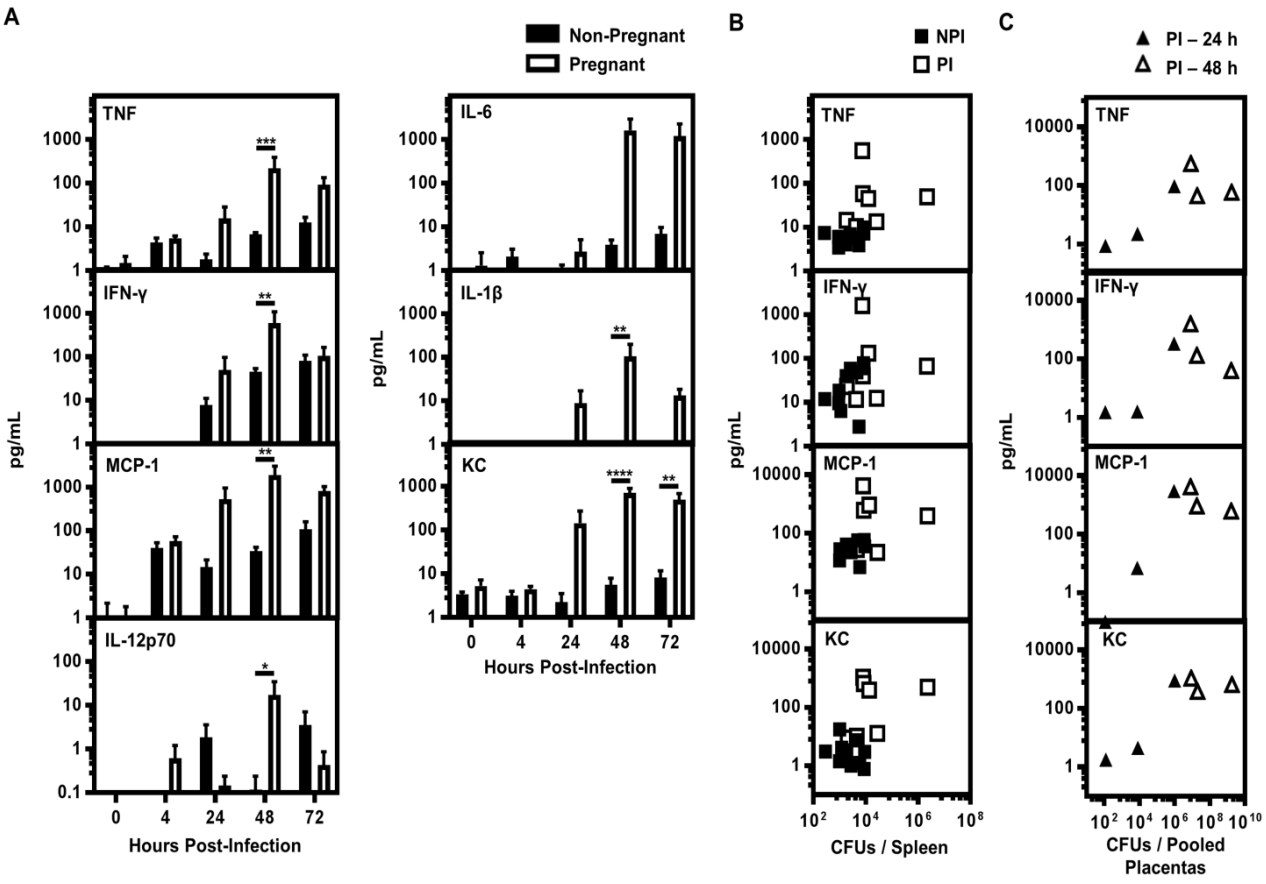
A
Non-Pregnant
Pregnant
TNF
IFN-γ
MCP-1
IL-12p70
IL-6
IL-1β
KC
pg/mL
Hours Post-Infection
B
NPI
PI
CFUs / Spleen
C
PI – 24 h
PI – 48 h
CFUs / Pooled Placentas

Figure 3.2. Pregnant mice exhibit increased serum levels of pro-inflammatory cytokines during ST infection. (A) Serum was collected from non-pregnant and pregnant mice at various time points post-infection for quantification of pro-inflammatory cytokine levels. Serum from healthy, age-matched, non-pregnant and pregnant (day 11 to 13 of gestation) 129/Sv-E mice were used as non-infected controls (0 h post-infection). Data are presented as mean ± SEM. Statistical significance in cytokine expression was analyzed by one-way ANOVA. Data show a significant increase in TNF ($P \leq 0.001$), IFN-γ ($P \leq 0.01$), MCP-1 ($P \leq 0.001$), IL-12p70 ($P \leq 0.05$), IL-6 ($P \leq 0.05$), IL-1β ($P \leq 0.01$) and KC ($P \leq 0.0001$) from the non-infected to infected state. *Statistical significance in cytokine expression between non-pregnant and pregnant mice per time point was calculated using Tukey's multiple comparisons test. *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$, ****$P \leq 0.0001$. N = 3-8 mice per group per time point. (B) Spleens were collected for bacterial enumeration at 48 h post-infection. Data points represent serum cytokine level versus total splenic bacterial burden of individual mice. (C) Bacteria were enumerated in pooled placentas at 24 and 48 h post-infection. Data points represent serum cytokine level versus total placental bacterial burden of individual mice. Placental bacterial numbers in one mouse at 24 h post-infection was beyond the limit of detection and was plotted as 10^6 CFUs. N = 7-10 mice per group. NPNI, non-pregnant non-infected; NPI, non-pregnant infected; PI, pregnant infected.

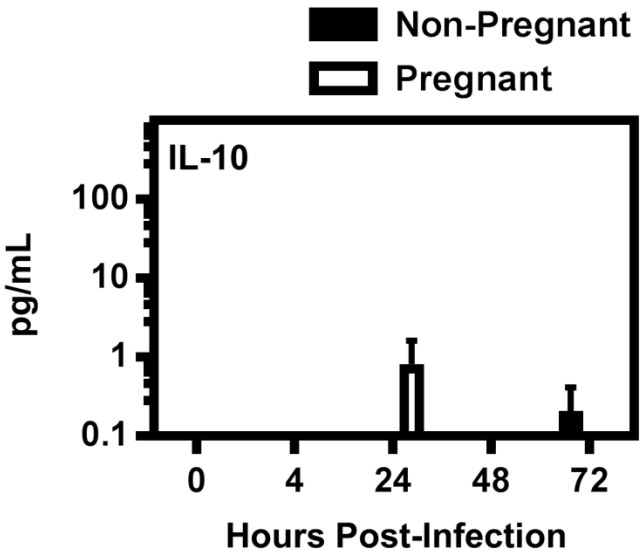
Non-Pregnant
Pregnant
IL-10
100
10
1
0.1
pg/mL
0
4
24
48
72
Hours Post-Infection

Figure 3.3. Maternal serum IL-10 profiles of non-pregnant and pregnant mice in the presence or absence of ST infection. Serum was collected from non-pregnant and pregnant mice at various time points post-infection for quantification of IL-10 expression levels. To comply with allowable serum collection amounts from mice as per approved protocol, in 1 group of mice, we collected serum at 4 and 48 h, and in another 24 and 72 h. Serum from healthy, age-matched, non-pregnant and pregnant (day 11-13 of gestation) 129/Sv-E mice were used as non-infected controls (0 h post-infection). Data are presented as mean ± SEM. Statistical significance in cytokine expression was analyzed by one-way ANOVA. Statistical significance in cytokine expression between non-pregnant and pregnant mice per time point was calculated using Tukey's multiple comparisons test. N = 3-8 mice per group per time point.

pregnant mice compared to non-pregnant mice. TNF, IFN-γ and IL-12p70 expression levels were ~10-fold higher in PI mice relative to NPI mice at 48 h post-infection. Furthermore, ~100-fold difference in MCP-1 and KC expression was observed between the two groups at 48 h post-infection. A similar trend was observed in KC expression at 72 h post-infection. IL-1β was only detected in the serum of PI mice, consistent with a pronounced pro-inflammatory state. In contrast, serum IL-10 levels remained similar in all groups (Figure 3.3). These results are suggestive of a rapid serum inflammatory cytokine storm in ST-infected pregnant mice by 24-48 h (current study) which is maintained at 72 h post-infection (Chattopadhyay et al., 2010). The increased serum cytokines in PI mice correlated to the exacerbated infection in placental and systemic tissues seen during pregnancy relative to the controls (Figure 3.1A and 3.1B) (Chattopadhyay et al., 2010).

We also analyzed the relationship between serum cytokine expression and splenic and placental bacterial burden in individual mice at 24 and/or 48 h post-infection (Figure 3.2B and 3.2C). Analysis of splenic CFU vs serum levels of TNF, IFN-γ, MCP-1 and KC at 48 hours post-infection indicated that mice with higher bacterial burdens in the spleen also produced high levels of serum cytokines (Figure 3.2B). Similarly, a positive correlation was seen between increased serum cytokine levels and higher placental bacterial burden from 24 to 48 h among individual mice (Figure 3.2C). Thus, our results suggest that increased bacterial burden contributes to an enhanced serum inflammatory cytokine response.

3.4.3 TLR4 enhances systemic *ST* growth in pregnant mice

Toll-like receptor 4 (TLR4) is involved in innate immune activation through recognition of lipopolysaccharide (LPS) expressed by Gram-negative bacteria, such as

Salmonella (Takeda and Akira, 2003). To examine the contribution of TLR4-mediated inflammation in modulating maternal susceptibility to systemic infection, we performed *in vivo* blockade of TLR4 using anti-mouse TLR4/MD-2 antibody prior to ST infection (Figure 3.4). At 72 h post-infection, no significant difference was observed in splenic bacterial numbers between non-treated and treated non-pregnant mice (~10^4 CFU). As expected, non-treated pregnant mice exhibited significantly increased bacterial numbers (~100-fold) relative to non-treated non-pregnant mice. However, treatment with the anti-TLR4 antibody resulted in a decreased splenic bacterial burden (10- to 100-fold decrease) in pregnant infected mice compared to non-treated pregnant infected mice. These results suggest that TLR4 signaling contributes to the exacerbation of ST infection, associated cytokine storm and maternal illness during pregnancy.

3.4.4 ST infection during pregnancy is associated with an increased splenic IL-10 response

We next examined the profile of cytokine expression in the splenic compartment. Previously, we have shown that ST infection in pregnant mice results in reduced recruitment of innate immune cells in the spleen (Pejcic-Karapetrovic et al., 2007). IL-10 and TNF play antagonistic anti-inflammatory and pro-inflammatory roles, respectively, and an appropriate ratio of these cytokines may be required for successful maintenance of pregnancy (Brogin et al., 2012). We quantified IL-10 and TNF mRNA levels in the spleen through qRT-PCR (Figure 3.5A and 3.5B). Pregnant mice exhibited increased IL-10 expression relative to non-pregnant mice even in the non-infected state. At 72 h post-infection, we observed a significant increase in IL-10 mRNA levels in both non-pregnant and pregnant groups. In contrast, expression levels of TNF showed an increased trend upon infection in non-pregnant mice, but this trend was not

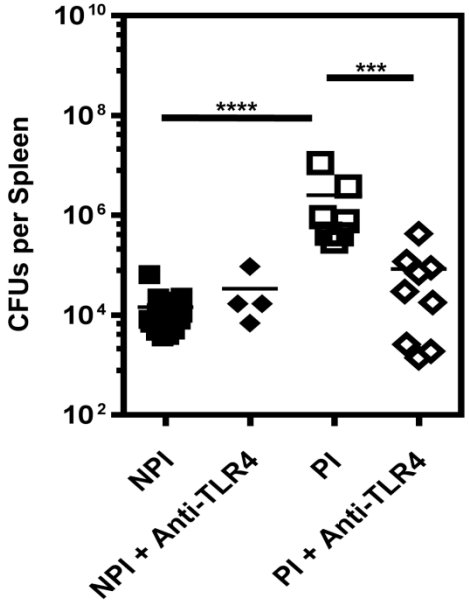
10^{10}
10^{8}
10^{6}
10^{4}
10^{2}
CFUs per Spleen
****
***
NPI
NPI + Anti-TLR4
PI
PI + Anti-TLR4

Figure 3.4. TLR4 blockade leads to decreased systemic ST growth in pregnant mice. Anti-mouse TLR4/MD-2 complex (MTS510) functional grade purified was administered to non-pregnant and pregnant 129x1/SvJ mice before infection with ST 10^3 i.v. At 72 h post-infection, bacteria in the spleen was quantified through CFU assay. Non-treated non-pregnant and pregnant mice were used as controls. Data are presented as mean ± SEM, and statistical significance was analyzed by Mann-Whitney test, ***$P \leq 0.001$, ****$P \leq 0.0001$. N = 4-14 mice per group. NPI, non-pregnant infected; PI, pregnant infected.

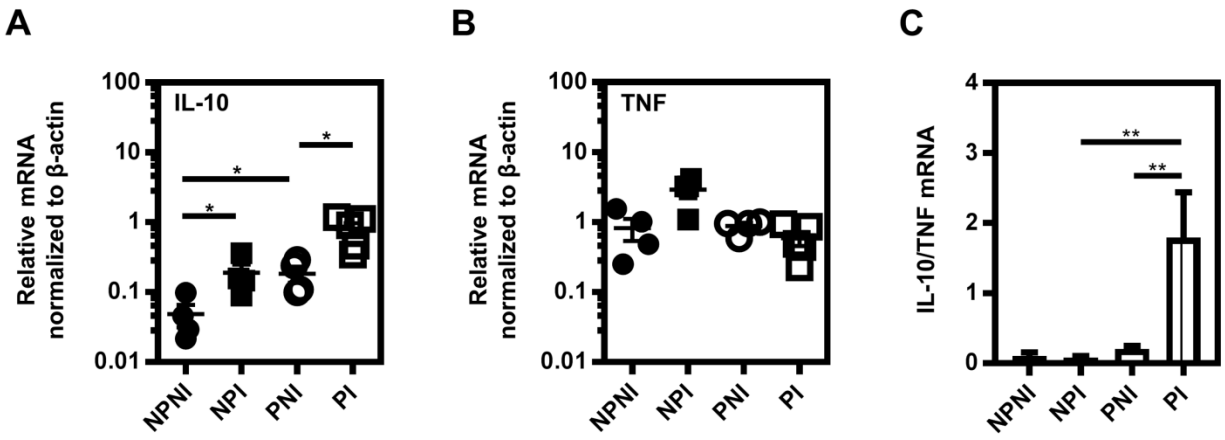
A
IL-10
Relative mRNA normalized to β-actin
100
10
1
0.1
0.01
*
*
*
NPNI
NPI
PNI
PI
B
TNF
Relative mRNA normalized to β-actin
100
10
1
0.1
0.01
NPNI
NPI
PNI
PI
C
IL-10/TNF mRNA
4
3
2
1
0
**
**
NPNI
NPI
PNI
PI

Figure 3.5. Splenic IL-10 responses are increased during pregnancy. At 72 h post-infection, spleens from non-pregnant and pregnant mice were harvested for quantification of IL-10 (A) and TNF (B) mRNA levels through qRT-PCR. (C) The ratio of IL-10 to TNF mRNA expression in the spleen was evaluated. Spleens from healthy, age-matched, non-pregnant and pregnant (day 14 and 15 of gestation) 129x1/SvJ mice were used as controls. Relative mRNA levels were normalized to β-actin expression. Data are presented as mean ± SEM, and statistical significance was analyzed by Mann-Whitney test, $*P \leq 0.05$, $**P \leq 0.01$. N = 4-5 mice per group. NPNI, non-pregnant non-infected; NPI, non-pregnant infected; PNI, pregnant non-infected; PI, pregnant infected.

marked in pregnant mice. Furthermore, the ratio of IL-10 to TNF mRNA expression was significantly increased in pregnant infected mice relative to their non-pregnant counterparts (Figure 3.5C). A similar trend was observed between pregnant non-infected and pregnant infected mice. Overall, our results suggest a skewed IL-10 splenic response to infection during pregnancy.

3.4.5 Pregnant mice exhibit decreased splenic Th17 to Treg ratios during ST infection

Treg cells can suppress other inflammatory cell types, including T and B lymphocytes, NK cells and DCs (Chapoval et al., 2010; Josefowicz et al., 2012; Saito et al., 2010). In contrast, Th17 cells contribute to protective immunity against *Salmonella* infection. We quantified splenic mRNA expression of IL-17A and RORγ-t mRNA expression at 24 h post-infection (Figure 3.6A). Upon infection, non-pregnant mice exhibited a significant increase in splenic IL-17A mRNA levels. Pregnant mice exhibited slightly increased basal levels of splenic IL-17A mRNA compared to non-pregnant mice. However, ST infection did not evoke an increase in IL-17 mRNA levels. Splenic RORγ-t mRNA expression was similar, and overall very low in most groups, except for a slight increase observed in pregnant infected mice, despite having decreased IL-17A mRNA levels relative to non-pregnant infected mice.

We then examined the role of pregnancy in modulating the ratio of Th17 cells to Tregs during ST infection through flow cytometry. Th17 cells and Tregs were identified as $CD4^+$ IL-$17A^+$ and $CD4^+$ $Foxp3^+$ cells, respectively (Figure 3.6B and 3.6C). Based on numbers, the ratio of Th17 to Treg cells was close to 1 in non-pregnant mice before and after infection (Figure 3.6D). In contrast, the number of Tregs outnumbered the number of Th17 cells in pregnant mice, both in the non-infected and infected state. Moreover, as expected, the numbers

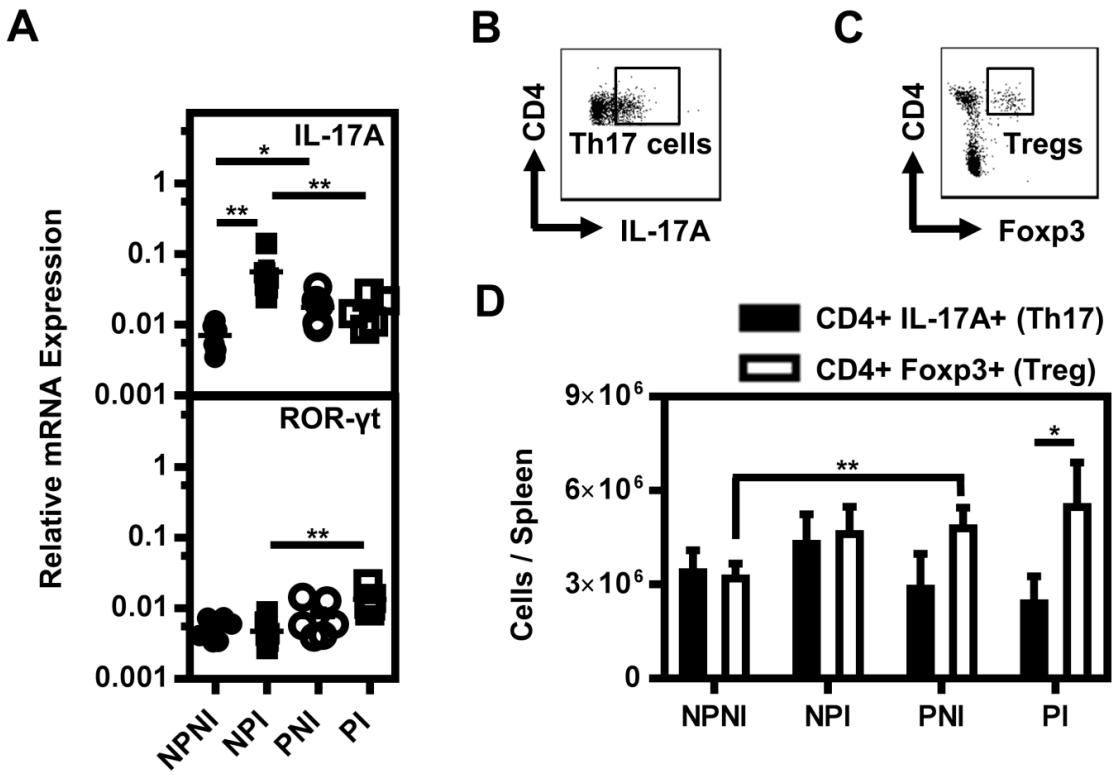
A
IL-17A
ROR-γt
Relative mRNA Expression
1
0.1
0.01
0.001
*
**
**
**
NPNI
NPI
PNI
PI
B
CD4
Th17 cells
IL-17A
C
CD4
Tregs
Foxp3
D
CD4+ IL-17A+ (Th17)
CD4+ Foxp3+ (Treg)
Cells / Spleen
9×10^6
6×10^6
3×10^6
0
**
*
NPNI
NPI
PNI
PI

Figure 3.6. Pregnancy decreases Th17 to Treg cell ratios during ST infection. Non-pregnant and pregnant 129/Sv-E mice (day 11 and 12 of gestation) were infected with ST 10^3 CFUs i.v. (A) At 24 h post-infection, spleens were harvested for quantification of IL-17 and RORγ-t mRNA expression levels through qRT-PCR. Spleens from healthy, age-matched, non-pregnant and pregnant (day 12 and 13 of gestation) 129/Sv-E mice were used as controls. IL-17 and RORγ-t mRNA levels were normalized to β-actin. (B,C) Flow cytometry gating strategies for identifying $CD4^+$ $IL\text{-}17A^+$ Th17 cells and $CD4^+$ $FoxP3^+$ Tregs. (D) Th17 and Treg cell numbers were quantified through flow cytometry at 48 h post-infection. Data are presented as mean ± SEM, and statistical significance was analyzed by Mann-Whitney test, $*P \leq 0.05$, $**P \leq 0.01$. N = 4-11 mice per group. NPNI, non-pregnant non-infected; NPI, non-pregnant infected; PNI, pregnant non-infected; PI, pregnant infected.

of Tregs in pregnant mouse spleens were significantly higher than those in non-pregnant mice. Our results suggest an altered Treg/Th17 ratio during pregnancy, a trend that was not reversed after infection.

3.4.6 Anti-CD25 treatment reduces ST-associated disease severity during pregnancy

Anti-CD25 treatment has been widely used for Treg depletion and functional inactivation in mice *in vivo*. We therefore administered anti-CD25 antibody to non-pregnant and pregnant mice at days 7-8 of gestation. Mice were infected with ST 4 days after antibody treatment. Thereafter, we measured the bacterial burden in the spleen and pooled placentas at 48 h post-infection. As expected, non-treated pregnant mice exhibited an increased splenic bacterial burden relative to non-pregnant controls (Figure 3.7A). In contrast, anti-CD25 antibody treatment resulted in a dramatic reduction in the bacterial burden in pregnant infected mice compared to non-treated infected pregnant mice. In the placenta, anti-CD25 treatment did not provide a statistically discernable reduction in bacterial numbers relative to non-treated mice (Figure 3.7B). However, treated pregnant mice showed decreased fetal resorption rates relative to non-treated pregnant controls following infection (Figure 3.7C). Lastly, anti-CD25 treatment in pregnant infected mice significantly increased splenic Th17 cell numbers (Figure 3.7D), effectively restoring the Th17/Treg ratios (Figure 3.7E) to levels similar to that observed in non-treated pregnant non-infected (PNI) controls (Figure 3.6D). Our results suggest that Tregs dampen the beneficial Th17 response to ST infection during pregnancy.

3.4.7 Placental Th17 responses are reduced during ST infection

We further examined the altered distribution of Th17 cells in the placentas of PI mice relative to PNI controls. IL-17A and RORγ-t mRNA expression at 24 h post-infection was

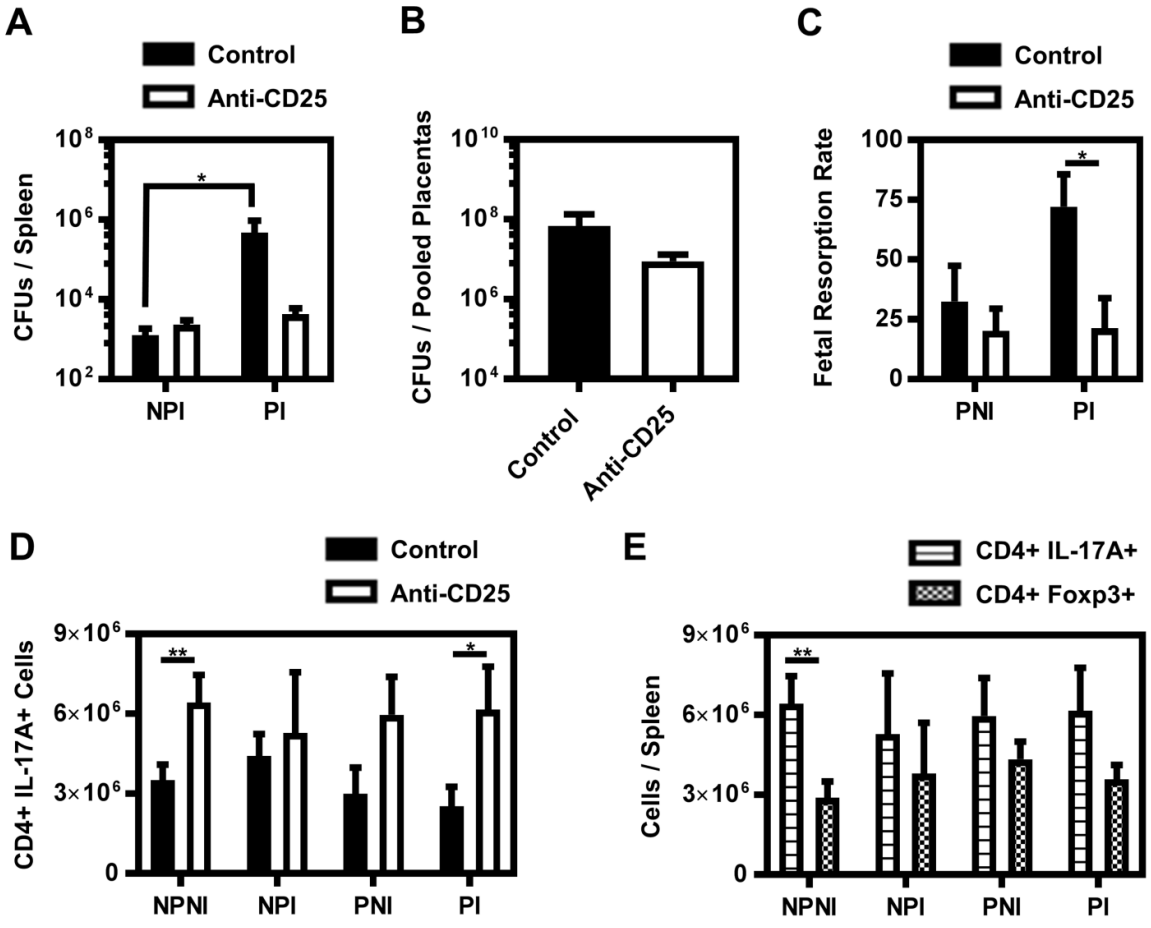
A
Control
Anti-CD25
CFUs / Spleen
10^8
10^6
10^4
10^2
*
NPI
PI
B
CFUs / Pooled Placentas
10^{10}
10^8
10^6
10^4
Control
Anti-CD25
C
Control
Anti-CD25
Fetal Resorption Rate
100
75
50
25
0
*
PNI
PI
D
Control
Anti-CD25
CD4+ IL-17A+ Cells
9×10^6
6×10^6
3×10^6
0
**
*
NPNI
NPI
PNI
PI
E
CD4+ IL-17A+
CD4+ Foxp3+
Cells / Spleen
9×10^6
6×10^6
3×10^6
0
**
NPNI
NPI
PNI
PI

Figure 3.7. Anti-CD25 treatment reduces maternal and fetal disease progression associated with ST infection. Anti-mouse CD25 (PC61.5) functional grade purified antibody was administered to non-pregnant and pregnant 129/Sv-E mice 4 d prior to infection with ST 10^3 i.v. (A,B) Spleens and placentas were harvested at 48 h post-infection for bacterial enumeration. Control spleens were obtained from non-treated non-pregnant and pregnant mice (day 13 and 14 of gestation). (C) Fetal resorption rates were determined using the formula $R/(R + V) \times 100$, where R is the number of resorbing fetuses and V is the number of viable fetuses per mouse. (D) Splenic Th17 cell numbers in control and anti-CD25-treated mice were measured through flow cytometry at 48 h post-infection. (E) Comparisons of splenic Th17 and Treg cell numbers in anti-CD25-treated mice at 48 h post-infection. Data are presented as the mean ± SEM, and statistical significance was analyzed by Mann-Whitney test, $*P \leq 0.05$, $**P \leq 0.01$. N = 4-11 per group. NPNI, non-pregnant non-infected; NPI, non-pregnant infected; PNI, pregnant non-infected; PI, pregnant infected.

determined (Figure 3.8). Low levels of IL-17A and RORγ-t mRNA levels were detected in the placentas of PNI mice, but even this level was significantly reduced in the placentas of PI mice (Figure 3.8). Our results suggest that the Th17 response to ST infection may be dampened in the placental compartment despite significant bacterial burden.

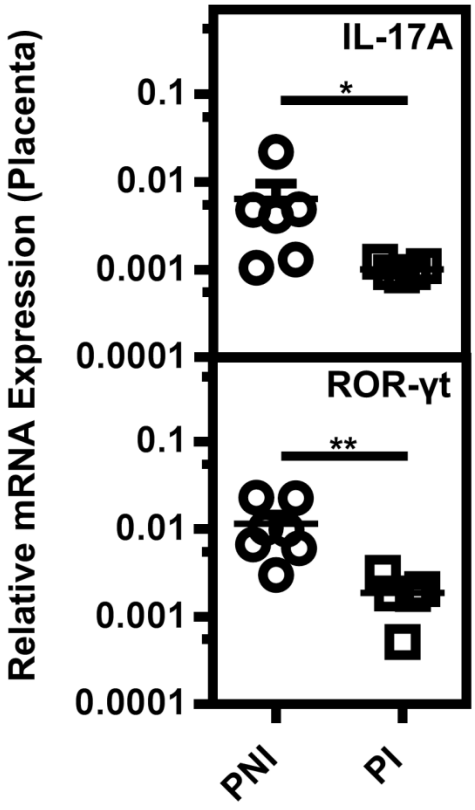
IL-17A
ROR-γt
Relative mRNA Expression (Placenta)
0.1
0.01
0.001
0.0001
*
**
PNI
PI

Figure 3.8. Pregnant mice exhibit reduced placental Th17 cell responses during infection. Non-pregnant and pregnant (day 11 and 12 of gestation) 129/Sv-E mice were infected with ST 10^3 i.v. At 24 h post-infection, placental IL-17 and RORγ-t mRNA levels were measured through qRT-PCR and normalized to β-actin expression. Placentas from healthy, age-matched 129/Sv-E mice (day 12 and 13 of gestation) were used as non-infected controls. Data are presented as mean ± SEM, and statistical significance was analyzed by Mann-Whitney test, $*P \leq 0.05$, $**P \leq 0.01$. N = 4-6 mice per group. PNI, pregnant non-infected; PI, pregnant infected.

3.5 Discussion

In this study, we demonstrate the Th17-Treg immune crosstalk during intracellular ST infection in the maternal host. Maintenance of an appropriate ratio of pro- and anti-inflammatory responses at the feto-maternal interface is a hallmark of successful pregnancy which is perturbed by the intracellular pathogen such as ST, leading to not only fetal loss but also ineffective systemic immunity in the maternal host.

Efficient control of *Salmonella* infection is associated with expression of pro-inflammatory cytokines, including TNF, IFN-γ and IL-12 (Gulig et al., 1997; Jouanguy et al., 1999). Nevertheless, an overt cytokine storm in response to infection may have deleterious effects. Indeed, ST infection during pregnancy leads to profound placental inflammatory cytokine response, which may contribute to the detrimental effects on the fetus (Chattopadhyay et al., 2010; Pejcic-Karapetrovic et al., 2007). We also observed that the profound increase in serum pro-inflammatory cytokine expression in pregnant mice relative to non-pregnant mice during ST infection was associated with increased bacterial numbers in placental tissues. It appears that recognition of pathogen-specific molecular patterns at the feto-maternal interface evokes a profound inflammatory response that contributes to increase in circulating cytokines in the serum. Indeed, from 24 to 72 h with steadily increasing placental bacterial burden, we noticed a parallel increase in serum inflammatory cytokines with a peak occurring around 48 h post-infection. We also noted that splenic bacterial burden was higher in pregnant infected mice relative to non-pregnant group, and this was consistent with the increased serum cytokine response in the former group. However, we noted that ratio of splenic IL-10 vs TNF production was skewed in favor of a reduced inflammatory response. This latter observation is consistent with an ineffective maternal splenic immunity to ST infection and

suggests that compartment-specific dichotomy may occur between pro- and anti-inflammatory responses (Pejcic-Karapetrovic et al., 2007). In murine *Porphyromonas gingivalis* infection, increased production of maternal TNF and decreased IL-10 production was shown to be associated with increased fetal growth restriction and/or resorption. However, in human placental malaria, the activation of peripheral blood T cells dually expressing IFN-γ and IL-10 was indicative of an improved fetal outcome. Expression of the anti-inflammatory cytokine IL-10 in placental tissues occurs during the first and second trimesters of normal pregnancy relative to the third trimester (Hanna et al., 2000). In mice, enhanced IL-10 expression in gestational tissues is associated with decreased susceptibility to pre-term labor (Robertson et al., 2006). Disruption of the balance between IL-10 and TNF has been implicated in pregnancy complications, including pre-term birth, pre-eclampsia and gestational diabetes mellitus (Brogin et al., 2012). However, the profound replication of ST in the placental milieu may mask the beneficial effect of IL-10 for down-modulating inflammation locally. Moreover, we have observed that IL-10 production by trophoblast-derived choriocarcinoma cells *in vitro* leads to ineffective control of intracellular replication of ST (Nguyen et al., 2013). Taken together, our data suggest a negative regulatory role for IL-10 in the control of ST infection within the splenic compartment as well.

Lipopolysaccharide recognition by TLR4 leads to activation of macrophage pro-inflammatory responses, chemokine production and nitric oxide synthesis (Vazquez-Torres et al., 2004). TLR4 is expressed by macrophages, DCs as well as by non-immune cells such as endothelial, endometrial and trophoblast cells (Hirata et al., 2005; Hijiya et al., 2002). Deficiency in TLR4 is associated with impaired bacterial clearance and cytokine responses against *Salmonella* infection in non-pregnant mice (Weiss et al., 2004). However, ST infection

has also been shown to exploit TLR4 signaling in macrophages for increased intracellular survival (Wong et al., 2009). In a similar fashion, we observed that *in vivo* TLR4 blockade decreased bacterial burden in pregnant infected mice. Other studies have implicated TLR4 in the induction of pre-term labor in response to LPS (Elovitz et al., 2003). Thus, TLR4 signaling evoked by ST infection during pregnancy may be contributing to increased intracellular bacterial survival, consequent overt local inflammation and maternal pathology induced by a systemic cytokine storm.

Successful pregnancy is associated with increased numbers of $CD4^+CD25^+$ $Foxp3^+$ Tregs, which enable maternal tolerance towards fetal alloantigens (Shima et al., 2010). A study by Rowe *et al*. addressed the role of Tregs in maternal immunity to pre-natal pathogens (Rowe et al., 2011). C57BL/6 ($H\text{-}2^b$) female mice were mated with BALB/c ($H\text{-}2^d$) male mice to compare the impact of allogeneic pregnancies on Treg expansion. Pregnant Treg-ablated mice infected with either LM or ST exhibited increased resistance to infection compared to non-Treg ablated controls. Treg-derived IL-10 expression was also critical in enhancing susceptibility to LM infection. In our study, we observed that syngeneic pregnancies also lead to increased splenic levels of $CD4^+Foxp3^+$ Tregs (Figure 3.6D). Furthermore, consistent with the previous report of Rowe *et al*., anti-CD25 antibody treatment curtailed the increase in bacterial burden seen in pregnant infected mice relative to non-pregnant mice (Rowe et al., 2011). It also reduced fetal resorption rates associated with ST infection. Taken together, we provide further evidence that Tregs may prevent an effective immune response to ST, leading to increased disease severity during pregnancy.

Th17 cells are effector lineage $CD4^+$ T cells that can contribute to immunity to microbial pathogens but also to harmful autoimmune and inflammatory conditions (Curtis and

Way, 2009; Khader et al., 2009; Ghoreschi et al., 2010; Langrish et al., 2005). IL-17 expression promotes neutrophil recruitment and expression of pro-inflammatory cytokines against extracellular bacteria (Jin and Dong, 2013). Furthermore, Th17 cells play a crucial role in controlling *Salmonella* infection. Oral infection of streptomycin-treated mice results in enhanced IL-17 expression and increased $CD3^+$ T-cell function in the cecal mucosa. IL-17A deficiency leads to reduced mucosal protection from disseminated disease (Godinez et al., 2008; Raffatellu et al., 2008). Similarly, reduced IL-17A expression was associated with increased splenic and liver bacterial burden during systemic *S*. Enteritidis infection (Schulz et al., 2008). Our data demonstrate decreased IL-17 mRNA levels in the spleen and placenta in pregnant infected mice relative to non-pregnant controls. Additionally, RORγ-t expression was lower in the placenta of pregnant infected mice consistent with a reduced Th17 response. However, splenic RORγ-t expression appeared to be marginally higher in the pregnant infected group. Nevertheless, overall the RT-PCR assay for RORγ-t expression yielded low sensitivity with detection in late cycles. It is possible that an intracellular RORγ-t flow cytometry based assay may have provided increased sensitivity and stringency for comparisons among groups. Nevertheless, splenic Treg cells outnumbered Th17 cells in pregnant infected mice. As anti-CD25 treatment increased Th17 cell numbers, it suggests that the Treg response during pregnancy dampened the Th17 response to infection. It is possible that the developmental plasticity of T cell subsets plays a role in modulating the Th17/Treg balance in ST-infected pregnant mice. Inflammatory cytokines such as IL-6 can drive the differentiation of Th17 cells from Tregs (Saito et al., 2010). Nevertheless, Tregs induced by IL-2 and TGF-β are resistant to Th17 conversion by cytokines such as IL-6 (Zheng et al., 2008). Th17 cells are also plastic: expression of IL-12 or IL-4 enables Th17 cell conversion into IFN-γ-producing Th1 cells or IL-4-producing Th2 cells, respectively (Hirahara et al., 2013). Signaling components such as

DC-derived retinoic acid and Itk (IL2-inducible T cell kinase) have also been shown to offset Th17/Treg ratios, leading to altered immune responses (Mucida et al., 2007). Recently, High mobility group box 1 (HMGB1) expression was shown to drive the Th17/Treg ratio towards a Th17 response by augmenting IL-6 production through TLR4 signaling (Li et al., 2014). Increased proportions of peripheral blood Th17 cells relative to Tregs have been correlated with pregnancy complications including pre-term labor, pre-eclampsia, and unexplained early recurrent miscarriage (Wang et al., 2010). Furthermore, Th17 cell frequencies in peripheral blood are reported to remain unchanged in successful pregnancy through all three trimesters (Nakashima et al., 2010). In model systems, altered Treg/Th17 ratios and adverse pregnancy outcomes have also been associated with infections by prenatal pathogens, including LM and *Toxoplasma gondii* (Rowe et al., 2011; Zhang et al., 2012). Lastly, enhanced IL-10 expression in pregnant mice may promote Treg expansion and offset the effects of pro-inflammatory response to infection. Our study suggests an inverse correlation between Treg and Th17 responses in pregnant mice infected with ST, which may contribute to exacerbated maternal infection.

Our study provides a model for modulation of Th17/Treg balance and inflammatory responses during pregnancy, leading to reduced resistance to systemic ST infection. Firstly, we surmise that rapid replication of ST in the placental milieu may drive an overt TLR4-mediated local inflammation. This in turn may trigger a local aberrant cytokine storm that also results in high levels of circulating serum inflammatory cytokines. The maternal host may counter this response with enhanced Treg expansion, which then specifically halts the differentiation of Th17 cells necessary for the effective control of ST infection. Overall, a

complex interplay of compartment-specific ineffective immune responses appears to enhance the pathology of ST infection during pregnancy.

3.6 Acknowledgments

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4.0 MANUSCRIPT THREE

Type I interferons differentially modulate maternal host immunity to infection by *Listeria monocytogenes* and *Salmonella enterica* serovar Typhimurium during pregnancy

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The specific contributions of each author in the research article are listed below:

Gerard Agbayani: Designed and performed the experiments, analyzed the data, wrote and edited the manuscript

Kristina Wachholz: Designed and assisted in performing the experiments, and analyzed the data

Shawn P. Murphy: Contributed to the discussion and ideas, and edited the manuscript

Subash Sad: Obtained the IFNAR$^{-/-}$ mice, and edited the manuscript

Lakshmi Krishnan: Conceptualized the study, designed the experiments, wrote and edited the manuscript, and contributed reagents

4.1 Abstract

Problem: IFN-alpha receptor deficiency ($IFNAR^{-/-}$) enhances immunity to *Listeria monocytogenes* (LM) and *Salmonella enterica* serovar Typhimurium (ST) in the non-pregnant state by inhibiting pathogen-induced immune cell death. However, the roles of IFNAR signaling in modulating immunity to infection during pregnancy are not well-understood.

Method of Study: WT and $IFNAR^{-/-}$ mice were infected systemically with LM or ST. Bacterial burden in spleen and individual placentas was enumerated at day 3 post-infection. Immune cell numbers and percentages were quantified in spleen and individual placentas, respectively, through flow cytometry. Cytokine expression in serum, spleen and individual placentas was measured through cytometric bead array.

Results: $IFNAR^{-/-}$ mice exhibited decreased splenic monocyte numbers in non-pregnant and pregnant state, and an altered distribution of placental immune cell types in the non-infected state. $IFNAR^{-/-}$ mice controlled LM infection more effectively than wild-type even during pregnancy, and this correlated with enhanced serum IL-12 expression, despite reduced splenic monocyte numbers relative to WT controls. In contrast, pregnant $IFNAR^{-/-}$ mice unlike their non-pregnant counterparts exhibited increased susceptibility to ST infection which was associated with decreased serum IL-12 expression.

Conclusion: Type I IFN responses differentially impact host resistance to LM and ST infection during pregnancy through modulation of immune cell distribution and cytokine responses.

4.2 Introduction

Type I interferons (IFNs) are a large family of cytokines that modulate innate and adaptive immune responses, including inflammatory cytokine expression, macrophage activation and T cell function (Stenger and Rollinghoff, 2001). The IFN-α and IFN-β subtypes are well-characterized for their roles in regulating host immunity to infections, and will be referred to as Type I IFNs in this study (Alsharifi et al., 2008). Type I IFNs signal through the ubiquitous transmembrane IFN-alpha receptor (IFNAR) (Ivashkiv and Donlin, 2014). They are produced by innate immune cell types, including macrophages and dendritic cells (DCs) upon pathogen detection by pattern recognition receptors and cytokine stimulation (Ivashkiv and Donlin, 2014; Trinchieri, 2010). Non-immune cells, including epithelial cells and fibroblasts, also promote activation of anti-microbial mechanisms through IFN-β production (Ivashkiv and Donlin, 2014). Type I IFNs induce differential effects on host immunity to viral infections. IFNAR signaling typically exerts anti-viral functions through induction of IFN-stimulated genes (ISGs), which encode proteins that interfere with various stages of the viral life cycle, regulate host cell death and promote activation of innate and adaptive immune mechanisms (Schoggins and Rice, 2011; Boxx and Cheng, 2016). However, IFNAR signaling enhances susceptibility to chronic infection by lymphocytic choriomeningitis virus (LCMV) infection. This is associated with increased serum expression of anti-inflammatory interleukin-10 (IL-10) and programmed death-ligand 1, and decreased splenic numbers of cytokine-producing LCMV-specific $CD4^+$ T cells (Teijaro et al., 2013; Wilson et al., 2013). IFNAR signaling also induces differential host responses to bacterial infections. Type I IFNs enhance resistance to macrophage infection by *Legionella pneumophila* bacteria by promoting polarization of classically-activated M1 macrophage responses and production of anti-

microbial nitric oxide (Plumlee et al., 2009). Protection mediated by type I IFNs from invasive lung infection by *Streptococcus pneumoniae* bacteria is associated with reduced lung epithelial permeability and enhanced alveolar epithelial type II cell survival (LeMessurier et al., 2013; Maier et al., 2016). In contrast, IFNAR signaling compromises innate immunity to intracellular bacterial infection by *Listeria monocytogenes* (LM) and *Salmonella* Typhimurium by sensitizing immune cells to pathogen-induced death (Robinson et al., 2012; O'Connell et al., 2004; Auerbuch et al., 2004). Thus, the complex network of immune activation and regulatory roles mediated by type I IFNs differentially impact host immunity to infection.

Pregnancy features the complex interplay between inflammatory and immune-regulatory T-cell mechanisms to promote maternal tolerance to the semi-allogeneic fetus. As a consequence, this immune-altered condition can also modulate host susceptibility to infections. We previously showed that pregnancy does not compromise the inherent resistance of C57BL/6J wild-type (WT) mice to systemic LM infection (Krishnan et al., 2010). This is associated with robust systemic innate immune and antigen-specific $CD8^+$ T-cell responses by pregnant mice similar to those of non-pregnant mice. However, pregnant mice exhibited progressive bacterial growth in the placenta and enhanced fetal loss in the course of LM infection. Thus, preferential placental colonization by LM appeared to be differentially regulated by maternal systemic and local placental immunity in response to infection. In contrast, ST infection during pregnancy leads to profound systemic maternal and fetal disease (Agbayani et al., 2017; Chattopadhyay et al., 2010; Pejcic-Karapetrovic et al., 2007). Pregnant mice exhibited reduced maternal splenic innate immune responses to infection, which was associated with decreased ratio of pro-inflammatory Th17 cells to anti-inflammatory regulatory T cells (Tregs) and enhanced IL-10 responses (Agbayani et al., 2017; Pejcic-

Karapetrovic et al., 2007). In contrast, profound placental infection correlated with enhanced expression of pro-inflammatory cytokines, including interleukin-6 (IL-6), TNF and IL-18, leading to fetal loss (Pejcic-Karapetrovic et al., 2007; Chattopadhyay et al., 2010). Thus, compartment-specific dichotomy between pro- and anti-inflammatory responses in pregnant mice impacted systemic and placental ST burden and disease progression.

The role of type I IFN responses in modulating innate immunity to intracellular bacterial infections during pregnancy, are not well-understood. In this study, we show that IFNAR deficiency modulates splenic and placental immune cell distribution in the non-infected state, and differentially modulates disease progression in response to LM and ST during pregnancy. Resistance to LM infection in non-pregnant IFNAR$^{-/-}$ mice is retained during pregnancy and is associated with increased serum IL-12 expression relative to the C57BL/6J wild-type (WT) controls. In contrast, protection conferred by IFNAR deficiency against ST infection in the non-pregnant state is abrogated during pregnancy which correlated with reduced serum IL-12 expression. Taken together, our study shows the critical role for IL-12 in modulating host immunity to infection. Furthermore, the differential impact of type I IFNs on immunity to LM and ST during pregnancy are attributed to specific host-pathogen interactions.

4.3 Materials and Methods

4.3.1 Mice and Matings

C57BL/6J wild-type (WT) mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA). IFNAR$^{-/-}$ mice bred on the WT background were obtained from Dr. Kaja Murali-Krishna (Emory University, Atlanta, GA, USA). Matings were performed by housing one male with one to two females for two nights. The presence of a copulatory plug after each day of mating was designated as day 0 of pregnancy. The rates of fetal resorptions, which are defined as uterine necrotic scars or as necrotic and/or hemorrhagic fetuses and placentas, were calculated using the formula $R/(R + V) \times 100$, where R is the number of resorbing fetuses and V is the number of viable fetuses per mouse. The study was performed in accordance with the guidelines of the Canadian Council on Animal Care and the protocols approved by National Research Council Canada in Ottawa, Canada.

4.3.2 Infections and Bacterial Enumeration

Listeria monocytogenes 10403S (LM) and *Salmonella enterica* serovar Typhimurium SL1344 (ST) were grown to mid-log phase at $OD_{600} = 1.0$ and $OD_{600} = 0.8$, respectively, under constant shaking in Bacto™ brain-heart infusion (BHI) medium (BD, Mississauga, ON, Canada) containing 50 μg/mL streptomycin (Sigma-Aldrich, Oakville, ON, Canada). Bacterial stocks were maintained in 20% glycerol at -80ºC. For mouse infections, frozen bacterial stocks were thawed and diluted in 0.9% sodium chloride (saline) solution. Mice were injected intravenously (i.v.) via the lateral vein with $5x10^4$ LM colony-forming units (CFUs), or $1x10^3$ ST CFUs suspended in 200 μL of saline solution.

Spleen and placentas were homogenized in RPMI 1640 medium containing 8% fetal bovine serum (FBS). Serial 10-fold dilutions of tissue homogenates were plated onto BHI or

Lysogeny broth agar plates (BD, Mississauga, ON, Canada) to enumerate LM and ST CFUs, respectively. Plates were incubated at 37^{o}C overnight and bacterial colonies were enumerated. Data were presented as CFUs per spleen or placenta.

4.3.3 Flow Cytometric Analysis

Spleens and individual placentas (with intact deciduas) were homogenized and passed through a 100 μM cell strainer to obtain a single-cell suspension. Total splenic cell numbers were determined by staining cells with the Acridine orange/propidium iodide (AO/PI) solution (Nexcelom Bioscience, Lawrence, MA, USA) and counting live cells using the Cellometer Auto 2000 (Nexcelom Bioscience, Lawrence, MA, USA). Placental homogenates were incubated in Hanks' Balanced Salt Solution (Gibco; Thermo Fisher Scientific, Burlington, ON, Canada) containing 0.2% Collagenase Type IV (Worthington Biochemical Corporation; Cedarlane, Burlington, ON, Canada). Immune cells were then isolated through Percoll® (GE Healthcare; Sigma-Aldrich, Oakville, ON, Canada) density gradient centrifugation. Briefly, placental homogenates were re-suspended in 5 mL 40% Percoll® and underlaid with 5 mL 70% Percoll®. Cells were then centrifuged at 2,000 rpm for 25 min without braking using the Heraeus Megafuge 40R (Thermo Fisher Scientific, Burlington, ON, Canada). Immune cells at the interphase between the 40%/70% gradient were isolated using plastic Pasteur pipettes.

Splenic and placental cells were re-suspended in 1x phosphate-buffered saline containing 2% FBS (staining buffer) prior to incubation with unconjugated anti-CD16/CD32 (FcγRIII/II) antibody for 10 min. Cells were then stained with the following fluorochrome-conjugated antibodies for 30 min: CD45 (30-F11), CD11b (M1/70), Ly-6G (1A8), CD8a (53-6.7), CD45R/B220 (RA3-6B2) and CD11c (HL3) (BD Biosciences, Mississauga, ON,

Canada); TCR-ß (H57-597), CD4 (RM4-5) and CD49b (DX5) (eBioscience, Burlington, ON, Canada); F4/80 (BM8) and Ly-6C (HK1.4) (BioLegend; Cedarlane, Burlington, ON, Canada). Lastly, cells were washed and re-suspended in staining buffer prior to flow cytometry acquisition using the BD LSR Fortessa™. Flow cytometry data were analyzed using FlowJo® 7.6.5 (FlowJo, LLC, Ashland, OR, USA) software. Major immune cell types were identified as described previously (Agbayani et al., 2018).

4.3.4 Cytokine Measurements

Serum was obtained from blood using Microtainer® serum separator tubes (BD, Mississauga, ON, Canada). Splenic and placental tissues were collected and homogenized in 1x lysis buffer (10 mM Tris-HCl pH 8.0, 150 mM sodium chloride, 1% NP-40 v/v, 10% glycerol v/v, 5 mM EDTA) containing cOmplete™, Mini, EDTA-free Protease Inhibitor Cocktail (Roche; Sigma-Aldrich, Oakville, ON, Canada) (Amsen et al., 2009). Total protein concentration per spleen or placenta was determined using the Pierce™ BCA Protein Assay Kit (Thermo Scientific™, Burlington, ON, Canada). Tissue homogenates were diluted to a final protein concentration of 500 µg/mL. Cytokine expression in splenic and placental tissues was presented as pg/mg of protein.

Expression levels of IL-6, IL-10, MCP-1 (CCL2), IFN-γ, TNF, IL-1β, IL-12p70 (IL-12) and KC (CXCL1) cytokines in the serum and tissue homogenates were measured using the Cytometric Bead Array (CBA) Mouse Inflammation Kit and Flex Set (BD Biosciences, Mississauga, ON, Canada), as described previously (Agbayani et al., 2017). Samples were acquired using the BD LSR Fortessa™. Data were analyzed using FCAP Array™ software (Soft Flow Hungary Ltd., Pécs, Hungary).

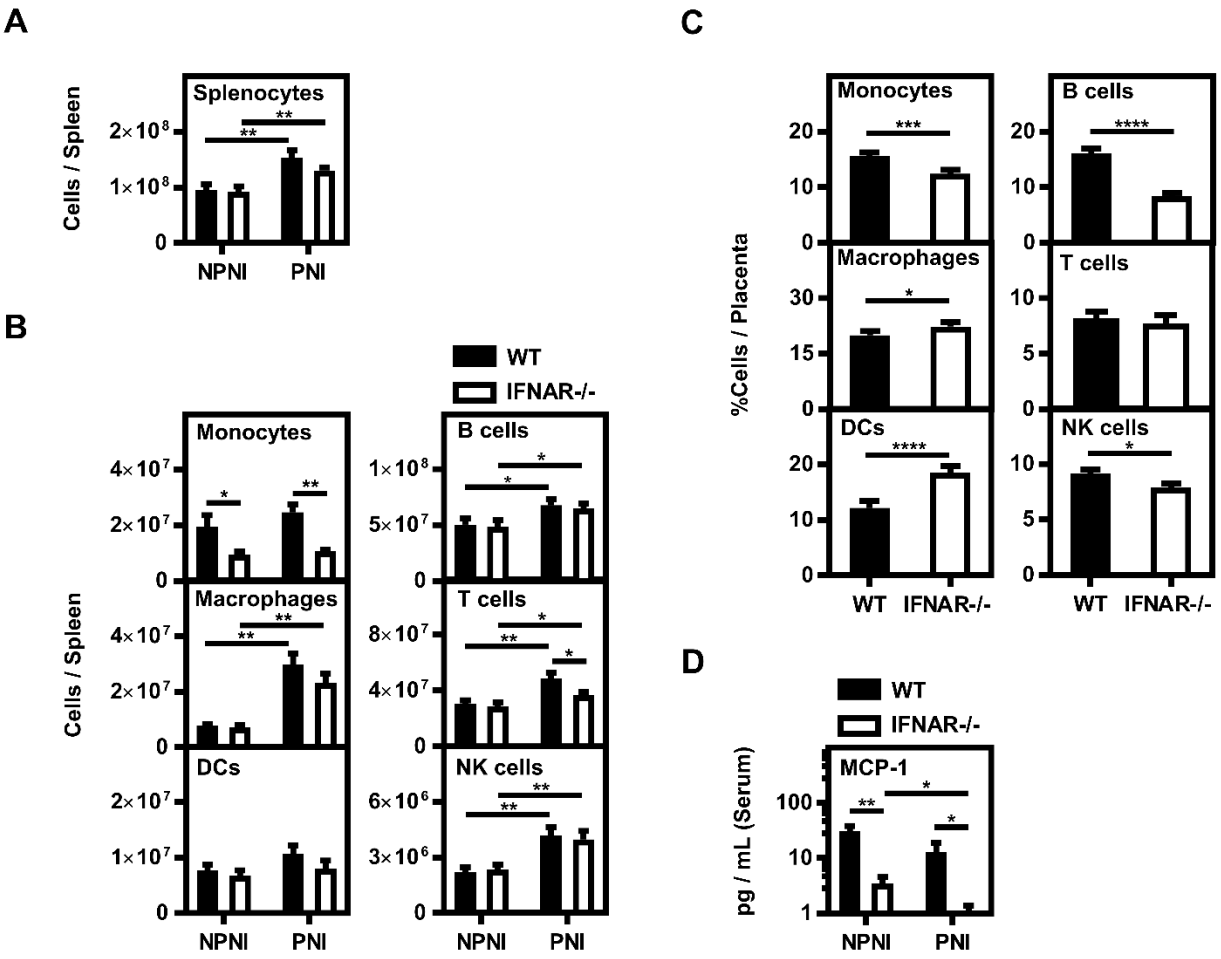
4.3.5 Statistical Analysis

All statistical analyses were performed using GraphPad® Prism 7 (GraphPad, La Jolla, CA, USA) software. Bacterial CFUs, immune cell numbers and cytokine expression levels were presented as mean ± SEM. Statistical significance of CFU, flow cytometry and cytokine expression data was determined through Mann-Whitney U test. Survival curves were analyzed using the Gehan-Breslow-Wilcoxon test. P values of ≤ 0.05 were considered statistically significant. *: $p \leq 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ****: $p < 0.0001$.

4.4 Results and Discussion

4.4.1 Type I IFNs modulate splenic and placental immune cell distributions and serum cytokine expression

We first determined the total splenic numbers in non-pregnant and pregnant mice WT and IFNAR mice by counting live cells using the Cellometer. In the non-infected state, pregnant WT and IFNAR$^{-/-}$ mice exhibited elevated total splenic cell at day 14-15 of pregnancy relative to their non-pregnant counterparts (Figure 4.1A). We then analyzed the distribution of immune cell subsets in the spleen and individual placentas through flow cytometry. The increased total splenic numbers in WT and IFNAR$^{-/-}$ mice during pregnancy correlated with a general increase in splenic myeloid (macrophages and neutrophils) and lymphoid (B cells, T cells and NK cells) immune cell subsets during normal gestation (Figure 4.1B and 4.2A) (Norton et al., 2009). It is proposed that the elevation of circulating granulocyte and monocyte numbers in the maternal host acts as a compensatory mechanism for pregnancy-related changes in Th17 cells and Tregs (Faas et al., 2014). Furthermore, the spleen contains a reserve of monocytes that are rapidly recruited to inflammatory sites during infection (Swirski et al., 2009). We observed a decrease in splenic monocyte numbers in non-pregnant IFNAR$^{-/-}$ mice compared to their WT counterparts, consistent with a previous study (Figure 4.1B) (Seo et al., 2011). We also show that the reduced splenic monocyte numbers in IFNAR$^{-/-}$ mice is retained during pregnancy. Thus, our results further highlight the role of type I IFNs in regulating monocyte distribution and that this mechanism is not impacted during pregnancy. Splenic T cell (CD4^{+} and CD8^{+}) numbers were also decreased in pregnant IFNAR$^{-/-}$ mice relative to pregnant WT controls, indicating a potential role for IFNAR signaling in modulating T cell distribution during pregnancy (Figure 4.1B and 4.2A). We also show for the first time an


A
Splenocytes
Cells / Spleen
2×10^8
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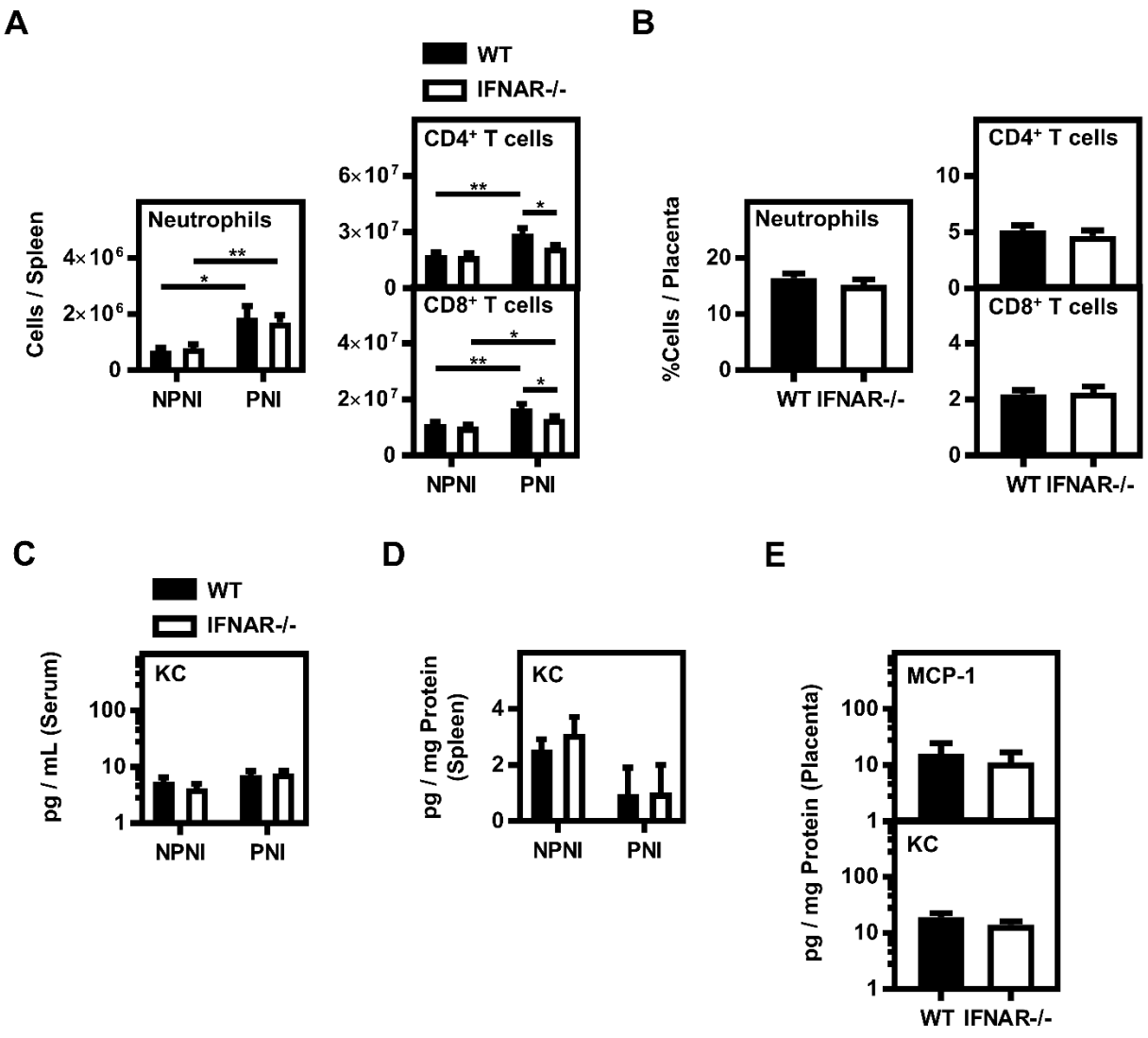
0
NPNI
PNI
B
WT
IFNAR-/-
Monocytes
Macrophages
DCs
B cells
T cells
NK cells
Cells / Spleen
4×10^7
2×10^7
1×10^7
6×10^6
3×10^6
8×10^7
5×10^7
1×10^8
C
%Cells / Placenta
Monocytes
Macrophages
DCs
B cells
T cells
NK cells
WT
IFNAR-/-
D
pg / mL (Serum)
MCP-1
100
10
1

Figure 4.1. IFNAR$^{-/-}$ mice exhibit altered distributions of splenic and placental immune cell types relative to WT mice. Spleen, individual placentas and serum were collected from non-pregnant and pregnant (day 14-15 of gestation) WT and IFNAR$^{-/-}$ mice in the non-infected state. (A) Total splenic cell numbers were determined by counting live cells using the Cellometer. N = 6 mice per group. Immune cell populations in the spleen and individual placentas were identified through flow cytometry. (B) Splenic numbers of monocytes, macrophages, DCs, B cells, T cells and NK cells. N = 6 mice per group. (C) Placental percentages of monocytes, macrophages, DCs, B cells, T cells and NK cells. N = 51-52 individual placentas per pregnant group. (D) MCP-1 expression levels were determined through CBA. N = 4-5 mice per group. Data are presented as mean ± SEM. Statistical significance was analyzed by Mann Whitney U test. *: $p \leq 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ****: $p < 0.0001$. NPNI, non-pregnant non-infected; PNI, pregnant non-infected.


A
WT
IFNAR-/-
Neutrophils
Cells / Spleen
4×10^6
2×10^6
0
NPNI
PNI
CD4+ T cells
6×10^7
3×10^7
0
CD8+ T cells
4×10^7
2×10^7
0
B
Neutrophils
%Cells / Placenta
20
10
0
WT IFNAR-/-
CD4+ T cells
10
5
0
CD8+ T cells
4
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C
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pg / mL (Serum)
100
10
1
D
KC
pg / mg Protein (Spleen)
4
2
0
E
MCP-1
pg / mg Protein (Placenta)
100
10
1
KC
100
10
1

Figure 4.2. Immune cell populations and cytokine expression profiles of non-pregnant and pregnant WT and IFNAR$^{-/-}$ mice in the non-infected state. Spleen (A) and/or individual placentas (B) were collected from non-pregnant and pregnant (day 14-15 of gestation) WT and IFNAR$^{-/-}$ mice for flow cytometric analysis of immune cell populations. N = 6 mice per group; 51-52 individual placentas per pregnant group. Cytokine expression levels in serum (C), spleen (D) and individual placentas (E). N = 3-5 mice per group; 3 individual placentas per pregnant group. Data are presented as mean ± SEM. Statistical significance was analyzed by Mann Whitney U test. *: $p \leq 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ****: $p < 0.0001$. NPNI, non-pregnant non-infected; PNI, pregnant non-infected.

analysis of immune cell populations in the individual placenta. IFNAR$^{-/-}$ mice exhibited reduced frequencies of monocytes, B cells and NK cells, and increased levels of macrophages and DC in the placenta relative to WT mice (Figure 4.1C and 4.2B). No significant differences were observed in placental neutrophil and T cell ($CD4^+$ and $CD8^+$) percentages between the two mouse strains. We then examined cytokine expression in the maternal systemic and placental compartments. Baseline levels of MCP-1 and KC were observed in the serum (Figure 4.1D and 4.2C). MCP-1 (CCL2) is a chemoattractant of monocytes, macrophages, NK cells and memory T cells (Deshmane et al., 2009). Furthermore, KC promotes neutrophil recruitment and regulates neutrophil and T cell functionality during infection (Jin et al., 2014). Non-pregnant and pregnant IFNAR$^{-/-}$ mice exhibited decreased baseline levels of serum MCP-1 relative to their WT counterparts (Figure 4.1D). This correlated with reduced monocyte numbers and percentages in spleens and individual placentas, respectively (Figure 4.1B-D). Thus, modulation of monocyte distribution in the absence of IFNAR signaling is associated with downregulation of serum MCP-1 expression. Serum MCP-1 levels were also further reduced in pregnant IFNAR$^{-/-}$ mice compared to non-pregnant controls (Figure 4.1D). In contrast, we did not observe a statistically discernable reduction in serum MCP-1 expression levels between non-pregnant and pregnant WT mice. These results indicate that IFNAR signaling contributes to the regulation of baseline serum MCP-1 expression during pregnancy. Furthermore, baseline KC expression levels in the serum and spleen were similarly low in non-pregnant and pregnant IFNAR$^{-/-}$ mice compared to WT controls (Figure 4.2C and 4.2D). A similar trend was observed in the placental expression of MCP-1 and KC between the two mouse strains (Figure 4.2E). Lastly, IL-6, IL-10, IFN-γ, TNF, IL-1β and IL-12p70 expression levels in the serum, spleen and individual placentas were below the limits of detection (data

not shown). Thus, IFNAR signaling modulates splenic and placental immune cell distributions and serum cytokine expression in the non-infected state.

4.4.2 IFNAR deficiency confers host protection from LM infection during pregnancy

The protective role of IFNAR deficiency against systemic LM infection in non-pregnant mice is associated with reduced expression of IFN-inducible apoptosis-associated genes, decreased lymphocyte apoptosis and increased splenic numbers of TNF-producing macrophages (Auerbuch et al., 2004; O'Connell et al., 2004; Carrero et al., 2004). Furthermore, genetic resistance of C57BL/6J (WT) mice to LM infection is primarily attributed to the resistant allele at the *Hc* locus on chromosome 2 (Gervais et al., 1984). To determine whether this protection is retained by the maternal host during pregnancy, non-pregnant and pregnant WT and IFNAR$^{-/-}$ mice were infected with LM 5x10^4 CFUs i.v. at mid-gestation (day 11-12 of gestation). Bacteria were enumerated in the spleen and individual placentas 3 days post-infection. IFNAR$^{-/-}$ mice showed reduced bacterial burden compared to WT mice in the non-pregnant state, as observed in previous studies (Figure 4.3A) (Auerbuch et al., 2004; O'Connell et al., 2004). Pregnant WT mice also controlled splenic LM infection more efficiently compared to non-pregnant controls, as we observed previously (Krishnan et al., 2010). Next, pregnant IFNAR$^{-/-}$ mice showed significantly reduced splenic bacterial burden relative to pregnant WT mice. A similar trend in bacterial numbers was also observed in individual placentas (Figure 4.3B). Lastly, no significant differences were observed in fetal resorption rates among pregnant mice from both mouse strains (Figure 4.3C). These results suggest that the protection conferred by IFNAR deficiency against LM infection is not adversely impacted by pregnancy. Furthermore, increased control of infection in non-pregnant IFNAR$^{-/-}$ mice is associated with elevated splenic cell numbers relative to their WT counterparts (Figure 4.4A).

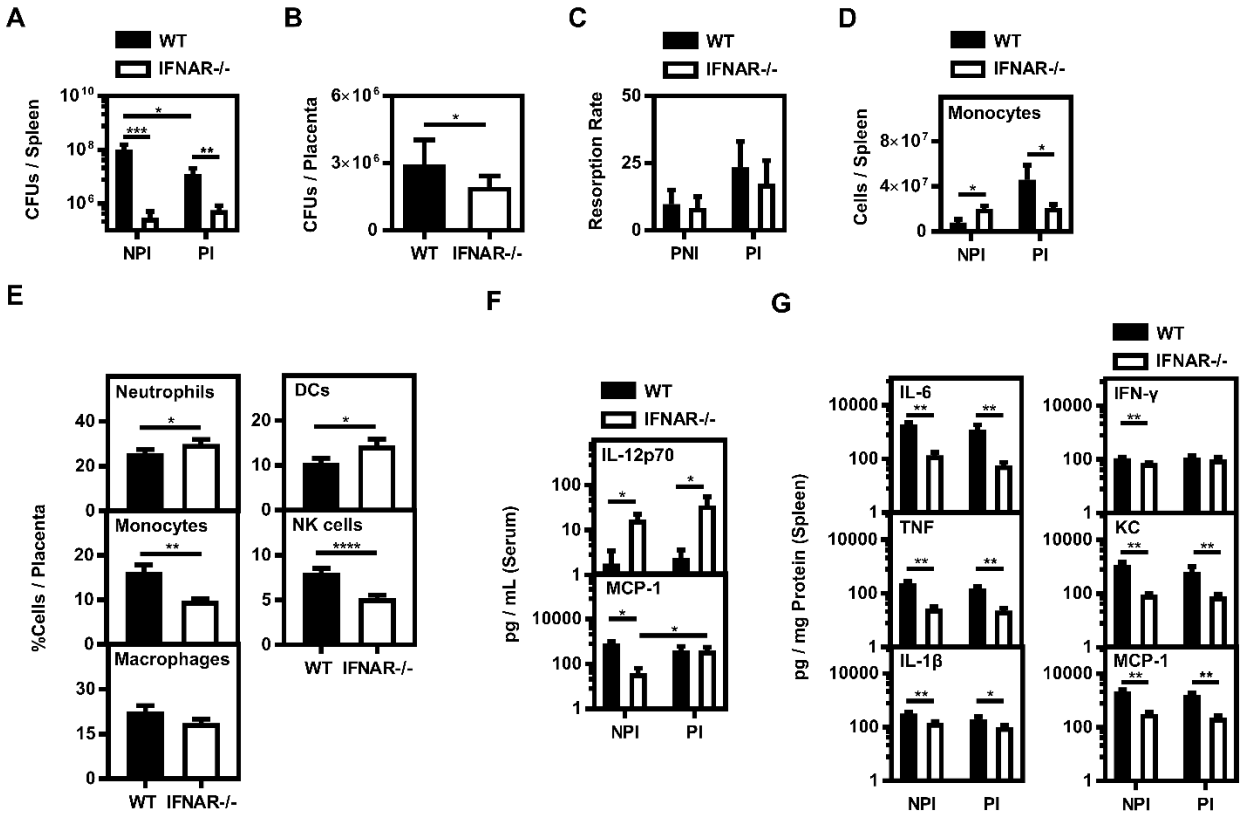
A
WT
IFNAR-/-
CFUs / Spleen
NPI
PI
B
CFUs / Placenta
WT
IFNAR-/-
C
Resorption Rate
PNI
PI
D
Monocytes
Cells / Spleen
E
Neutrophils
DCs
Monocytes
NK cells
Macrophages
%Cells / Placenta
F
IL-12p70
MCP-1
pg / mL (Serum)
G
IL-6
IFN-γ
TNF
KC
IL-1β
MCP-1
pg / mg Protein (Spleen)

Figure 4.3. IFNAR$^{-/-}$ mice retain increased resistance to LM infection during pregnancy. Non-pregnant and pregnant (day 11-12 of gestation) WT and IFNAR$^{-/-}$ mice were infected with LM 5x10^4 CFUs i.v. Spleens, individual placentas and serum were collected at day 3 post-infection. (A and B) Bacterial burdens in spleens and individual placentas were enumerated. N = 6-8 mice per group; 47-63 individual placentas per pregnant group. (C) Fetal resorption rates were determined using the formula $R/(R + V) \times 100$, where R is the number of resorbing fetuses and V is the number of viable fetuses per mouse. N = 3-11 mice per group. Immune cell populations in the spleen and individual placentas were identified through were evaluated through flow cytometry. (D) Monocyte numbers in the spleen in non-pregnant and pregnant WT and IFNAR$^{-/-}$ mice at day 3 post-infection. N = 4 mice per group. (E) Percentages of neutrophils, monocytes, DCs and NK cells in the placenta at day 3 post-infection. N = 24-27 individual placentas per pregnant group. Cytokine expression levels in the serum (F) and spleen (G) at day 3 post-infection. N = 4-5 mice per group. Data are presented as mean ± SEM. Statistical significance was analyzed by Mann Whitney U test. *: $p \leq 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ****: $p < 0.0001$. NPI, non-pregnant infected; PNI, pregnant non-infected; PI, pregnant infected.

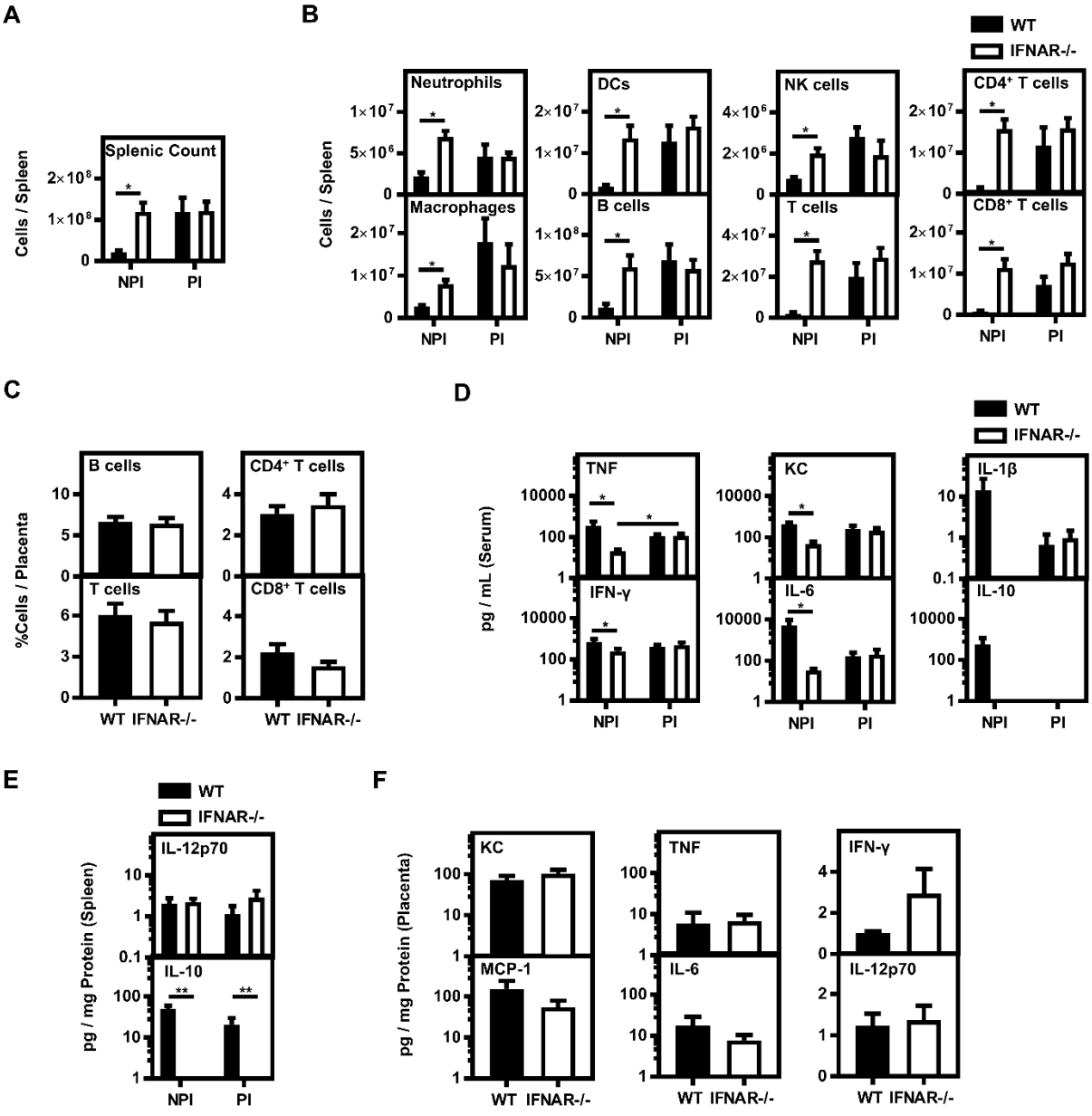
A
Splenic Count
Cells / Spleen
NPI
PI
B
WT
IFNAR-/-
Neutrophils
DCs
NK cells
CD4+ T cells
Macrophages
B cells
T cells
CD8+ T cells
Cells / Spleen
C
B cells
CD4+ T cells
T cells
CD8+ T cells
%Cells / Placenta
WT IFNAR-/-
D
TNF
KC
IL-1β
IFN-γ
IL-6
IL-10
pg / mL (Serum)
E
IL-12p70
IL-10
pg / mg Protein (Spleen)
F
KC
TNF
IFN-γ
MCP-1
IL-6
IL-12p70
pg / mg Protein (Placenta)

Figure 4.4. Immune cell distributions and cytokine expression profiles in non-pregnant and pregnant WT and IFNAR$^{-/-}$ mice during systemic LM infection. Non-pregnant and pregnant (day 11-12 of gestation) WT and IFNAR$^{-/-}$ mice were infected LM 5x10^4 CFUS i.v. Spleen, individual placentas and serum were collected at day 3 post-infection. (A) Total splenic cell counts. N = 4 mice per group. Numbers of splenic (B) and placental (C) immune cell types. N = 4 mice per group; 33-34 individual placentas per pregnant group. Cytokine expression levels in the serum (D), spleen (E) and individual placentas (F). N = 4-5 mice per group; 8 individual placentas per pregnant group. Data are presented as mean ± SEM. Statistical significance was analyzed by Mann Whitney U test. *: $p \leq 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ****: $p < 0.0001$. NPI, non-pregnant infected; PI, pregnant infected.

Reduced splenic cell numbers in non-pregnant WT mice is consistent with accelerated macrophage and lymphocyte death in response to LM, as observed previously, leading to impaired innate immune cell responses (Carrero et al., 2004; Auerbuch et al., 2004). Furthermore, high LM burden enhances BM neutrophil exhaustion and reduces neutrophil infiltration into infected tissues (Navarini et al., 2009). In contrast, pregnant IFNAR$^{-/-}$ mice exhibited reduced monocyte numbers, despite lower bacterial numbers in the spleen and individual placentas relative to WT controls (Figure 4.3A, 4.3B, 4.3D). We surmise that pregnancy-associated increase in splenic immune cell numbers in the non-infected state may be sufficient to control bacterial growth in IFNAR$^{-/-}$ mice upon infection, despite reduced monocyte numbers relative to WT mice (Figure 4.1A, 4.1B, 4.3D). Reduced placental bacterial numbers were also associated with increased percentages of neutrophils and DCs, and decreased percentages of monocytes and NK cells in the placenta (Figure 4.3B, 4.3E). Thus, our results suggest that deficiency in IFNAR signaling modulates splenic and placental immune cell distribution during LM infection. It is also possible that differential cytokine regulation during pregnancy is responsible for altered kinetics of infection in IFNAR mice relative to WT mice. Accordingly, we examined cytokine responses in the serum, spleen and individual placentas at day 3 post-infection. Increased control of LM infection in non-pregnant IFNAR$^{-/-}$ mice relative to WT controls was associated with enhanced IL-12p70 expression in the serum at day 3 post-infection, consistent with previous studies (Figure 4.3F) (Way et al., 2007; Auerbuch et al., 2004). IL-12p70 is an immune-regulatory cytokine produced primarily by innate immune cells, including neutrophils, monocytes, macrophages and DCs (Hamza et al., 2010). It plays critical roles in innate and adaptive immunity by promoting IFN-γ production upon NK cell activation and naïve CD4^{+} T cell differentiation into Th1 cells (Hamza et al., 2010). IFN-α/β has been shown to inhibit IL-12 and IFN-γ expression during

viral infection, further highlighting the immune-regulatory roles of IFNAR signaling in host immunity (Cousens et al., 1997). Non-pregnant IFNAR$^{-/-}$ mice also exhibited reduced LM-induced expression of TNF, MCP-1, IFN-γ and KC in the serum, correlating with decreased bacterial burden in the spleen compared to their WT counterparts (Figure 4.3A, 4.3F, 4.4D). No significant differences were observed in IL-1β and IL-10 expression in the serum among the non-pregnant groups (Figure 4.4D). During pregnancy, increased control of systemic LM infection in IFNAR$^{-/-}$ mice was similarly associated with enhanced IL-12p70 expression compared to WT mice (Fig. 4.3A, 4.3F). However, serum expression levels of TNF, MCP-1, IFN-γ, IL-6 and KC were similar between pregnant WT and IFNAR$^{-/-}$ groups (Figure 4.3F and 4.4D). IL-10 expression levels were similarly low in in all non-pregnant and pregnant groups (Figure 4.4D). Interestingly, MCP-1 and TNF expression levels increased from the non-pregnant to pregnant state in IFNAR$^{-/-}$ mice, indicating a potential role for type I IFN signaling in regulating bacterial-induced inflammation during pregnancy (Figure 4.3F and 4.4D). However, this enhanced inflammatory state was not detrimental to maternal and fetal protection from infection, suggesting a potential mechanism of homeostatic control during pregnancy. Analysis of splenic cytokine expression at day 3 post-infection showed reduced IL-6, IL-1β, KC, MCP-1, TNF, IL-10 and IFN-γ expression in non-pregnant IFNAR$^{-/-}$ mice relative to WT mice (Figure 4.3G and 4.4E). It is likely that the reduced splenic expression of these cytokines is attributed to decreased bacterial burden in pregnant IFNAR$^{-/-}$ mice relative to their WT counterparts. Furthermore, deficiency in IFNAR signaling is associated with downregulation of MCP-1 production by BM-derived macrophages in response to LM infection (Jia et al., 2009). Consistent with a previous study, we show that non-pregnant IFNAR$^{-/-}$ mice exhibit enhanced control of LM infection despite reduced splenic MCP-1 expression relative to WT controls (Figure 4.3A and 4.3G) (Jia et al., 2009). A similar trend

in splenic bacterial burden and cytokine expression was observed in pregnant WT and IFNAR$^{-/-}$ mice, except in IFN-γ expression where no significant differences were observed between the two groups (Figure 4.3G). Splenic IL-12p70 expression was similarly low in all non-pregnant and pregnant groups (Figure 4.4E). Furthermore, non-pregnant and pregnant IFNAR$^{-/-}$ mice showed reduced splenic IL-10 expression levels compared to their WT counterparts. In the placenta, KC, MCP-1 TNF, IL-6, IFN-γ and IL-12p70 expression were similar between the two mouse strains at day 3 post-infection (Figure 4.4F). This indicates that increased control of placental infection in IFNAR$^{-/-}$ mice is not due to an altered placental inflammatory profile, but may be attributed to efficient initial control of infection in the systemic compartment. Thus, our results indicate compartment-specific regulation of cytokine responses in non-pregnant and pregnant hosts during systemic LM infection. Furthermore, enhanced serum IL-12 expression associated with IFNAR deficiency may be a critical indicator of host resistance to LM infection during pregnancy.

4.4.3 Pregnancy compromises host protection conferred by IFNAR deficiency against ST infection

The increased resistance of non-pregnant IFNAR$^{-/-}$ mice to ST infection is attributed to reduced bacterial-induced macrophage death (Robinson et al., 2012). In the current study, we determined the role of IFNAR signaling in host immunity to ST infection during pregnancy. Non-pregnant and pregnant (day 11-12 of gestation) WT and IFNAR$^{-/-}$ mice were infected with ST 10^3 CFUs i.v. As expected, non-pregnant IFNAR$^{-/-}$ mice exhibited increased survival rates (median survival: 15 days) relative to their WT counterparts (median survival: 7 days) (Figure 4.5A). However, the enhanced resistance of IFNAR$^{-/-}$ mice against infection in the non-pregnant state was significantly reduced during pregnancy (median survival: 9

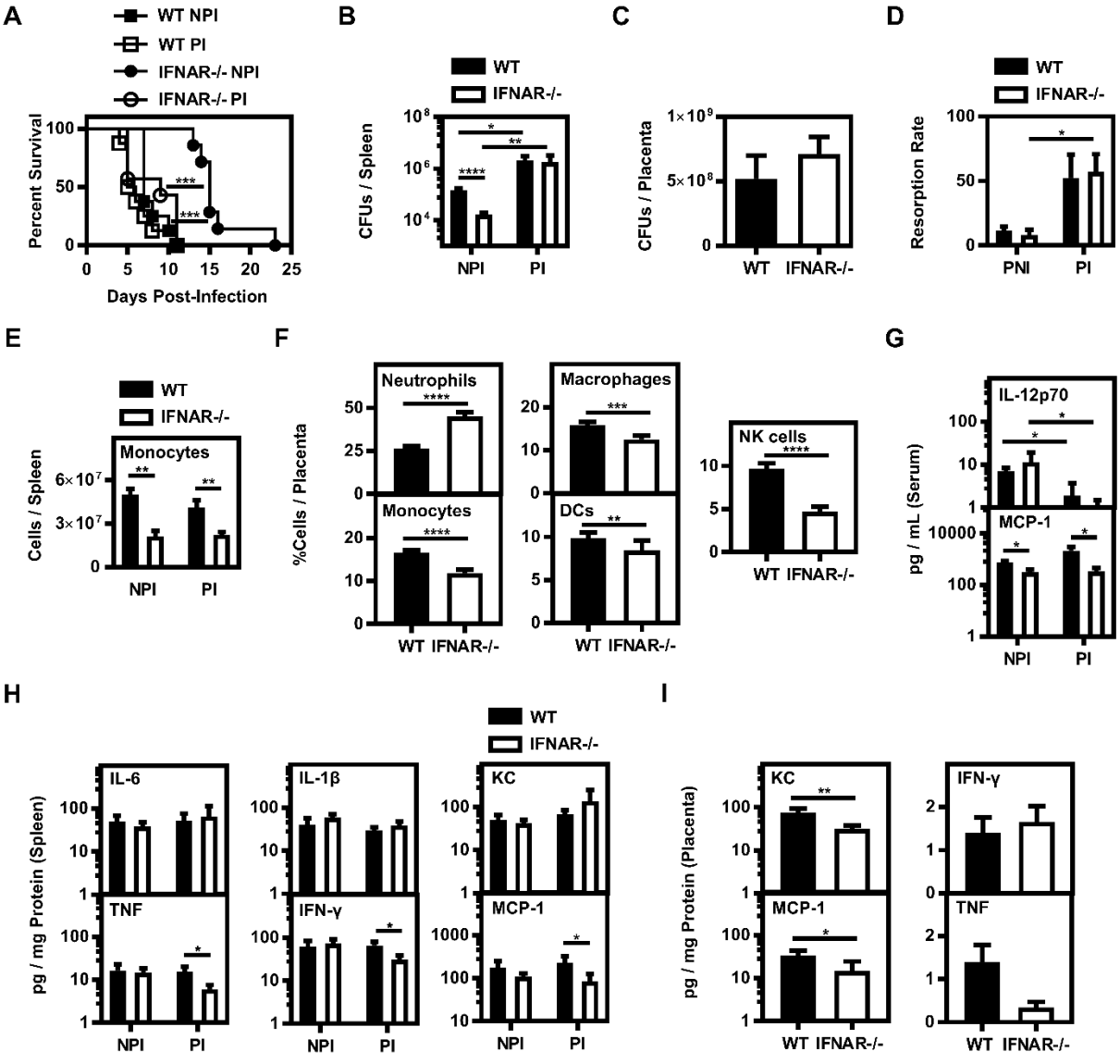
A
WT NPI
WT PI
IFNAR-/- NPI
IFNAR-/- PI
Percent Survival
Days Post-Infection
B
WT
IFNAR-/-
CFUs / Spleen
NPI
PI
C
CFUs / Placenta
WT
IFNAR-/-
D
Resorption Rate
PNI
PI
E
Cells / Spleen
Monocytes
NPI
PI
F
%Cells / Placenta
Neutrophils
Monocytes
Macrophages
DCs
NK cells
G
pg / mL (Serum)
IL-12p70
MCP-1
H
pg / mg Protein (Spleen)
IL-6
IL-1β
KC
TNF
IFN-γ
MCP-1
I
pg / mg Protein (Placenta)
KC
IFN-γ
MCP-1
TNF

Figure 4.5. IFNAR$^{-/-}$ mice exhibit enhanced susceptibility to ST infection during pregnancy. Non-pregnant and pregnant (day 11-12 of gestation) WT and IFNAR$^{-/-}$ mice were infected with ST 10^3 CFUs i.v. (A) Mice were monitored every day following infection. Mouse deaths were recorded upon observing >20% loss in body weight and/or 2 or more severe signs of clinical illness, including piloerection, lack of grooming and morbidity. N = 7-8 mice per group. Spleen, individual placentas and serum were collected at day 3 post-infection. (B and C) Bacterial burden in spleen and individual placentas were enumerated. N = 7-13 mice per group; 35-70 individual placentas per pregnant group. (D) Fetal resorption rates were determined using the formula $R/(R + V) \times 100$, where R is the number of resorbing fetuses and V is the number of viable fetuses per mouse. N = 5-13 mice per group. Immune cell populations in the spleen and individual placentas were identified through were evaluated through flow cytometry. (E) Monocyte numbers in the spleen in non-pregnant and pregnant WT and IFNAR$^{-/-}$ mice at day 3 post-infection. N = 4-7 mice per group. (F) Percentages of neutrophils, monocytes, macrophages, DCs, B cells, T cells ($CD4^+$ and $CD8^+$) and NK cells in the placenta at day 3 post-infection. N = 38-40 individual placentas per pregnant group. Cytokine expression levels in the serum (G), spleen (H) and placenta (I) at day 3 post-infection. N = 5-9 mice per group; 10 individual placentas per pregnant group. Bacterial burden, resorption rates, cell numbers and cytokine expression levels are presented as mean ± SEM. Statistical significance was analyzed by Mann Whitney U test. *: $p \leq 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ****: $p < 0.0001$. NPI, non-pregnant infected; PNI, pregnant non-infected; PI, pregnant infected.

days). Furthermore, no significant differences in survival rates were observed between the two pregnant groups. Bacterial enumeration at day 3 post-infection showed reduced splenic bacterial numbers in non-pregnant IFNAR$^{-/-}$ mice compared to WT controls (Figure 4.5B). However, IFNAR$^{-/-}$ mice showed a dramatic increase in splenic bacterial burden from the non-pregnant to pregnant state, to levels similar to those of pregnant infected WT mice. Splenic cell numbers from the non-pregnant to pregnant state were similar in both mouse strains during infection (Figure 4.6A). Furthermore, placental CFUs and fetal resorption rates were similarly high in pregnant WT and IFNAR$^{-/-}$ mice upon infection (Figure 4.5C and 4.5D). Thus, in contrast to LM infection, our results indicate that the protection conferred by IFNAR deficiency against ST infection is adversely impacted by pregnancy. Flow cytometric analysis of splenic immune cell populations at day 3 post-infection showed decreased monocyte percentages in non-pregnant and pregnant IFNAR$^{-/-}$ mice relative to their WT counterparts (Figure 4.5E). In a similar study, impaired bone marrow monocyte progenitor differentiation and reduced splenic accumulation of Ly6C^{+} monocyte-derived antigen presenting cells in response to lipopolysaccharide (LPS) was attributed to IFNAR deficiency (Lasseaux et al., 2017). LPS expressed by Gram-negative bacteria, such as *Salmonella*, induces immune activation upon recognition by host cells via Toll-like receptor 4 (TLR4) (Takeda and Akira, 2003). These results indicate that defective monocyte recruitment and/or differentiation during ST infection may be associated with IFNAR deficiency, leading to altered splenic distribution of monocytes. Furthermore, no differences were observed in the splenic numbers of neutrophils, macrophages, DCs, NK cells, B cells and T cells (CD4^{+} and CD8^{+}) in non-pregnant and pregnant IFNAR$^{-/-}$ mice during infection relative to their WT counterparts (Figure 4.4B). We also observed a ~2-fold increase in neutrophil percentages in the placentas of IFNAR$^{-/-}$ mice, which corresponded to an overall decrease in the percentages of other

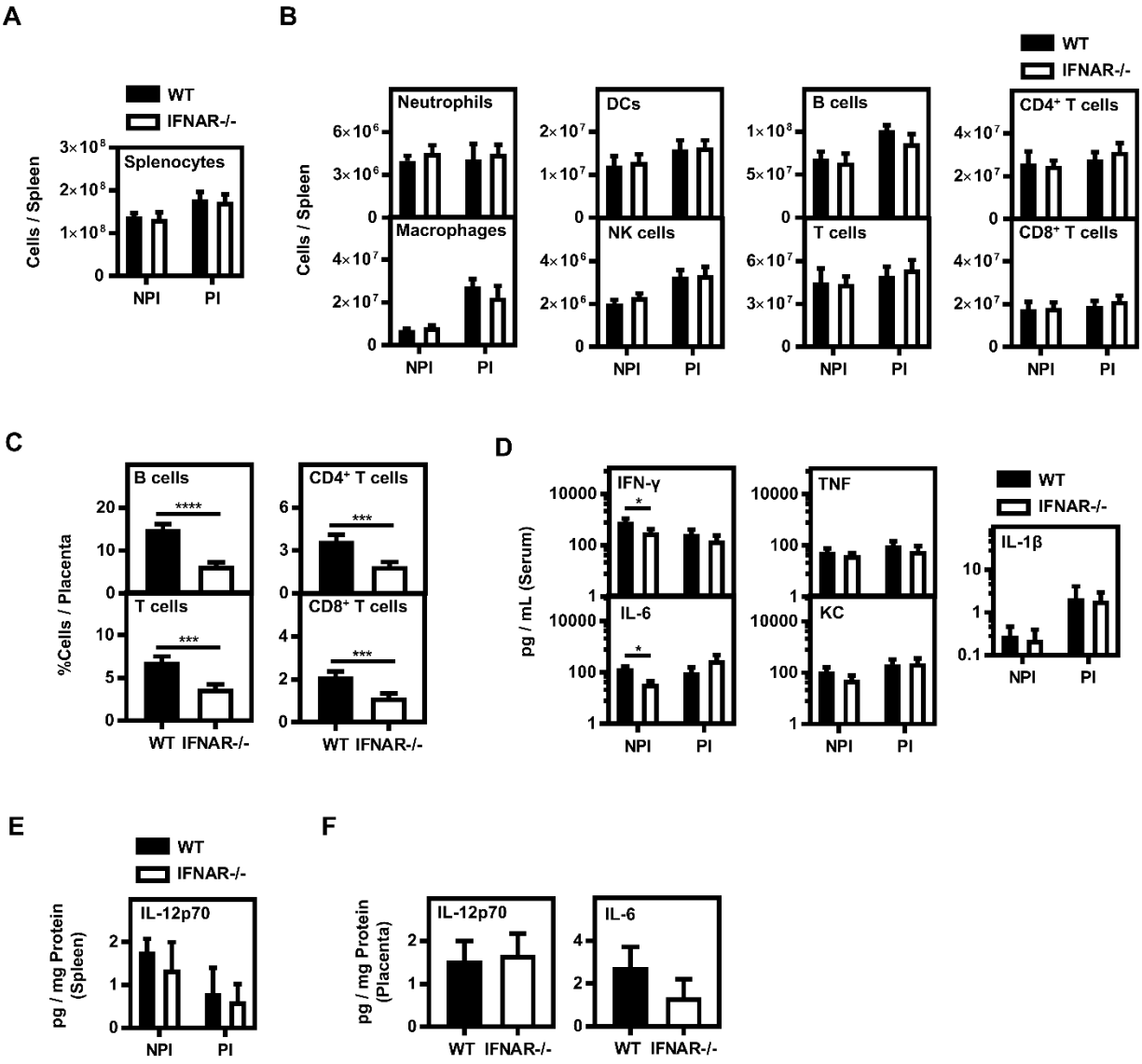
A
WT
IFNAR-/-
Splenocytes
Cells / Spleen
NPI
PI
B
Neutrophils
Macrophages
DCs
NK cells
B cells
T cells
CD4+ T cells
CD8+ T cells
C
B cells
T cells
CD4+ T cells
CD8+ T cells
%Cells / Placenta
WT IFNAR-/-
D
IFN-γ
IL-6
TNF
KC
IL-1β
pg / mL (Serum)
E
IL-12p70
pg / mg Protein (Spleen)
F
IL-12p70
IL-6
pg / mg Protein (Placenta)

Figure 4.6. Immune cell distributions and cytokine expression profiles in non-pregnant and pregnant WT and IFNAR$^{-/-}$ mice during systemic ST infection. Non-pregnant and pregnant (day 11-12 of gestation) WT and IFNAR$^{-/-}$ mice were infected ST 10^3 CFUS i.v. Spleen, individual placentas and serum were collected at day 3 post-infection. (A) Total splenic cell counts. N = 4-6 mice per group. Numbers of splenic (B) and placental (C) immune cell types. N = 4-7 mice per group; 38-40 individual placentas per pregnant group. Cytokine expression levels in the serum (D), spleen (E) and individual placentas (F). N = 5-9 mice per group; 10 individual placentas per pregnant group. Data are presented as mean ± SEM. Statistical significance was analyzed by Mann Whitney U test. *: $p \leq 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ****: $p < 0.0001$. NPI, non-pregnant infected; PI, pregnant infected.

placental immune cell types relative to WT mice (Figure 4.5F). Placental necrosis associated with neutrophil infiltration is a hallmark of immune pathology in ST infection during pregnancy (Chattopadhyay et al., 2010). IFNAR deficiency has also been implicated in increased neutrophil migration but reduced monocyte infiltration into inflamed tissues during parasitic infection by *Leishmania amazonensis* (Xin et al., 2010). Thus, our results suggest that type I IFNs play critical roles in modulating innate immune cell distribution in both splenic and placental compartments during infection. Analysis of serum cytokine expression at day 3 post-infection showed a reduction in IL-12p70 expression by pregnant WT and IFNAR$^{-/-}$ relative to their non-pregnant counterparts (Figure 4.5G). This indicates that regulatory mechanisms associated with pregnancy may be exploited by ST, leading to impaired innate immune responses associated with decreased serum IL-12 expression. Splenic expression of IFN-γ, TNF and MCP-1 remained similar between non-pregnant WT and IFNAR$^{-/-}$ mice (Figure 4.5H). In contrast, the expression levels of these cytokines were reduced in pregnant IFNAR$^{-/-}$ mice relative to their WT counterparts. No significant differences were observed in the splenic expression levels of IL-6, KC, IL-1β and IL-12p70 among all non-pregnant and pregnant groups (Figure 4.5H and 4.6E). Placental expression of KC and MCP-1 was reduced in IFNAR$^{-/-}$ mice, which correlated with decreased monocyte percentages in the placenta relative to WT mice (Figure 4.5I). Although KC is typically associated as a neutrophil chemoattractant, it has been shown to modulate monocyte recruitment using *in vivo* models of atherosclerosis and arteriogenesis (Vries et al., 2015; Huo et al., 2001). Therefore, it is possible that KC and MCP-1 expression contributed to the modulation of monocyte distribution in the placenta during ST infection. In contrast, placental expression levels of IFN-γ, TNF, IL-12p70 and IL-6 remained similar between the two mouse strains (Figure 4.5I and 4.6F). Thus, the protective role of IFNAR deficiency against LM and ST is largely dependent on pathogen-

specific interactions with the host. Fetal-placental expression of type I IFNs during viral infection has been attributed major roles in the control of fetal viremia and the modulation of maternal survival (Racicot et al., 2017). In our study, reduced maternal systemic LM infection in pregnant IFNAR$^{-/-}$ mice was associated with increased placental control of LM relative to their WT counterparts. In contrast, enhanced susceptibility of IFNAR$^{-/-}$ mice to ST infection during pregnancy correlated with profound placental infection similar to WT mice. Our results indicate that bacterial burden in the placenta may also provide an additional source of infection, as observed during viral infection, thereby promoting disease progression (Racicot et al., 2017). However, the extent of the protection mediated by fetal-placental-derived type I IFNs against intracellular bacterial infections remains to be elucidated. Induction of IFNAR signaling during viral infection has been shown to decrease the threshold for pro-inflammatory cytokine expression in systemic and uterine compartments, and enhance susceptibility to secondary bacterial infection-driven pre-term births (Cappelletti et al., 2017). This supports the prevailing "double-hit" hypothesis, wherein viral infection enhances the risk of invasive bacterial infection in the maternal host and adverse pregnancy outcomes (Cappelletti et al., 2017; Racicot et al., 2013; Cardenas et al., 2011). Furthermore, viral-induced inhibition of IFN-β expression enhances pro-inflammatory cytokine production and placental trophoblast cell sensitivity to LPS (Racicot et al., 2016). We have previously shown that TLR4 expression is associated with robust pro-inflammatory cytokine production in pregnant mice in response to ST infection, leading to increased maternal disease progression and fetal deaths (Agbayani et al., 2017). Taken together, these results highlight the complex signaling networks regulated by IFNAR and TLR-mediated inflammation in host immunity to infection during pregnancy. Achieving a balance between pro-inflammatory and anti-inflammatory responses in maternal systemic and fetal-placental compartments is critical in determining pregnancy outcomes.

4.5 Conclusion

Our study highlights the critical roles of IFNAR signaling in modulating immune cell distribution and cytokine expression, leading to differential responses to intracellular infections during pregnancy. The increased resistance to LM infection and enhanced serum IL-12 response associated with IFNAR deficiency is not adversely impacted by pregnancy, despite the altered distribution of monocytes in maternal systemic and placental compartments. In contrast, the reduced resistance of pregnant hosts to ST infection is due to pregnancy-associated impairment of innate immune responses, including serum IL-12 expression, irrespective of IFNAR signaling.

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5.0 GENERAL DISCUSSION

Innate immunity is often characterized as a primitive system involving rapid but non-specific and limited responses against pathogens, in contrast to antigen-specific and long-lived responses of adaptive immunity. Nevertheless, our studies highlight the complex regulatory networks that govern innate immunity to intracellular pathogens. We show the critical regulatory roles of functional selectin-ligand expression, from immune cell development to the efficiency of innate immune responses against infection. We also demonstrate the impact of T cell cross-talk with inflammatory mediators in shaping innate and adaptive immunity. Lastly, we provide insight into host-pathogen interactions that modulate innate immune responses to infection during pregnancy, which may be considered as an "altered-immune' host state.

6.1 FucT-IV and -VII expression shapes innate immune responses through modulation of hematopoiesis and kinetics of immune responses

The roles of FucT-IV and -VII in leukocyte migration and lymphocyte homing have been extensively described. Leukocyte tethering is an essential initial step towards cellular adhesion, activation and extravasation into tissues during leukocyte recruitment (Lowe, 1997). P-selectin ligand synthesis by FucT-VII mediates leukocyte tethering to the endothelial wall, while control of leukocyte rolling velocities by E-selectin ligands is dependent primarily on FucT-VII and supported by FucT-IV (Weninger et al., 2000). Functional inactivation of PSGL-1 in humans and mice abrogates neutrophil and Th1 cell migration into the inflamed peritoneum and skin, respectively (Borges et al., 1997a; Borges et al., 1997b). Although L-selectin is typically associated as a lymphocyte homing receptor, it also mediates neutrophil and monocyte rolling on the endothelial wall and adherent leukocytes (Bargatze et al., 1994).

The lack of functional selectin ligand interactions also impairs T cell homing to draining LNs, consequently, promoting redistribution of T cells into the blood and non-lymphoid organs, including the lungs (Harp and Onami, 2010). Deficiency in both FucTs leads not only to defective leukocyte migration to inflammation sites but also neutrophilia, which is attributed to increased production and reduced turnover of neutrophils (Homeister et al., 2001; Maly et al., 1996). Thus, FucT-IV and -VII expression plays a role in immune homeostasis through regulation of leukocyte numbers and distribution. However, the precise contributions of functional selectin ligand expression in hematopoiesis are not well-understood. In adult mammals, self-renewing BM HSCs differentiate into progenitors that progressively develop lineage restriction. These progenitors then give rise to lineage-committed precursors that form mature immune and non-immune cells (Orkin and Zon, 2008). BM stromal cells support HSC function through production of cytokines, including stem cell factor, granulocyte-colony stimulating factor (G-CSF) and CXCL12 (Anthony and Link, 2014). Lévesque *et al.* established the crucial link between selectin-ligand interactions and regulation of hematopoiesis (Levesque et al., 1999). Adhesion of P-selectin and E-selectin to PSGL-1 expressed by human $CD34^+$ hematopoietic progenitor cells (HPCs) resulted in HPC growth inhibition and apoptosis *in vitro*, indicating a critical role for selectin/PSGL-1 interactions in the regulation of hematopoiesis (Levesque et al., 1999; Winkler et al., 2004). A similar phenomenon was also observed in murine HPCs upon adherence to E-selectin and to a lesser extent, P-selectin (Winkler et al., 2004). Given that FucT-IV and -VII expression regulates PSGL-1 interactions with E-selectin and P-selectin in humans and mice, it is clear that functional selectin ligand expression is important in hematopoiesis (Homeister et al., 2001; Martinez et al., 2005). Our progenitor expansion experiments using whole BM cultures showed increased GMP numbers in FtDKO mice. However, *in vitro* culture of sorted GMPs

showed no significant differences between WT and FtDKO GMP frequencies, indicating that functional selectin ligand expression does not modulate progenitor expansion on a per-cell basis. Increased GMP numbers, and consequently, elevated innate immune cell numbers in FtDKO mice may then be attributed to FucT-IV and -VII-mediated adhesive interactions between progenitors and BM stromal cells. Furthermore, FucT-VII has been shown to play an indirect role in T cell lymphopoiesis through regulation of PSGL-1 expression required by hematopoietic progenitors for homing to the thymus (Sultana et al., 2012). It is then possible that increased splenic T cell numbers in FtDKO mice is attributed to defective thymic reconstitution, leading to progenitor homing to the spleen. Taken together, our results highlight the critical roles of FucT-IV and -VII in regulating hematopoiesis, apart from their traditional role in leukocyte recruitment during inflammation.

Leukocytosis or elevated leukocyte numbers in circulation is a common predictor of infection severity. It is proposed that vascular aggregation of leukocytes during infection plays a role in contributing to disease progression (Pierce et al., 2000; Williams et al., 1998). Individuals with leukocyte adhesion deficiency II (LAD II) syndrome exhibit neutrophilia, defective neutrophil and monocyte migration and increased incidence of recurrent bacterial infections (Becker and Lowe, 1999). P-selectin has also been shown to modulate the numbers of circulating leukocytes through delayed neutrophil clearance (Johnson et al., 1995). However, leukocytosis is not sufficient to cause spontaneous disease or enhance susceptibility to environmental infection in FtDKO mice, suggesting a mechanism of homeostatic control that has yet to be identified (Homeister et al., 2001). Furthermore, our model shows that the protective advantage conferred by FucT-IV and -VII deficiency against intracellular infection is associated with increased innate immune cell numbers. Despite the essential role of

inflammatory monocytes against LM, our FtDKO model demonstrates that neutrophils are not dispensable in the innate immune response to infection (Shi et al., 2011). We show that neutrophil-specific depletion in FtDKO mice compromises bacterial control in the liver, indicating a compartment-specific role for neutrophils in mediating protection from infection. Efficient control of LM infection in FtDKO mice is not attributed to increased LM-induced serum pro-inflammatory cytokine production. In order to determine the role of functional selectin ligands in modulating cytokine production on a per-cell basis, we performed LM infections on neutrophils and macrophages *in vitro*. WT and FtDKO neutrophils generated similar basal levels of TNF (Appendix Figure 1A). No significant differences in TNF expression levels were also observed between WT and FtDKO neutrophils in response to infection. Furthermore, basal levels of TNF, IL-6 and MCP-1 were similar between WT and FtDKO macrophages (Appendix Figure 1B). During infection, WT and FtDKO macrophages showed a similar increase in TNF and IL-6 expression levels. However, MCP-1 expression levels were reduced in FtDKO macrophages relative to WT controls. Our results suggest that functional selectin ligand deficiency may adversely impact cytokine signaling by macrophages on a per-cell basis *in vitro*. However, it is clear that increased innate immune cell numbers in FtDKO mice results in rapid cellular responses and early control of bacterial growth *in vivo*, eliminating the need for the sequelae of inflammation. It is possible that increased systemic inflammation during LM infection may contribute to disease progression in WT mice. Monocyte priming with high levels of pro-inflammatory cytokines enhances intracellular bacterial growth, indicating a role for inflammation in infection persistence (Kanangat et al., 1999). Taken together, our study highlights the critical role of functional selectin ligand deficiency in modulating immunity beyond leukocyte recruitment, particularly in enhancing hematopoiesis and the early innate immune response to infection.

6.2 T cells play critical regulatory roles in innate and adaptive immunity to intracellular pathogens

T cells provide cell-mediated immune responses as part of the adaptive arm of immunity. Effector T cell functions, such as cytotoxic killing and cytokine production are elicited in a highly-specific manner through TCR recognition of specific antigens presented via MHC. However, T cells also aid innate immune functions in response to infection. IFN-γ production by $CD8^+$ T cells is induced by macrophage-derived IFN-α/β, IL-12, and IL-18 upon LPS stimulation (Kambayashi et al., 2003). T cell depletion leads to a marked decrease in pro-inflammatory cytokine expression and neutrophil recruitment during ST infection, indicating a critical role for T cells in amplifying inflammatory responses (Godinez et al., 2008). LM infection also induces non-antigen-specific IFN-γ production by $CD8^+$ T cells in the presence of IL-12, IL-15 and IL-18 (Yajima et al., 2001; Berg et al., 2002). In FtDKO mice, it is possible that the skewed T cell distribution observed predominantly in the spleen indirectly aids innate immune responses against LM infection, despite defective leukocyte migration. This response is further augmented by increased splenic levels of innate immune cells, including neutrophils, monocytes and DCs. In contrast, functional selectin ligand deficiency impairs T-cell mediated responses in other inflammatory conditions. Our previous study demonstrated the compromised immunity of FtDKO mice to B16-OVA melanoma tumor model, despite robust responses to previous LM-OVA vaccination (Stark et al., 2012). This is associated with reduced OVA-specific $CD8^+$ T cell infiltration into draining LNs and tumors, and not due to impaired $CD8^+$ T cell function in FtDKO mice (Stark et al., 2012). In another study, FucT-VII deficiency also impairs effector T cell migration to inflamed cutaneous sites upon hapten antigen challenge, indicating a critical role for functional selectin ligand interactions in skin

immune surveillance (Smithson et al., 2001). Thus, functional selectin ligand deficiency plays dichotomous roles in immunity to infectious and non-infectious diseases.

Tregs and Th17 cells play critical roles in immunity to intracellular infection. Treg-derived IL-10 has been shown to promote memory $CD8^+$ T cell maturation, in addition to regulating DC maturation and inflammation during acute viral infection (Laidlaw et al., 2015). However, Tregs can contribute to disease progression by promoting pathogen persistence within the host. Delayed early control of *Salmonella* infection is associated with enhanced Treg suppressive potency, while increased bacterial control in the late phase of infection corresponds with a reciprocal decrease in immune-suppression (Johanns et al., 2010). Similarly, Th17 cells play beneficial and pathogenic roles in inflammation. As the major producers of IL-17, Th17 cells contribute to innate immune responses by promoting activation of macrophages and the recruitment of neutrophils and macrophages to inflammation sites (Khader and Gopal, 2010). IL-17 also promotes Th1 responses during infection by regulating DC activation and cytokine expression (Bai et al., 2009). Deficiency in IL-17 or IL-17R expression is associated with decreased bacterial control and mucosal barrier function in response to intracellular infection by ST (Raffatellu et al., 2008). However, Th17 cells induced in the presence of IL-23 are considered pathogenic in various autoimmune conditions, including inflammatory bowel disease, multiple sclerosis and rheumatoid arthritis (Lee et al., 2012b). Similarly, increased serum IL-23 concentrations and hepatic accumulation of Th17 cells are associated with liver damage during chronic hepatitis B virus infection (Zhang et al., 2010). While IL-23 is also produced by macrophages during *Salmonella* infection, it plays a beneficial role by promoting IL-17 expression by memory T cells towards bacterial control (van Beelen et al., 2007; van de Wetering et al., 2009). The mechanisms behind the differential

regulation of pathogenic and protective functions of Th17 cells in autoimmunity and infection remain to be elucidated. However, it is increasingly clear that Th17 cell and Treg balance plays a crucial role in determining disease outcomes. As expected, an imbalance towards pro-inflammatory Th17 cell responses relative to immune-suppressive Treg responses is observed in autoimmune conditions. In contrast, reduced Th17 to Treg ratios are observed in patients with latent *Mycobacterium tuberculosis* (Mtb) infection (Babu et al., 2010). Treg depletion significantly increased antigen-specific Th17 responses, suggesting a role for Treg-mediated downregulation of Th17 cell responses in Mtb infection latency (Babu et al., 2010). Thus, the Th17/Treg balance often modulates pathologies associated with chronic inflammation, and my thesis shows it may also play a subtle role during states of "altered health" such as pregnancy.

6.3 Pregnancy determines maternal and fetal outcomes during infection through modulation of Th17/Treg balance

Pregnancy adds a layer of complexity in delineating the roles of T cells in immunity to infection due to dynamic immunological changes occurring within the maternal host. Early pregnancy is characterized by immune cell recruitment to the implantation site and a transient cytokine shift to an inflammatory profile in preparation for implantation and placentation (Robertson et al., 1997). This is attributed in part to cytokines present in seminal fluid, including regulated upon activation normal T-cell expressed and secreted (RANTES), macrophage inflammatory protein (MIP)-1α and MIP-1β, MCP-1 and TGF-β1 (Robertson et al., 1997). Induction of fetal tolerance is attributed in part to the expression of Th2 cytokines, including IL-3, IL-4, IL-5 and IL-10 at the fetal-maternal interface. In mice, peak levels of Th2 cytokines in decidua and/or placenta are observed on day 12 of pregnancy (Lin et al., 1993). The last phase of pregnancy results in restoration of an inflammatory cytokine profile

in preparation for parturition. Immune cell infiltration into the myometrium and cervix during labor is associated with increased levels of IL-1β, IL-6 and IL-8 (Osman et al., 2003; Young et al., 2002). Thus, the dynamic cytokine changes corresponding with the phases of pregnancy indicates a critical role for timing of infection in maternal and fetal disease progression. We previously showed that infection of mice with ST at mid-pregnancy (days 10-12 of pregnancy) leads to exacerbated systemic infection relative to non-pregnant mice (Pejcic-Karapetrovic et al., 2007). Furthermore, this is associated with robust placental colonization by ST (Pejcic-Karapetrovic et al., 2007). Rowe *et al*. established that increased Treg numbers associated with pregnancy enhances maternal susceptibility to intracellular infection by LM and ST (Rowe et al., 2011). We also show that reciprocal downregulation of Th17 responses with increased Treg numbers reduces bacterial control in maternal systemic and placental tissues during ST infection. Anti-CD25 treatment restored Th17/Treg balance and enhanced the control of infection to levels similar to those of non-pregnant mice. In contrast, reduced resistance to *Toxoplasma gondii* infection during pregnancy and increased fetal loss correlates with increased Th17 cell to Treg ratios (Zhang et al., 2012). It is possible that differences in pathogen induction of Treg proliferation and cell death, and cytokine-mediated differentiation of Tregs into Th17 cells may account for contrasting Th17 cell-to-Treg ratios between ST and *T. gondii* infection. Nevertheless, it is clear that dysregulation of Th17/Treg balance is a crucial factor in determining maternal and fetal outcomes during various infections.

6.4 Type I IFNs regulate maternal and fetal susceptibilities to intracellular bacterial pathogens during pregnancy

The ubiquitous expression of IFNAR highlights the extensive roles of type IFN signaling in homeostasis and immunity to infection. However, identifying the mechanisms

behind the divergent roles of type I IFNs in host immunity to pathogens remains a challenge. Type I IFNs mediate protection from extracellular infection by *Streptococcus* species through enhanced bacterial clearance, maintenance of epithelial barrier integrity and regulation of inflammatory responses (Castiglia et al., 2016; LeMessurier et al., 2013; Maier et al., 2016; Mancuso et al., 2007; Gratz et al., 2011). In contrast, intracellular bacterial pathogens such as *Francisella tularensis*, *Coxiella burnetti*, LM and ST exploit inflammatory and/or cell death mechanisms mediated by type IFNs to promote virulence and survival (Robinson et al., 2012; Auerbuch et al., 2004; Carrero et al., 2004; O'Connell et al., 2004; Henry et al., 2007). Thus, determining key factors involved in pathogen sensing and the various type I IFN induction pathways utilized by pathogens may provide insight into differential impact of IFNAR signaling on host responses to infection.

The regulatory roles of IFNAR signaling extend into the complex immune network of pregnancy. Baseline levels of IFN-β in trophoblasts and murine placentas may contribute to fetal tolerance by regulating T cell function via induction of IL-10 and IDO (Mor et al., 2017; Racicot et al., 2016). During viral infection, IFNAR signaling induced through PAMP recognition reduces the threshold for pro-inflammatory cytokine production in systemic circulation and reproductive tissues, and increases susceptibility to secondary bacterial infection-driven pre-term births in mice (Cappelletti et al., 2017). It is proposed that maternal macrophages contribute to TLR9-induced pre-term births during infection, given their constitutive expression of IFNAR and TLRs (Cappelletti et al., 2017; Huynh et al., 2012; Thaxton et al., 2009). In another study, dysregulation of TLR4-mediated inflammation in human trophoblast cells through viral inhibition of the IFN-β pathway enhances pro-inflammatory cytokine expression in response to LPS (Racicot et al., 2016). Thus, targeting

the type I IFN signaling pathway presents a therapeutic strategy in limiting adverse pregnancy outcomes. Recent studies have focused on the roles of type I IFNs in maternal and fetal immunity to infections during pregnancy using the Zika virus (ZIKV) model. ZIKV is a mosquito-borne flavivirus that poses a high risk of congenital and intrauterine transmission from the infected maternal host to the fetus. Deficiency in IFNAR expression has been shown to promote placental and fetal infection by ZIKV, leading to fetal deaths and congenital abnormalities, including microcephaly and intrauterine growth restriction (Coyne and Lazear, 2016). Placental inflammation induced by viral infection rather than viral presence in the fetal tissue is sufficient in altering neurological development of the fetus (Cardenas et al., 2010; Mor, 2016). ZIKV is a potent inducer of pro-inflammatory IL-12 production in the umbilical cord and fetal blood, correlating with placental dysfunction and immunopathology in infected pregnant rhesus macaques (Hirsch et al., 2018). In contrast, less is known about the role of type I IFN signaling in modulating maternal and fetal immunity to intracellular bacterial pathogens. Our results indicate that the protection conferred by IFNAR deficiency against LM and ST in the non-pregnant state is differentially modulated during pregnancy. Enhanced resistance to LM infection in non-pregnant mice is associated with increased serum IL-12 expression compared to WT controls. This protection from infection is retained during pregnancy. In contrast, compromised control of ST infection in pregnant WT and IFNAR$^{-/-}$ mice relative to non-pregnant controls correlates with reduced serum IL-12 expression. IL-12 is an immune-regulatory cytokine that provides a critical link between innate and adaptive immunity. It promotes macrophage activation, IFN production by NK cells and IL-12 production by DCs during the innate immune response (Hamza et al., 2010). IL-12 then shapes adaptive immunity by promoting Th1 cell differentiation (Hamza et al., 2010). Consistent with our study, type I IFN signaling has been shown to negatively regulate IL-12 expression in non-

pregnant hosts during bacterial and viral infections (Auerbuch et al., 2004; Cousens et al., 1997). Our results also suggest that the differential impact of LM and ST infections on maternal innate immunity is attributed to pathogen-dependent modulation of immune-regulatory mechanisms associated with pregnancy. IL-12 administration presents therapeutic potential against intracellular infection in non-pregnant hosts. Treatment of LM-infected mice with recombinant murine IL-12 is associated with enhanced control of systemic infection, while the opposite trend is observed upon administration of anti-IL-12 (Wagner et al., 1994). Combinatorial IL-12/antibiotic treatment has also been shown to significantly improve disease outcomes in mice infected with intracellular pathogens *Mycobacterium avium* or *Francisella tularensis* subsp. *novicida,* when compared to treatment with IL-12 or antibiotic alone (Doherty and Sher, 1998; Pammit et al., 2004). Validation of IL-12 therapies against infection is critical in pregnant hosts, given the complex regulation of inflammatory and immune-suppressive mechanisms involved in the maintenance of pregnancy.

6.0 CONCLUSION

This study provides a mechanistic insight into the roles of different components of the immune system (functional selectin ligand interactions, Th17/Treg balance, and IFNAR signaling) in modulating innate immunity to two bacterial pathogens with differing intracellular lifestyles. Functional selectin ligand deficiency is characterized by defective leukocyte migration and lymphocyte homing to inflammation sites, and elevated leukocyte levels in circulation. In Chapter 3, I showed that functional selectin ligand deficiency confers innate immune advantage against systemic LM infection through increased circulation of innate immune cells, including neutrophils, monocytes and DCs. Enhanced bacterial control in FtDKO mice was retained upon adoptive transfer to WT hosts with normal functional selectin ligand expression. These results demonstrated that factors inherent to FtDKO immune cells shape innate immune responses and mediate early protection from infection, despite impaired leukocyte migration kinetics. In Chapter 4, I evaluated the impact of $CD4^+$ T cell responses on the modulation of innate immunity to infection during pregnancy. Successful pregnancy is attributed in part to the maintenance of appropriate ratio of pro- and anti-inflammatory responses by Th17 cells and Tregs, respectively. We showed that enhanced maternal susceptibility to ST infection during pregnancy is associated with reduced splenic Th17 cell-to-Treg ratios. Efficient bacterial control and reduced fetal loss were restored in pregnant mice upon functional inactivation of Tregs *in vivo* prior to infection. Thus, Treg-mediated suppression of Th17 cell responses during pregnancy impairs immunity to ST infection, leading to adverse maternal and fetal outcomes. Lastly, Chapter 5 demonstrated the roles of IFNAR signaling in differential regulation of maternal immunity to infection during pregnancy. Increased protection from LM infection in $IFNAR^{-/-}$ mice during pregnancy is

associated with increased serum IL-12 responses. In contrast, impaired innate immunity to ST infection during pregnancy is associated with reduced serum IL-12 responses, indicating a critical role for host-pathogen interactions in differentially augmenting the role of IFNAR deficiency. Taken together, this study highlights the complex interplay of immune interactions among different cellular immune components, host environment and pathogen challenge (Figure 6.1). The knowledge of specific components of leukocyte recruitment, T cell responses and cytokine signaling could yield future therapeutic targets for augmenting innate immunity against intracellular pathogens.

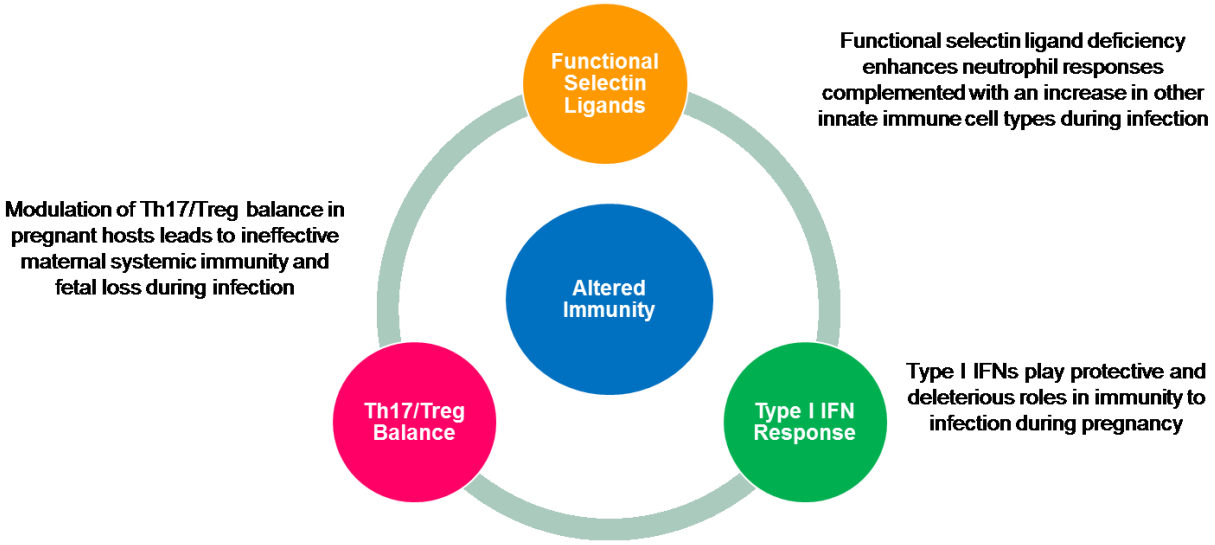
Functional Selectin Ligands
Functional selectin ligand deficiency enhances neutrophil responses complemented with an increase in other innate immune cell types during infection
Modulation of Th17/Treg balance in pregnant hosts leads to ineffective maternal systemic immunity and fetal loss during infection
Altered Immunity
Th17/Treg Balance
Type I IFN Response
Type I IFNs play protective and deleterious roles in immunity to infection during pregnancy

Figure 6.1. Interactions among cellular immune components, host environment and pathogen challenge modulate host resistance to intracellular infections.

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8.0 CONTRIBUTIONS OF COLLABORATORS

Collaborator	Affiliation	Contribution
Dr. Subash Sad	University of Ottawa	• Co-Supervisor • Thesis advisory committee member • Provided significant input on experiments (Chapter 3) • Edited manuscripts for publications (Chapters 3 and 5) • Provided IFNAR$^{-/-}$ mice (Chapter 5)
Dr. Seung-Hwan Lee	University of Ottawa	• Thesis advisory committee member
Dr. Ashok Kumar	University of Ottawa	• Thesis advisory committee member
Dr. Lionel Filion	University of Ottawa	• Thesis advisory committee member
Dr. Julie Joseph	University of Ottawa	• Assisted in setting up qRT-PCR (Chapter 4)
Dr. Shawn P. Murphy	University of Rochester	• Provided input on experiments (Chapters 4 and 5) • Edited manuscripts for publications (Chapters 4 and 5)
Komal Gurnani	National Research Council Canada	• Maintained ST stocks • Performed i.v. injections, mouse irradiations and GMP CFU scoring (Chapters 3, 4 and 5) • Performed bacterial enumeration and flow cytometry experiments (Chapters 3 and 4)
Kristina Wachholz	National Research Council Canada	• Maintained ST stocks • Performed bacterial enumeration and flow cytometry experiments (Chapters 3, 4 and 5)
Ahmed Zafer	National Research Council Canada	• Performed cell sorting and i.p. injections (Chapters 3 and 4)
Anindita Chattopadhyay	National Research Council Canada	• Performed qRT-PCR experiments (Chapter 4)
Renu Dudani	National Research Council Canada	• Maintained LM stocks • Performed i.v. injections (Chapters 4 and 5)
Susanne MacLean	National Research Council Canada	• Assisted in some bacterial enumeration experiments (Chapter 5)
Dr. Tina Nguyen	National Research Council Canada	• Assisted in some bacterial enumeration experiments (Chapter 3)
Janet Clark	National Research Council Canada	• Performed i.v. injections (Chapter 5)

9.0 APPENDIX

9.1 Materials and Methods

9.1.1 Neutrophil Sorting

BM cells were resuspended in 1x PBS containing 2% FBS and 1 mM EDTA (cell sorting buffer) and incubated with anti-CD16/32 (FcγRIII/II) antibody for 10 min at 4ºC in the dark. Neutrophils were stained with anti-Ly-6G (1A8) as described previously. Purity of sorted fraction was 97-99%.

9.1.2 Generation of Bone Marrow-Derived Macrophages

Tibias and femurs were processed to obtain a single-cell suspension of BM cells, as described previously. Cells were resuspended in R8 media containing 5 ng/mL M-CSF (R&D Systems Inc.; Cedarlane, Burlington, ON, Canada) and 50 μg/mL gentamicin (Gibco; Thermo Fisher Scientific, Burlington, ON, Canada) and plated onto 100 mm x 15mm non-tissue culture-treated polystyrene petri-dishes. Media was replaced at day 2 (with gentamicin) and day 4 (without gentamicin) of culture. At day 5, media was removed and adherent cells were washed once with 10 mL 1x PBS. Afterwards, adherent cells were serum-starved by adding 10 mL 1x PBS prior to incubation at 37ºC for 5 minutes. Bone marrow-derived macrophages (BMDMs) were then lifted off using a Falcon™ cell scraper and plated onto 24-well plates for subsequent assays. Macrophage purity of ≥90% was determined through flow cytometric analysis of F4/80 expression.

9.1.3 *In Vitro* Cytokine Measurements

BMDMs or neutrophils were seeded in 24-well plates at $3x10^5$ cells per well in R8 medium. Cells were centrifuged at 500 g for 5 min at room temperature and resuspended in

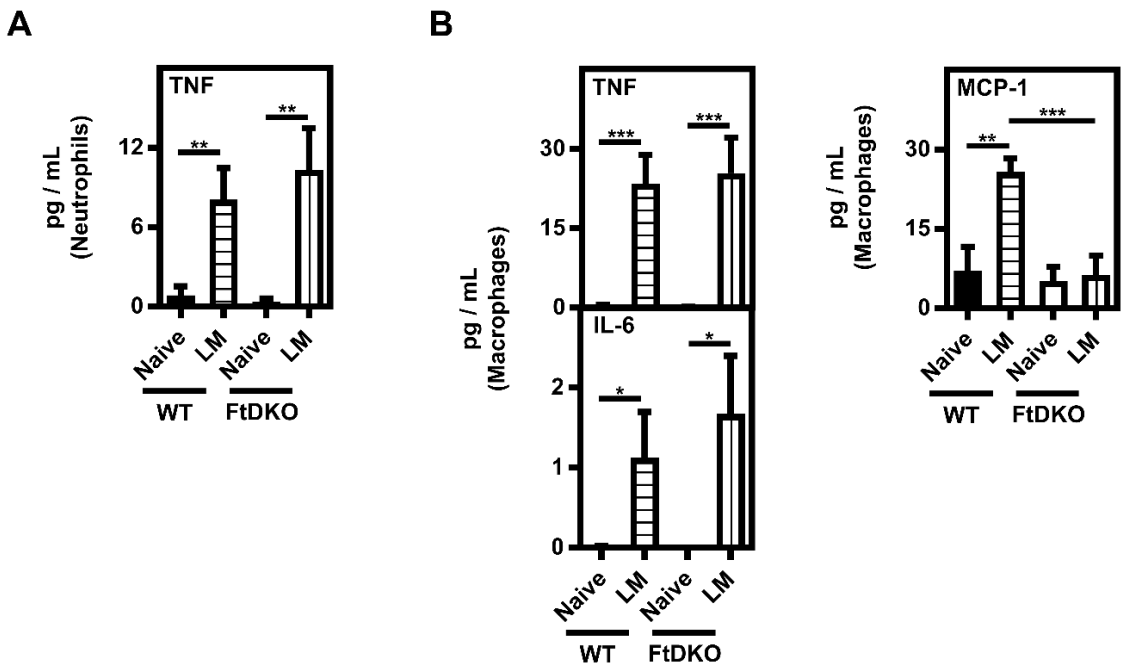
300 μL R8 medium containing LM $3x10^4$ CFUs to obtain a 0.1 bacteria-to-cell ratio or multiplicity of infection (MOI). Bacteria and cells were centrifuged prior to incubation at 37°C at 5% CO_2 to maximize bacterial contact with cells. After 30 min, cells were washed three times with 2 mL R8 medium containing 50 μg/mL gentamicin. Cells were then incubated for 2 h in 300 μL R8 medium containing 50 μg/mL (high) gentamicin to remove extracellular bacteria. After 2 h, high gentamicin medium was replaced with 300 μL R8 medium containing 10 μg/mL (low) gentamicin. At 24 h post-infection, supernatants were collected by centrifuging cells at 500 g for 5 min at 4°C. Cytokine measurements were performed through CBA.

9.2 Results

9.2.1 Functional selectin ligand deficiency impacts inflammatory cytokine production by macrophages in response to LM infection

We first examined the role of functional selectin ligands in cytokine expression by neutrophils in response to LM infection (Appendix Figure 1A). Neutrophils were infected at 0.1:1 MOI. TNF levels significantly increased from the non-infected to infected state in both WT and FtDKO neutrophils at 20-24 h post-infection. However, no significant differences were observed in neutrophil TNF and IL-10 levels between the two mouse strains. These results suggest that WT and FtDKO mice have similar neutrophil cytokine functionality on a per-cell basis.

Next, we assessed whether functional selectin ligand deficiency modulates macrophage response to infection using bone marrow (BM)-derived macrophages (Appendix Figure 1B). BM cells were harvested and cultured in RPMI-1640 with 8% fetal bovine serum and 5 ng/mL M-CSF for 5 days. Macrophages were infected at 0.1:1 MOI at day 6 of culture. Supernatants were collected for cytokine measurements through CBA at 20-24 h post-infection. WT macrophages showed a significant increase in MCP-1 upon infection, while no changes were observed in FtDKO macrophages. Furthermore, MCP-1 expression levels of FtDKO macrophages were significantly lower relative to WT controls in the infected state. These results suggest a role for functional selectin ligand expression in modulating MCP-1 expression by macrophages.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Appendix Figure 1. FtDKO macrophages express reduced levels of MCP-1 compared to in response to LM. Neutrophils and macrophages were infected *in vitro* with LM at 0.1:1 cell-bacteria ratio. Inflammatory cytokine expression levels in supernatants were measured through CBA at 20-24 hours post-infection. (A) TNF and IL-10 production by neutrophils. (B) TNF, IL-10, IL-12p70, IL-6 and MCP-1 production by macrophages. Results are pooled from 3 experiments each having 3 replicates. Cytokine expression levels were analyzed by Mann Whitney U test. *: $p \leq 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ****: $p < 0.0001$.

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GERARD PATRICK AGBAYANI

HIGHLIGHTS

- Ph.D. candidate in Microbiology and Immunology at the University of Ottawa
- Over six years of research experience in host-pathogen interactions using intracellular bacterial infection and *in vivo* animal models
- Significant experience in designing and optimizing multi-colour flow cytometry panels for immune cell phenotyping and functional characterization

EDUCATION

2012 – Present University of Ottawa, Ottawa, Ontario, Canada
Doctorate in Microbiology and Immunology
Relevant Coursework: Immunology, Host/Pathogen Interactions and Molecular Immunology, Immunochemistry, Advanced Topics in Immunology, Advanced Topics in Cell Death

2005 – 2009 Ateneo de Manila University, Manila, Philippines
Bachelor of Science in Biology (Honours Program)
Cumulative GPA: 3.37/4.00 (Honourable Mention Award)
Relevant Coursework: Biochemistry, Microbiology, Cellular and Molecular Biology

RESEARCH EXPERIENCE

2012 – Present **Ph.D. Student**, Dr. Lakshmi Krishnan's Group, Centre of Human Health Therapeutics, Department of Immunobiology, National Research Council Canada, Ottawa, Ontario

- Evaluated the role of functional selectin ligands in host immunity to intracellular bacterial infection by *Listeria monocytogenes* (LM) using *in vivo* murine models and *in vitro* techniques
- Examined the contributions of T cell responses and type I interferons in modulating innate immunity to infection by LM and/or *Salmonella enterica* serovar Typhimurium (ST) during murine pregnancy

PROFESSIONAL EXPERIENCE

2014 – Present **Technical Officer (Part-Time)**, Dr. Lakshmi Krishnan's Group, Centre of Human Health Therapeutics, Department of Immunobiology, National Research Council Canada, Ottawa, Ontario

- Design multi-colour flow cytometry panels for internal projects and client work
- Provide technical assistance to multiple collaborators within the program
- Assist in general laboratory maintenance

2009 – 2011 **Laboratory Technician**, NutriChem Compounding Pharmacy and Clinic, Ottawa, Ontario

- Prepared customized medicines and supplements, including creams, capsules, and injectables in accordance with Good Manufacturing Practice (GMP) standards

SKILLS AND TECHNIQUES

- Designing and optimizing 15-colour flow cytometry panels using the BD LSRFortessa™
- Conducting and troubleshooting flow cytometry-based bioassays, including immune cell phenotyping and cytokine analysis through cytometric bead array
- Analyzing flow cytometry data using FlowJo and FCAP Array™ software
- Performing microbiological techniques, including bacterial colony-forming unit assay, and growth of LM and ST cultures
- Complying with operational procedures outlined by Biosafety Level 2 safety standards
- Broad experience in mouse breeding and manipulation, including euthanasia, blood and tissue collection, clinical monitoring and genotyping
- Maintaining mouse cell cultures using sterile techniques and performing *in vitro* immunological assays, such as Annexin V apoptosis assay and reactive oxygen species measurement
- Operating and maintaining basic laboratory equipment
- Analyzing data and presenting research progress in lab meetings and conferences
- Proficient with GraphPad Prism, and Microsoft Office software, including Word, Excel and PowerPoint

PEER-REVIEWED PUBLICATIONS

Agbayani G., Gurnani K., Zafer A., Sad S., Krishnan L. 2018. Lack of functional selectin-ligand interactions enhances innate immune resistance to systemic *Listeria monocytogenes* infection. J. Leukoc. Biol. 103:355-368.

Agbayani G., Wachholz K., Chattopadhyay A., Gurnani K., Murphy S.P., Krishnan L. 2017. Modulation of Th17 and regulatory T-cell responses during murine pregnancy contributes to increased maternal susceptibility to *Salmonella* Typhimurium infection. Am. J. Reprod. Immunol. 78:e12742.

Joseph J., Ametepe E.S., Haribabu N., **Agbayani G.**, Krishnan L., Blais A., Sad S. 2016. Inhibition of ROS and upregulation of inflammatory cytokines by FoxO3a promotes survival against *Salmonella* Typhimurium. Nat. Commun. 7:12748.

MANUSCRIPT IN PREPARATION

Agbayani G., Wachholz K., Krishnan L. 2018. Type I interferon responses differentially modulate maternal host immunity to infection by *Listeria monocytogenes* and *Salmonella enterica* serovar Typhimurium during pregnancy.

CONFERENCE ABSTRACTS

Agbayani G., Wachholz K., Chattopadhyay A., Gurnani K., Murphy S.P., Krishnan L. 2017. Modulation of Th17 and Regulatory T cell responses during murine pregnancy contributes to increased maternal susceptibility to *Salmonella* Typhimurium infection. Placenta. 57:227.

Agbayani G., Sad S., Krishnan L. 2016. α1,3-Fucosyltransferase-IV and -VII deficiency enhances innate immunity against systemic *Listeria monocytogenes* infection. 29th CSI Annual Meeting, Ottawa, ON Canada.

Agbayani G., Wachholz K., Chattopadhyay A., Gurnani K., Krishnan L. 2015. Regulation of T regulatory cell and Th17 responses in pregnant mice infected with *Salmonella* Typhimurium. Am. J. Reprod. Immunol. 73:S2.

Krishnan L., Nguyen T., **Agbayani G.**, Wachholz K. 2015. The battle of wits: the placental barrier and virulent *Salmonella enterica* Typhimurim infection. Am. J. Reprod. Immunol. 73:S2.

Agbayani G., Sad S., Krishnan L. 2014. Lack of functional selectin ligand interactions enhances inherent neutrophil function leading to increased resistance to systemic *Listeria monocytogenes* infection. J. Immunol. 192:186.9.

Wachholz K., **Agbayani G.**, Nguyen T., Gurnani K., Krishnan L. 2014. Placental infection by *Salmonella enterica* Typhimurium in a murine model: mechanisms of pathogenesis and role of inflammatory cell death. Placenta. 35:A64.

UNDERGRADUATE THESIS

2008 – 2009 **"Identification and Molecular Characterization of a Plant Defensin Gene from *Capsicum frutescens* L. (Chili Pepper)"** (Supervisor: Maria Katrina Constantino; Thesis Partner: Jan Abigail Elizabeth Garcia)

- Submitted a putative defensin gene sequence (Accession Number FJ649598) that was identified and reconstructed from the isolated genomic DNA of *C. frutescens* L. (Chili Pepper), as contribution to the global gene database of the National Center for Biotechnology Information (NCBI), 8600 Rockville Pike, Bethesda, MD, USA

SCHOLARSHIPS

2016
2013 – 2014 **Ontario Graduate Scholarship** (CDN $15,000 for one year), University of Ottawa and Government of Ontario

2013 – 2017 **Admission Scholarship – Ph.D. Program** (CDN $9,000 per year for four years), University of Ottawa

2013 **Dean's Scholarship** (CDN $1,500), Ph.D. Program, University of Ottawa

2012 – 2013 **Admission Scholarship – M.Sc. Program with Thesis** (CDN $7,500 per year for two years), University of Ottawa

HONOURS AND AWARDS

2016 **Cedarlane Laboratories Poster Award**, 29th Annual Canadian Society for Immunology Conference, Ottawa, ON, Canada

2016 **Second Place**, Research Poster Competition, Ph.D. Microbiology and Immunology Category, Department of Biochemistry, Microbiology and Immunology, University of Ottawa

2015 **Third Place**, Research Seminar Competition, Ph.D. Microbiology and Immunology Category, Department of Biochemistry, Microbiology and Immunology, University of Ottawa

2014 **Trainee Abstract Award – First-Year Members**, Immunology 2014 Conference, The American Association of Immunologists, Bethesda, MD, USA

2014 **Best Flow Cytometry Figures**, Flow Cytometry Symposia Day Poster Competition, Department of Biochemistry, Microbiology and Immunology, University of Ottawa

2013 **First Place**, Research Seminar Competition, M.Sc. Microbiology and Immunology, Department of Biochemistry, Microbiology and Immunology, University of Ottawa

2012 **Second Place**, Research Poster Competition, M.Sc. Microbiology and Immunology, Department of Biochemistry, Microbiology and Immunology, University of Ottawa

2008 **Departmental Research Award**, Department of Biology, Ateneo de Manila University, Philippines

2008 **Finalist**, Undergraduate Thesis Poster Competition, 35th Philippine Society for Biotechnology and Molecular Biology Annual Convention

2008 **Finalist**, School of Science and Engineering Awards for Outstanding Student Research, Science and Mathematics Category, Ateneo de Manila University, Philippines

ORGANIZATION MEMBERSHIPS

2016 – 2017 Canadian Society for Immunology, Canada
2013 – 2014

2015 – 2016 American Society for Reproductive Immunology, USA

2013 – 2014 The American Association of Immunologists, USA

2008 – 2009 Philippine Society for Biotechnology and Molecular Biology, Philippines

2005 – 2009 Pre-Medical Society of the Ateneo, Ateneo de Manila University, Philippines

2005 – 2009 Ateneo Biological Organization, Ateneo de Manila University, Philippines

VOLUNTEER EXPERIENCE

2012 **Judge**, 51st Ottawa Regional Science Fair, Ottawa, Ontario

- Evaluated science projects of grades 7-12 students from public and private schools in the Ottawa-Carleton region