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On the role of immature myeloid cells in the host response to *Salmonella* infection

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Abstract of the Dissertation

On the role of immature myeloid cells in the host response to *Salmonella* infection

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Immature myeloid cells in the bone marrow are a heterogeneous population of cells that, under normal conditions, provide tissues with protective cell types such as granulocytes, dendritic cells, and macrophages. Under certain pathological conditions, myeloid cell homeostasis is altered and immature forms of these cells appear in significant numbers in tissues. Murine immature myeloid cells that express CD11b and Ly6C or Ly6G have been associated with immunosuppression in cancer and, more recently, infection. Using a murine model of persistent salmonellosis, we found that $CD11b^{+}Ly6C^{hi}Ly6G^{-}$ mononuclear and $CD11b^{+}$ $Ly6C^{int}$ $Ly6G^{+}$ polymorphonuclear cells accumulate and persist in tissues of mice infected with the bacterial pathogen *Salmonella*. The $CD11b^{+}Ly6C^{hi}Ly6G^{-}$ cells could differentiate into macrophage-like cells *ex vivo* and present antigen to T cells *in vitro*. However, significant proliferation of the T cells was observed only when the ability of the $CD11b^{+}Ly6C^{hi}Ly6G^{-}$ cells to produce nitric oxide was blocked. Thus, $CD11b^{+}Ly6C^{hi}Ly6G^{-}$ cells recruited in response to persistent salmonellosis exhibit protective and immunosuppressive properties, suggesting that these cells may have a complex role in the host response to infection. Emigration of $Ly6C^{hi}$ monocytes from the bone marrow is dependent on CC-chemokine receptor 2 (CCR2) and largely mediated by CC-chemokine ligand 2 (CCL2, also referred to as MCP1). Here, we found that *Ccr2* is required and *Ccl2* is important for the recruitment of $CD11b^{+}$ $Ly6C^{hi}$ $Ly6G^{-}$ monocytic cells into tissues during persistent salmonellosis. In addition, we found that *Ccr2*- and *Ccl2*-deficient mice are more susceptible to persistent *Salmonella* infection than wild-type mice, with

Ccr2-deficient mice being more susceptible than *Ccl2*-deficient mice. Depletion of $CCR2^+$ cells during the first or third week of *Salmonella* infection increased susceptibility to persistent salmonellosis, indicating that $CCR2^+$ cells, including $CD11b^+$ $Ly6C^{hi}$ $Ly6G^-$ monocytic cells, play an essential role in early and late control of *Salmonella* infection. We propose a model in which $CD11b^+$ $Ly6C^{hi}$ $Ly6G^-$ monocytes provide protective functions in the host response to infection, where accumulation and persistence of these cells beyond a certain threshold level may cause collateral effects that prolong or exacerbate disease.

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List of Abbreviations

PMN	Polymorphonuclear
MN	Mononuclear
MHC	Major Histocompatibility Complex
DC	Dendritic Cell
PRR	Pattern Recognition Receptor
PAMP	Pathogen-associated Molecular Pattern
LPS	Lipopolysaccharide
DAMP	Damage-associated Molecular Pattern
TLR	Toll-like Receptor
CLR	C-type Lectin Receptor
RIG	Retinoic Acid-inducible Gene
RLR	RIG-I-like Receptor
NLR	Nucleotide-binding Domain, Leucine-rich Repeat Protein
CD	Cluster of Differentiation
BCR	B Cell Receptor
Ig	Immunoglobulin
V	Variable
D	Diversity
J	Joining
RAG	Recombination Activating Gene
CSR	Class Switch Recombination
SHM	Somatic Hypermutation
AID	Activation-induced Cytidine Deaminase
TCR	T Cell Receptor
Treg	T Regulatory Cell
IMC	Immature Myeloid Cell
IM	Immature Monocyte
TipDC	TNF-α and Nitric Oxide Synthase-producing DC
MDSC	Myeloid-derived Suppressor Cell
CXCR	CXC Chemokine Receptor

CXCL	CXC Chemokine Ligand
CCR	CC Chemokine Receptor
CCL	CC Chemokine Ligand
NO	Nitric Oxide
ROS	Reactive Oxygen Species
IL	Interleukin
NK	Natural Killer
STING	Stimulator of Interferon Genes
S. Typhimurium	*Salmonella enterica* subspecies *enterica* serovar Typhimurium
T3SS	type III secretion system
SPI	*Salmonella* Pathogenicity Island
PPAR	Peroxisome Proliferator-activated Receptor
LB	Lysogeny Broth
Tg	Transgenic
OVA	Ovalbumin
CFU	Colony Forming Units
FACS	Fluorescence Activated Cell Sorting
M-CSF	Macrophage Colony Stimulating Factor
iNOS	Inducible Nitric Oxide Synthase
ANOVA	Analysis of Variance
WT	Wildtype
BM	Bone Marrow
DTR	Diphtheria Toxin Receptor
CFP	Cyan Fluorescent Protein
PDA	Pancreatic Ductal Adenocarcinoma
IHC	Immunohistochemistry
BMDM	Bone Marrow-Derived Macrophages
NRAMP	Natural Resistance Associated Macrophage Protein
H&E	Hematoxylin and eosin
GFP	Green Fluorescent Protein
SEM	Standard Error of the Mean

TNF	Tumor Necrosis Factor
IFNγ	Interferon Gamma

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Chapter 1. Introduction.

1.1 The Immune System

The mammalian immune system is a complex network of cells that protect against environmental insults. These cells constantly monitor their surroundings, assess factors that may pose a threat, and communicate with other cells to coordinate a detection and defense grid. These processes are mediated by a myriad of biological switches and signaling molecules that form a measured response to a wide range of stimuli. The immune system can be divided into two parts - the innate and adaptive immune system. In the context of infection, the innate immune system detects features common to many microbes and rapidly and non-specifically defends against invading microbes. On the other hand, the adaptive immune system recognizes and adapts over time to specific microbes, and provides long-term protection against re-infection in the form of immunological memory. In concert, innate immunity serves to initially control invading microbes and later, along with adaptive immunity, eradicate it from the host. Immune system dysfunction can lead to defects in controlling invading microbes, overactive responses that can damage the host, reactions against normal host cells, and cancer. Because the vast majority of diseases have an immune system component, a great deal of attention has focused on the fundamental aspects of how immunity works and its implications as a therapeutic target.

1.1.1 The innate immune system

The cells of the innate immune system can be broadly divided into two categories: granulocytes or polymorphonuclear (PMN) cells, and mononuclear (MN) cells. PMN cells include mast cells, basophils, eosinophils, and neutrophils, and MN cells include macrophages, dendritic cells (DC), and monocytes. These cells arise from pluripotent hematopoietic stem cells in the bone marrow. Upon exposure to a variety of complex stimuli within the bone marrow and in the periphery, these stem cells differentiate, exit the bone marrow, and enter various tissues where they can develop into the mature cells of the innate immune system.[1]

1.1.1.1 Polymorphonuclear innate immune cells

Granulocytes or PMN cells of the innate immune system are granular cells that have a multi-lobed nuclear morphology, and a diverse role in innate immunity. Mast cells, and basophils

contain many preformed compounds that can quickly and dramatically affect the extracellular environment by inducing inflammation and recruiting other innate immune cells. In addition, mast cells are very effective phagocytes. Both mast cells and basophils play an important role in a wide array of pathological conditions including infection, having both beneficial and detrimental functions.[2,3] Eosinophils also contain many preformed compounds that are important in signaling to other immune cells. These cells are thought to play an important role in lung-associated diseases including defense against bacterial and viral infections. However, eosinophils are also thought to cause collateral damage, and contribute to the pathology of the infection.[4] Of the four, neutrophils are the most abundant granulocyte in the host and the most extensively studied. These cells are very effective phagocytes, and contain many preformed compounds that are proinflammatory and antimicrobial. Although it is well established that neutrophils play a major role in protecting the host against infection, recent work has suggested that their role is more complex in that they can also contribute to the pathology of various diseases.[5]

1.1.1.2 Mononuclear innate immune cells

MN cells of the innate immune system are very effective phagocytic cells that have a diverse role in innate immunity, and a critical role in engaging the adaptive immune system. Macrophages play important roles in development, homeostasis, and inflammation by secreting cytokines and presenting antigen to T cells. In the context of infection, these cells are proinflammatory, can activate immune responses, and are able to phagocytose and kill invading microbes. Macrophages can also have anti-inflammatory functions, regulate wound healing, and suppress or modulate adaptive immune responses. These opposing functions are dictated by the microenvironment of the macrophage.[6] DCs are highly phagocytic cells that are exceptionally efficient at capturing and processing both cytosolic and non-cytosolic antigens, and presenting them to T cells via major histocompatibility complex (MHC) class I and II, respectively. In the context of infection, DCs are able to phagocytose and kill invading microbes, and process and present the resulting antigens. In addition, DCs can produce a wide range of cytokines to regulate T cell responses.[7] Monocytes are precursor cells that primarily function to replenish tissue macrophages and DCs. During infection, these cells rapidly sense and migrate to sites of infection where they can differentiate into macrophages and DCs, produce cytokines, and

produce antimicrobial molecules.[8] Although macrophages, dendritic cells, and monocytes have protective roles, these cells can also contribute to the pathology of disease.[6]

1.1.1.3 Detection mechanisms of innate immune cells

The ultimate goal of innate immune cells is to detect, and attempt to eliminate environmental insults, such as invading microbes. In order to function properly and protect the host against microbes, these cells have innate pattern recognition receptors (PRR) that recognize pathogen-associated molecular patterns (PAMP). PAMPs are molecules commonly associated with microbes, such as lipopolysaccharide (LPS), which is found in gram-negative bacteria. Recent evidence has also suggested that PRRs can recognize damage-associated molecular patterns (DAMP), which are host molecules that are released from damaged cells. The major families of PRRs are the Toll-like receptors (TLR) and C-type lectin receptors (CLR) that are transmembrane proteins, and the Retinoic acid-inducible gene (RIG)-I-like receptors (RLR) and the nucleotide-binding domain, leucine-rich repeat proteins (NLR) that are cytoplasmic proteins. Activation of these receptors typically leads to activation of proinflammatory responses that are antimicrobial. TLRs are known to recognize extracellular or endosomal proteins, lipid-sugars, and nucleic acids derived from bacteria, viruses, parasites, protozoa, and the host. CLRs are known to recognize sugars and proteins derived from fungi and the host. RLRs are known to recognize cytosolic nucleic acids derived from viruses. NLRs are known to recognize cytoplasmic protein-sugars derived from bacteria.[9] Although cells of the innate immune system are capable of detecting and rapidly controlling microbial growth, eradication of many microbial pathogens require the adaptive immune system.

1.1.2 The adaptive immune system

The adaptive immune system is comprised of lymphocytes that can be broadly divided into B and T cells. These cells arise from pluripotent hematopoietic stem cells in the bone marrow. Upon exposure to a variety of complex stimuli within the bone marrow, these stem cells differentiate into committed cells and B cells continue to mature in the bone marrow while T cells mature in the thymus.[1] An important aspect of these cells is their ability to develop and retain recognition of specific molecules of invading pathogens that allows for life long protection against subsequent infection with the same or similar microbe.[10,11]

1.1.2.1 B cells

A hallmark of B cells is the expression of the B cell receptor (BCR) on its surface. The primary function of B cells is to produce and secrete antibodies, which are the soluble binding form of the BCR, also known as immunoglobulins (Ig). During its maturation in the bone marrow, there is an error prone combinatorial rearrangement of the multiple variable (V), diversity (D), and joining (J) genes that ultimately form the BCR. This rearrangement is mediated by the recombination activating genes 1/2 (RAG-1/2) and stops once a functional BCR is assembled. This process results in each B cell expressing a different BCR that can recognize a vast and diverse array of molecules. Positive and negative selection ensures that mature B cells can respond to foreign but not self-antigens. In the periphery, upon recognition of an antigen, B cells activate and differentiate. This triggers class switch recombination (CSR) and somatic hypermutation (SHM) that is mediated by the activation-induced cytidine deaminase (AID), and followed by affinity maturation to increase the affinity for the antigen. These processes create an enormous repertoire of antibodies that can rapidly be improved to recognize a single antigen with high affinity from an invading microbe. The innate immune system recognizes these antigen-antibody complexes and facilitates clearance of the foreign antigen. After clearance of the foreign antigen, these antigen specific B cells are retained in the host as memory B cells where subsequent exposure to this antigen results in a rapid response of these B cells. Thus, the host acquires production against subsequent infection with the same or similar pathogen.[11,12]

B cells are classically defined by their ability to produce antibodies, but these cells can also capture, process, and present antigens, and suppress or modulate the immune response. The ability for B cells to capture, process, and present the antigens recognized by the BCR to T cells is critical for the affinity maturation of the BCR, and the development of memory B cells. This B cell-T cell interaction is critical in activating both B cells and $CD4^+$ T cells.[13] However, B cells can also exert a regulatory function by suppressing pathogenic T cell responses, especially in autoimmune diseases.[14] Interestingly, recent work has shown that pathogens can modulate these regulatory B cells to suppress protective T cell responses.[15]

1.1.2.2 T cells

A hallmark of T cells is the expression of the T cell receptor (TCR) on its surface. T cells can be separated into two main classes based on the expression of the CD4 and CD8 co-receptor. The primary function of $CD8^+$ T cells is to lyse infected or abnormal cells and the primary function of $CD4^+$ T cells is to augment B cell and innate immune cell function. In order to perform its function, the TCR recognizes a molecule in the context of MHC molecules. During maturation in the thymus, the TCR is rearranged and assembled from multiple gene segments. This occurs by an error prone combinatorial rearrangement of the multiple V, D, and J genes mediated by RAG-1/2 and stops once a functional TCR is assembled. This process results in each T cell expressing a different TCR that can recognize a vast and diverse array of molecules. Positive and negative selection ensures that mature T cells can recognize foreign but not self-molecules bound to MHC class I or II. This process generates an enormous repertoire of T cells with distinct TCRs that can recognize molecules from invading microbes, and, along with the innate immune system and B cells, can effectively eradicate the microbe. After eradication, these antigen specific T cells are retained in the host as memory T cells where subsequent infection with the same or similar microbe would rapidly mobilize these cells. Thus, the host acquires production against subsequent infection.[12,16]

$CD8^+$ T cells, also known as cytotoxic T cells, are activated via recognition of molecules in the context of MHC class I from professional antigen presenting cells, such as macrophages and DCs. This leads to proliferation and the development of cytotoxic effectors that lyse cells with this molecule bound to MHC I. In the context of infection, these cells are damaged or contain intracellular pathogens, and thus, unlike most innate immune cells and B cells, are able to eradicate pathogens that reside within cells. In addition to lysing the infected cells, activated $CD8^+$ T cells can also produce cytokines to augment innate immune cell function. Emerging work has also suggested that $CD8^+$ T cells can also exert a regulatory and perhaps pathological function by suppressing immune responses.[17]

$CD4^+$ T cells, also known as T helper cells, are activated via recognition of molecules in the context of MHC class II from professional antigen presenting cells. This leads to proliferation and differentiation into distinct subsets that produce a specific array of cytokines depending on the microenvironment. These cytokines modulate other immune cells to augment their protective

abilities and regulate potentially harmful effects. The $CD4^+$ T cell subsets are involved in autoimmunity, protection against intracellular and extracellular pathogens, immune tolerance, and regulation of immune responses. However, it is well established that the T regulatory cell (Treg) subset can be manipulated by pathogens to suppress protective T cell responses.[18]

1.1.3 Immune response during infection

Optimal clearance of an infection requires both the innate and adaptive immune system. Upon encountering a microbe, host cells recognize general features of microbes via PRRs. This recognition triggers an anti-microbial response within these cells. Circulating innate immune cells that detect these microbes can phagocytose the invading microbe or their foreign antigens and can kill these microbes. Innate immune cells, especially DCs, can then process, display and present the antigens to T cells. T cells that recognize these antigens in the context of the MHC class I or II molecules become activated. At the same time, B cells that recognize foreign antigens of the microbe become activated. Ultimately, antigen specific $CD8^+$ T cells kill infected cells and thus effectively eradicates the microbe from intracellular compartments. The antibody response and augmentation of innate immune cells by antigen specific $CD4^+$ T cells eradicate extracellular microbes. After complete eradication of the microbe from the host, many of the T and B cells that develop against the microbes are eliminated. However, a small number of memory T and B cells persist often for the lifetime of the host; this allows for immunological memory.

1.1.4 Modulation of the immune response during infection

Although the innate and adaptive immune system collaborates to eradicate invading microbes, many pathogens have evolved mechanisms to overcome this formidable defense grid. Pathogens accomplish this by modulating, evading, and subverting the cells and processes of the innate and adaptive immune system, and the intimate cross talk between these two systems.

Since the innate immune cells are typically the first cells to encounter and rapidly respond to a pathogen, many successful microbial invaders have evolved mechanisms to avoid detection by the innate immune system by employing strategies to modify or shield the molecules that are commonly recognized by PRRs. For example, the LPS of *Francisella* and *Yersinia pestis*, both bacterial pathogens, do not strongly activate TLR4 because their LPS have properties that are

uncommon among other gram-negative bacteria and thus not strongly recognized by TLR4.[19,20] However, detection by cells of the innate immune system is important for many pathogens to gain a foothold in the host. These pathogens have mechanisms to limit host anti-microbial processes, such as enzymes that detoxify the damaging radical oxygen species produced by the host. These pathogens have also evolved virulence proteins that are present on the surface of pathogens, are injected into host cells, and mimic host molecules. These proteins function to evade detection by the immune system, modify normal host processes, subvert or kill immune cells, block adaptive immunity, and inhibit or interfere with intercellular communication between immune cells.[21] This allows for the pathogen to enter the host and create an amenable environment to survive, grow, and persist. For example, the type 3 secretion system and its associated virulence effectors in pathogens such as *Salmonella* can facilitate invasion into and promote survival inside host cells.[22,23]

The ability for pathogens to subvert and suppress host immune responses to avoid clearance defines their ability to cause morbidity and mortality. However, equally important is how the host responds to immune modulation by the pathogen. These may include the use of redundant pathways, cryptic danger signals using host derived molecules, and patterns of pathogenesis, which are disease contributing, pathogen induced processes that may be sensed by the host.[24] Studying both the strategies and counter strategies used by the pathogen and the host to shape the immune response will lead to a greater understanding of the factors that dictate the outcome of infection. Furthermore, it would reveal potential therapeutic targets that could be harnessed in the fight against infection.

This dissertation focuses on the interface between the innate immune system and the bacterial pathogen *Salmonella.* Specifically, I focused on immature monocytes that are known to play a key role in initial control of infection but we found that these cells surprisingly also have important roles at later stages of infection. We also found that these cells may have immunosuppressive properties. A greater understanding of the double-edged properties of these cells may lead to novel therapeutics where tipping the balance of these two seemingly opposing roles can affect the outcome of infection.

1.2 Immature monocytes

In the bone marrow, immature monocytes (IM) are a heterogeneous population that, under normal conditions, can provide tissues with protective cell types such as dendritic cells and macrophages. Monocytes were thought to be the progenitor to all macrophages, and dendritic cells. However, recent work has suggested that monocytes are a distinct lineage of phagocytes that give rise to monocyte-derived dendritic cells or monocyte-derived macrophages. These monocyte-derived cells are distinct from tissue resident macrophages, and conventional and plasmacytoid DCs.[7,25] A major subset of monocytes can arise as a consequence of inflammation or infection.

During inflammatory diseases, myeloid cell homeostasis is altered and immature forms of these cells appear in peripheral tissue. During infection, immature monocytes can differentiate into cells that mediate innate protection against pathogens, such as TNF-α and nitric oxide synthase-producing DCs (TipDC).[26] However, under certain pathological conditions, including cancer and infection, these cells exit the bone marrow and enter into the periphery in an immature state where they can accumulate. The effect of this accumulation appears to be highly context dependent. In cancer, immature monocyte accumulation is typically associated with immunosuppression and, therefore, they have been referred to as myeloid-derived suppressor cells (MDSC).[27,28] In infection, IM accumulation is generally associated with inflammation and protection, but can also contribute to pathology.[29]

In humans, IMs are typically defined by the expression of CD11b and CD14. To study the role of IMs *in vivo*, work has focused on mouse models. In mice, studies have identified a subset of IMs by the expression of both CD11b and Gr1. The Gr1 antibody binds to two epitopes - namely Ly6C and Ly6G- that are encoded by different genes. Differential expression of these markers reveals that there are two subsets: PMN cells that are $CD11b^{+}$ $Ly6C^{int}$ $Ly6G^{+}$; and IMs that are $CD11b^{+}$ $Ly6C^{hi}$ $Ly6G^{-}$.[27,28] The recruitment of $CD11b^{+}$ $Ly6C^{int}$ $Ly6G^{+}$ cells are likely driven by the chemokines CXC-chemokine ligand (CXCL) 1, CXCL2, and CXCL5, and its receptor CXC-chemokine receptor (CXCR) 2. On the other hand, CXCR4 and its ligand CXCL12 are likely important for retention in the bone barrow.[5] Emigration of $CD11b^{+}$ $Ly6C^{hi}$ $Ly6G^{-}$cells from the bone marrow is dependent on CC chemokine receptor (CCR) 2 and its ligands CC chemokine ligand (CCL) 2, and CCL7.[29] By manipulating these chemokine ligands

and receptors, recent work has begun to dissect the role of these cells in infections with diverse pathogens.[29,30] Although the majority of the literature has shown a protective role during infection, an emerging body of work has demonstrated that these cells can also contribute to pathology. The role of IMs in infection can be divided into fungal, protozoan/helminth, viral, and bacterial infections.

1.2.1 Role of IMs in fungal infection

The vast majority of fungal infections occurs on the skin or hair and is resolved without or with little intervention. However, invasive fungal infections in humans have a high mortality rate; they are typically the result of opportunistic infections in the immunocompromised. The most common fungal pathogens are *Aspergillus*, *Cryptococcus*, *Candida*, and *Histoplasma*.[31] Innate immunity has become a focal point for fungal infection research and, recently, studies have examined the role of IMs in fungal infections.[32]

In a model of *Aspergillus fumigatus* infection, Hohl *et al*. found that infection induced recruitment of CCR2 and Ly6C expressing immature monocytes to the lungs and its draining lymph node, the mediastinal lymph nodes. Depletion of CCR2 expressing cells revealed that these cells were involved in the dissemination of *Aspergillus* from the lungs to the draining lymph nodes of the lung and were important in fungal clearance in the lungs. Furthermore, depletion resulted in the loss of antigen-specific $CD4^+$ T cell responses in the lung but not in the spleen.[33] In a separate study, Espinosa *et al*. found that CCR2 and Ly6C expressing immature monocytes contributed to augmenting neutrophil mediated killing of *Aspergillus.* Moreover, upon uptake of the fungi, these cells differentiated into monocyte-derived dendritic cells that were fungicidal.[34]

In a model of *Cryptococcus neoformans* infection, infection induced a CCR2-dependent accumulation of Ly6C expressing monocytes to the lung where the cells could differentiate into dendritic cells. This accumulation was found to be important for fungal clearance. Moreover, CCR2-dependent accumulation of Ly6C expressing monocytes was associated with an anti-fungal T cell response.[35,36] In a model of *Histoplasmsa capsulatum*, infection induced a CCR2-dependent accumulation of Ly6C expressing monocytes to the lungs that could abrogate the production of IL-4 by macrophages and dendritic cells. This abrogation was important for host

defense against *Histoplasma*.[37] In a model of *Candida albicans* infection, Ngo *et al.* found that depletion of CCR2 expressing cells during infection led to decreased fungal clearance in the kidneys and brain, and overall to increased mortality.[38]

Collectively, these studies suggest that IMs play a largely protective role in acute pulmonary infections. However, *Candida* infection could also lead to systemic accumulation of Gr-1 and CD80 expressing cells, and these cells were found to be able to suppress $CD4^+$ T cells.[39] However, because of the heterogeneous nature of Gr-1 expressing cells, the identity of the cells responsible for this suppression was not clear. Nonetheless, this suggests that although IMs play a largely protective role during fungal infection at specific sites, IMs may have an immunopathological effect.

1.2.2 Role of IMs in protozoan and helminth infection

The majority of protozoan and helminth infections occurs in the developing world and typically causes subclinical, life-long chronic infection that is non-life threatening in the immunocompetent. Of all protozoan and helminth infections, malaria is the most deadly worldwide. Malaria is typically caused by infections with the protozoan parasites *Plasmodium falciparum*, and *Plasmodium vivax*, which are responsible for the majority of malaria attributed morbidity and mortality. Mortality typically occurs in the young and patients with AIDS.[40] Although rare, protozoal infections with pathogens such as *Toxoplasma* and *Trypanosoma*, can be transmitted from pregnant women to their fetuses where it can have severe effects on the fetus.[41] A hallmark of these pathogens is their exceptional ability to evade and subvert the immune system, and thus, allows these pathogens to establish chronic infection. Recently, studies have focused on the role IMs in protozoan and helminth infection, especially on their role in immune evasion and chronicity.[42,43]

In a model of *Plasmodium* infection, CCR2-dependent recruitment of Ly6C expressing monocytes was found to be important in controlling the pathogen during the systemic blood-stage of malaria infection. These cells were found to produce nitric oxide (NO) and reactive oxygen species (ROS) and, thus, suggested a role for direct killing of the pathogen.[44] However, another study found that in a model of experimental cerebral malaria, CCR2-dependent

recruitment of monocytes did not affect susceptibility of mice to this infection.[45] This highlights the highly context-specific role of these cells during infection.

In models of *Toxoplasma* infection, CCR2-dependent recruitment of Gr1 expressing monocytes was important for control of the pathogen during the acute stage of systemic and gut mucosal infection.[46-49] In the gut mucosal infection, the lack of CCR2 mediated recruitment of cells resulted in mice succumbing to infection from acute intestinal inflammation. The acute inflammation was likely because of the failure to control *Toxoplasma* that caused a large increase in neutrophil recruitment and resulted in extensive tissue damage.[46] In another study, during an acute infection with *Toxoplasma,* Ly6C expressing monocytes were found to be able to inhibit neutrophil activation directly by producing prostaglandin E_2 and thereby limit the tissue damage caused by neutrophils. This resulted in improved survival as compared to mice where CCR2 mediated recruitment or prostaglandin E_2 production was abrogated. Interestingly, commensal bacteria in the gut, and not *Toxoplasma* directly, were responsible for this response of Ly6C expressing monocytes.[50] Thus, these studies suggest that IMs are important in controlling *Toxoplasma* infection but these cells can also limit damage to the gut by modulating the activity of neutrophils. Induction of Ly6C expressing monocytes may be advantageous to *Toxoplasma* since these cells mitigate excessive inflammation and prevent the death of the host. Indeed, recent evidence has indicated that *Toxoplasma gondii* Profilin could promote the recruitment of Ly6C expressing monocytes during infection.[51]

A host protective role of Ly6C expressing monocytes can also be found in helminth infections, such as in Schistosomiasis. In a model of *Schistosoma* infection, the CCR2-dependent recruitment of Ly6C expressing monocytes was found to contribute to containment of the pathogen and hepatic inflammation.[52] In another study, Ly6C expressing monocytes were found to be able to differentiate into alternatively activated macrophages that could play a role in limiting excessive hepatic damage that results from *Schistosoma* infection.[53] As in *Toxoplasma*, these studies suggest that Ly6C expressing monocytes both control *Schistosoma* infection and mitigate excessive inflammation.

Although many studies have shown a protective role for IMs in the host response to infection, IMs can also directly contribute to immunopathology. In a model of *Trypanosoma brucei* infection, migrating inhibitory factor was shown to be a mediator of the recruitment of

these Ly6C expressing monocytes and these cells were shown to contribute to pathogen induced liver damage.[54,55] Furthermore, several studies using infection models with pathogens such as *Toxoplasma*, *Trypanosoma*, and *Schistosoma* have suggested that IMs may function as MDSC-like cells and suppress T cell responses.[42]

1.2.3 Role of IMs in viral infection

Infection with viral pathogens is a major cause of morbidity and mortality worldwide. The innate immune system plays a major role in protection against these pathogens.[56] However, viruses have evolved a variety of subversion mechanisms to disarm, evade, or take advantage of the innate immune system.[21,57,58] Because of the emerging role of IMs in infection, many recent studies have focused on dissecting the role of these cells during viral infection.

In a model of West Nile virus and coronavirus infection, CCR2-dependent accumulation of Ly6C expressing monocytes were found to be important in protection against viral infection.[59,60] Interestingly, in a model of chikungunya virus infection, CCR2-dependent cell accumulation was found to be important in survival, but did not affect viral clearance. Instead, these cells were important for preventing excess damage caused by inflammation.[61] In a model of influenza infection, Aldridge *et al*. found that CCR2-dependent recruitment of monocytes was important in viral clearance by mediating activation of antigen-specific anti-influenza $CD8^+$ T cells. However, reducing, but not eliminating, CCR2-dependent recruitment of these cells by treating with pioglitazone resulted in improved protection against influenza infection.[62] Similarly, in two other studies, abrogation of the CCR2-dependent monocyte response during influenza infection led to enhanced survival and increased influenza specific $CD8^+$ T cell activity.[63,64] These results suggest that the CCR2-dependent recruitment of monocytes can negatively affect T cell responses. Indeed, in a model of murine retrovirus-induced immunodeficiency, IMs were shown to be able to suppress T and B cell responses.[65]

These studies suggest that the initial IM response is likely an innate immune response to viral pathogens in an attempt to contain the infection. However, many viral pathogens have evolved to manipulate and take advantage of the IM response. Some pathogens have evolved mechanisms to blunt the protective IM response. For example, murine gamma herpes virus 68 was found to encode M3 that was shown to be able to act as a chemokine decoy receptor. M3 can bind to

CCL2, one of the ligands of CCR2, and impair CCR2-dependent recruitment of monocytes.[66] Other pathogens have evolved mechanisms to induce the IM response to inhibit antigen-specific T cell responses and, thus, evade clearance. For example, human cytomegalovirus has been shown to be able to induce CCL2 expression during early stages of infection but inhibit its expression during the later stage of infection.[67] The murine cytomegalovirus encodes MCK2, a CCL like molecule that could induce CCR2-dependent recruitment of monocytes. In this study, Daley-Bauer *et al.* showed that MCK2 mediated CCR2-dependent recruitment of monocytes can impair antigen-specific antiviral $CD8^+$ T cell responses via nitric oxide production by monocytes; this diminished the ability for the host to clear the viral pathogen.[68]

Cumulatively, these studies suggest that the role of IMs in viral infection is highly context dependent and is often defined by the host-virus interaction. This is well illustrated in a recent study by Norris *et al*. where infection with two different strains of lymphocytic choriomeningitis virus revealed a difference in the role of IMs during acute and chronic infection. In this study, the Armstrong strain that causes acute infection and was rapidly cleared, and the Clone 13 strain that caused chronic infection was used. The early infection kinetics of both strains was similar and this included an accumulation of IMs. However, after 7 days, the IMs rapidly declined in mice infected with the Armstrong strain, but continued to increase and was sustained in mice infected with the Clone 13 strain. After 7 days of infection, but not before, the IMs of mice infected with the Clone 13 strain were found to be able to suppress T cells. Moreover, the CCR2-dependent accumulation of monocytes in the mice infected with the Clone 13 strain was found to impair antigen-specific antiviral $CD8^+$ T cell responses.[69] It is unclear whether viral elements directly drive this suppression, or if it is the result of the chronic nature of the infection.

1.2.4 Role of IMs in bacterial infection

Bacterial infections are a major cause of morbidity and mortality, especially in developing countries. In developed countries, the advent of antibiotics dramatically reduced the mortality and effectively cured diseases caused by bacterial pathogens. However, the alarmingly sharp increase in multi drug resistant bacterial pathogens has led to an urgent need for other therapeutics against bacterial infections. An important avenue of research focuses on modulating the immune system to augment anti-bacterial processes and limit damage mediated by excessive

inflammation.[70] Recent work has demonstrated that IMs have an important role during bacterial infection.

The role of IMs in bacterial infection has been most extensively studied in a murine model of *Listeria monocytogenes* infection. In a series of works by the Pamer laboratory, Ly6C expressing monocytes were found to require the CCR2/CCL2/CCL7 signaling axis to emigrate from the bone marrow into the periphery. Furthermore, the recruitment of these cells was found to be important in host protection against infection.[29,71-75] In other studies, interleukin (IL)-23 and the stimulator of interferon genes (STING) signaling pathway was found to be important in the recruitment of Ly6C expressing monocytes during *Listeria* infection.[76,77] In a recent study by Shi *et al*., depletion of CCR2 and Ly6C expressing monocytes revealed that these cells were important in controlling *Listeria* infection. Furthermore, it was found that depletion did not have a major effect on the antigen specific $CD8^+$ T cell response but did negatively affect the antigen specific $CD4^+$ T cell response against *Listeria.*[78] In contrast, depletion of Ly6G expressing cells had a minimal effect on the control of *Listeria* infection and T cell responses.[78] These data suggest that one of the functions of CCR2 and Ly6C expressing cells may be to directly present antigen to, and activate $CD4^+$ T cells. Alternatively, these cells may differentiate into cells that could then perform this function. Interestingly, a recent study has suggested that Ly6C and CCR2 expressing monocytes recruited during *Listeria* infection could produce IL-18 and IL-15, which can activate memory $CD8^+$ T cells and Natural Killer (NK) cells. This effect does not appear to depend on antigen presentation, but rather appears to depend on innate immune detection of the pathogen.[79] Furthermore, recent evidence has suggested that memory $CD8^+$ T cells increased the microbicidal activity of IMs during a recall infection with *Listeria*.[80]

A host protective role for IMs in the host response to *Mycobacterium* infection has also been observed. In this study, CCR2-dependent recruitment of cells was found to be important for protection.[81,82] Interestingly, a recent studied showed that a *Mycobacterium* associated glycolipid contributes to the CCR2-dependent recruitment of monocytes that were permissive for the bacterium.[83] In a depletion study, CCR2 expressing cells were shown to be important for controlling *Mycobacterium* in the lung and transporting the bacterium from the lung to the mediastinal lymph node. Furthermore, depletion resulted in a reduction of the total number of antigen specific $CD4^+$ T cells, suggesting that CCR2 expressing cells may be involved in T cell

priming. Interestingly, it was found that the CCR2 and Ly6C expressing cells and their derivatives do not prime T cells directly. Rather conventional DCs are responsible for T cell priming. This suggested that these cells only transport live bacteria and thus antigen to the lymph node. The antigen is then likely transferred to conventional DCs that could directly activate antigen specific T cells.[84] The cells also appeared to play a role in the induction of the humoral response by affecting B cells. In a model of *Streptococcus pneumoniae* infection, efficient production of pathogen derived-polysaccharide specific antibodies was found to require Ly6C expressing monocytes.[85]

1.2.5 Summary on the role of IMs in infection

The role of IMs in the host response to infection is complex and not well understood. The factors that drive IMs to exhibit protective or immunosuppressive properties during infection are not known. However, emerging evidence suggests that the role of IMs in the host response to infection can change over time. Available evidence suggests that the role of IMs during infection may be defined by whether the pathogen causes an acute or chronic infection. Many studies have focused on the role of IMs during acute infections, but the role of IMs during chronic or persistent infections, especially in the context of bacterial pathogens, is poorly defined. In this dissertation we focused on the role of IMs in the pathogenesis of and host response to the bacterial pathogen *Salmonella enterica* serovar Typhimurium.

1.3 *Salmonella*

Salmonella infection represents a major public health problem; it is a major cause of morbidity and mortality worldwide.[86-90] These bacteria are gram-negative, facultative intracellular pathogens. *Salmonella* are capable of causing a variety of diseases ranging from self-limiting gastroenteritis to enteric fever, depending on the serovar of *Salmonella*. Despite the advent of modern drugs that have lowered the overall incidence of infection, *Salmonella* infection still remains an alarming and often neglected problem, especially in the developing world, and with the rise of HIV/AIDS, and other immune compromising diseases.[91] Moreover, as antibiotic resistant bacteria become more prevalent, *Salmonella* is becoming an increasingly difficult pathogen to combat.[22,92-95]

1.3.1 Epidemiology

There are two species within the genus *Salmonella* – namely *Salmonella bongori* and *Salmonella enterica*. *Salmonella bongori* is frequently associated with reptiles and rarely causes disease in humans. On the other hand, *Salmonella enterica* can cause disease in a wide range of mammals, including humans. There are six *Salmonella enterica* subspecies and over 2500 serovars; serovars are based on the type of LPS and flagellar proteins present on the bacterium. *Salmonella enterica* subspecies *enterica* serovar Typhi and Paratyphi are the two most important disease causing *Salmonellae* in humans that cause typhoid fever. These bacteria are a major cause of morbidity and mortality in the developing world, especially in Asia, Latin America, and Africa. It is estimated that typhoid fever results in over 200,000 deaths and over 25 million illnesses per year.[96] Perhaps equally important in places such as sub-Saharan Africa, is the rise of infections with nontyphoidal *Salmonella* serovars that can become invasive and life threatening. This is especially problematic with immunocompromised humans, such as children, patients infected with HIV, and patients infected with malaria. The majority of these infections are caused by *Salmonella enterica* subspecies *enterica* serovar Typhimurium (*S.* Typhimurium).[22] Although vaccines exist for *Salmonella*, they are moderately effective in adults and induce a primarily B cell response, but do not provide lifelong protection and are ineffective in children.[97]

1.3.2 *Salmonella* Pathogenesis

Salmonella infection typically occurs by ingestion of contaminated food or water. The bacteria can then pass through the stomach and into the intestines where it can colonize and penetrate the gut epithelium. This can occur by direct invasion of epithelial cells or via microfold cells, which can transport contents, including bacteria, from the intestinal lumen across the gut epithelium. *Salmonella* can also cross the epithelium via CD18 and CX_3CR1 expressing phagocytes.[98-101] The penetration of the epithelium induces inflammation that produces reactive oxygen species, which can react with the luminal sulphur compound thiosulphate. This reaction forms tetrathionate that *Salmonella*, but not the microbiota, uses to grow and, thus provides a growth advantage in the gut for *Salmonella*.[102] Penetration of the gut epithelium leads to resident myeloid cells in the gut, especially in the Peyer's patches, to phagocytose *Salmonella* where they can be killed or trafficked to systemic sites. In immunocompetent hosts infected with non-typhoidal *Salmonella* serovars, the infection typically results in self-limiting gastroenteritis because *Salmonella* is efficiently controlled and thus does not disseminate beyond the intestine. However, infections with typhoidal serovars can result in typhoid fever. Phagocytes can harbor *Salmonella* and disseminate the bacteria into the mesenteric lymph node.[98] From this lymph node, *Salmonella* can then disseminate into the lymphatic and blood stream where it can colonize the spleen and liver and can persist in the gall bladder and bone marrow. After replication in target organs, *Salmonella* can re-enter the blood stream, cause bacteremia and can lead to specific shock and death. In a significant percentage of hosts, chronic carriage of *Salmonella* can occur. These hosts are typically asymptomatic and can shed bacteria in their feces and urine for their entire lifetime.[103,104]

1.3.3 Immune response to *Salmonella* infection

In order to study the immune response to *Salmonella*, experimental infection of mice with *S.* Typhimurium has been used as a model for the human disease caused by *S.* Typhi.[105-107] In susceptible strains of mice, *S.* Typhimurium is known to induce acute immunosuppression and delay protective immune responses.[105,106,108-110] The immunity that eventually develops requires both T and B cell responses. T cells, especially interferon gamma producing CD4+ T cells, have been shown to be critical in clearance of *Salmonella*. However, *Salmonella* dampens these T cell responses during infection.[105,106,108] In more resistant strains of mice, protective immunity to *S.*

Typhimurium also requires an unusually long time to develop. Despite the development of protective immune responses, *Salmonella* can exist in a latent-like stage in these mouse strains where the protective immune response prevents systemic re-infection.[103,104]

1.3.4 Mechanisms of immune evasion during *Salmonella* infection

Host defense against pathogens requires a concerted effort by both the innate and adaptive immune system. The first line of defense against infection is the cells of the innate immune system such as macrophages, neutrophils, and dendritic cells. This is followed by cells of the adaptive immune system that can ultimately lead to the eradication of the pathogen. However, many bacterial pathogens can subvert the innate and adaptive immune system, but the mechanisms that drive this evasion are not well understood.[108,111,112]

Salmonella have evolved mechanisms – namely two type III secretion systems (T3SS) – to evade the innate immune system.[105,106] The *Salmonella* pathogenicity island (SPI)-1 encoded T3SS enables invasion of epithelial cells and the SPI-2 encoded T3SS supports survival in macrophages.[22] Once inside phagocytes, *Salmonella* can avoid intracellular killing mechanisms and replicate within an altered vacuolar compartment known as the *Salmonella* containing vacuole. The T3SS prevents fusion of this vacuole with the lysosome and thus protects *Salmonella*.[113] In a recent study, Eisele *et al.* found that *Salmonella* is primarily associated with anti-inflammatory like macrophages and preferentially grows in these cells. Interestingly, peroxisome proliferator-activated receptor (PPAR) delta was found to be up regulated in macrophages infected with *Salmonella*. This caused more bioavailable glucose to be available for *Salmonella* to grow. Furthermore, PPAR delta was found to be critical for persistence of *Salmonella* during a chronic model of infection. These results suggest *Salmonella* may be actively modulating cells or the cellular environment to augment expression of PPAR delta and thus promote its long-term survival in the host especially during the chronic phase of infection.[114] *Salmonella* can also induce pro-inflammatory apoptosis in infected cells and thus allow *Salmonella* to escape and infect other cells.[115-117] Since these cells are antigen presenting cells that play an important role in inducing the adaptive immune response, killing them can affect the response of adaptive immune cells, such as T cells, to infection.[117] Furthermore, *Salmonella* can also down modulate antigen processing and presentation.[118-120] These findings

suggest that *Salmonella* can also manipulate the crosstalk between the innate and adaptive immune system.

T cells have been shown to be critical in the adaptive immune response and required for protection against many bacterial pathogens, including *Salmonella*.[105,106,121-125] However, *Salmonella* can affect the adaptive immune response and can lead to a slow and inefficient adaptive response. Studies have shown that, *in vitro, Salmonella* could directly inhibit T cells.[126,127] Although T cells are activated, studies showed that *Salmonella* could limit the *in vivo* proliferation of T cells.[128,129] However, *in vivo*, it is unclear if the affect on T cells is direct or indirect. Previous work has shown that immature $CD11b^{+}Gr1^{+}$ macrophage precursors could inhibit T cells but their role during infection is unclear.[28,130,131] Generally, studies reveal that *Salmonella* infection can lead to a state of immunosuppression in mice and induction of IL-10 and NO production, both of which have immunosuppressive activities.[110,132-134] *Salmonella* infection was also found to be able to induce the recruitment of Ly6C expressing monocytes to the gut associated lymphoid tissues. These cells were found to have an antimicrobial phenotype and could not efficiently induce antigen specific T cells.[135] Regardless, the nature of the immunosuppressive environment induced by *Salmonella* remains poorly characterized, especially in models of chronic *Salmonella* infection.

Together, these data suggest that *Salmonella* have evolved multiple mechanisms to manipulate both the innate and adaptive immune system to promote its growth, survival and persistence in the host. However, the mechanisms that underlie these mechanisms are not well understood. This dissertation focuses on the role of IMs in the pathogenesis of and host response to persistent *S*. Typhimurium infection. We have found that $CD11b^{+}Ly6C^{hi}Ly6G^{-}$ immature myeloid cells recruited in response to *Salmonella* Typhimurium infection exhibit protective and immunosuppressive properties.[136] Furthermore, we show that $CCR2^{+}$ cells, including $CD11b^{+}Ly6C^{hi}Ly6G^{-}$ cells, are essential in the early and late control of *Salmonella* infection. We propose a model in which $CD11b^{+}$ $Ly6C^{hi}$ $Ly6G^{-}$ monocytes provide protective functions in the host response to infection, where accumulation and persistence of these cells beyond a certain threshold level may cause collateral effects that prolong or exacerbate disease.

Chapter 2. $CD11b^{+}Ly6C^{hi}Ly6G^{-}$ immature myeloid cells recruited in response to *Salmonella enterica* serovar Typhimurium infection exhibit protective and immunosuppressive properties.

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2.1 Abstract

Immature myeloid cells in bone marrow are a heterogeneous population of cells that, under normal conditions, provide tissues with protective cell types such as granulocytes and macrophages. Under certain pathological conditions, myeloid cell homeostasis is altered and immature forms of these cells appear in tissues. Murine immature myeloid cells that express CD11b and Ly6C or Ly6G (two isoforms of Gr-1) have been associated with immunosuppression in cancer (in the form of myeloid-derived suppressor cells) and, more recently, infection. Here, we found that $CD11b^{+}$ $Ly6C^{hi}$ $Ly6G^{-}$ and $CD11b^{+}$ $Ly6C^{int}$ $Ly6G^{+}$ cells accumulated and persisted in tissues of mice infected with *Salmonella* Typhimurium (*S.* Typhimurium). Recruitment of $CD11b^{+}$ $Ly6C^{hi}$ $Ly6G^{-}$ but not $CD11b^{+}$ $Ly6C^{int}$ $Ly6G^{+}$ cells from bone marrow into infected tissues depended on chemokine receptor CCR2. The $CD11b^{+}$ $Ly6C^{hi}$ $Ly6G^{-}$ cells exhibited a mononuclear morphology, whereas the $CD11b^{+}$ $Ly6C^{int}$ $Ly6G^{+}$ cells exhibited a polymorphonuclear or band-shaped nuclear morphology. The $CD11b^{+}$ $Ly6C^{hi}$ $Ly6G^{-}$ cells differentiated into macrophage-like cells following *ex vivo* culture and could present antigen to T cells *in vitro*. However, significant proliferation of T cells was observed only when the ability of the $CD11b^{+}$ $Ly6C^{hi}$ $Ly6G^{-}$ cells to produce nitric oxide was blocked. $CD11b^{+}$ $Ly6C^{hi}$ $Ly6G^{-}$ cells recruited in response to *S.* Typhimurium infection could also present antigen to T cells *in vivo*, but increasing their numbers by adoptive transfer did not cause a

corresponding increase in T cell response. Thus, $CD11b^{+}$ $Ly6C^{hi}$ $Ly6G^{-}$ immature myeloid cells recruited in response to *S.* Typhimurium infection exhibit protective and immunosuppressive properties that may influence the outcome of infection.

2.2 Introduction

Salmonellae are a leading and increasing cause of morbidity and mortality in humans worldwide.[22,137] Infections with *Salmonellae* range in severity from self-limiting gastroenteritis to typhoid fever. Non-typhoidal *Salmonellae* such as *Salmonella enterica* serovar Typhimurium (*S*. Typhimurium) are a significant cause of inflammatory enterocolitis and death due to foodborne illness, and an emerging cause of invasive bacteremia in immunocompromised hosts. Typhoidal *Salmonellae* such as *Salmonella enterica* serovar Typhi (*S*. Typhi) cause systemic infections characterized by bacterial penetration of the intestinal barrier and extraintestinal dissemination to the liver and spleen, where the microorganisms survive and replicate in professional phagocytes. Septic shock and death can occur if systemic infections with *Salmonellae* are left untreated[22,137].

Much of what is known about immunity to *Salmonellae* comes from experimental infection of mice with *S*. Typhimurium, which has served as a useful model for the human disease caused by *S*. Typhi.[105,107] In humans and animal hosts, *S.* Typhimurium induce acute immunosuppression and delay onset of protective immune responses [105,107,138,139]. Immunity that eventually develops against *S*. Typhimurium requires humoral and cell-mediated immune responses.[105] T cells, particularly IFN-γ-producing $CD4^+$ T cells, play a critical role in the clearance of *S*. Typhimurium[105], but T cell responses to *S*. Typhimurium are thwarted during infection by mechanisms that, despite recent progress [140-145], are not well understood.

During early stages of infection, macrophages and neutrophilic granulocytes are critical for controlling spread and growth of *S*. Typhimurium.[105] These cells are produced by the differentiation of immature myeloid cells in infected tissues and bone marrow.[8,26,29] Although immature myeloid cells play a critical role in the protective host response to infection [26], several early studies have linked nitric oxide (NO)-producing macrophage precursors to immunosuppression in salmonellosis. [110] Murine immature myeloid cells express surface CD11b and Gr-1 [8], and have been found in spleens of mice infected with *S*. Typhimurium.[130,146] $CD11b^+$ $Gr\text{-}1^+$ immature myeloid cells are a heterogeneous population of cells that can be further divided based on the surface expression of Ly6C and Ly6G, two isoforms of Gr-1 that are differentially expressed on neutrophilic granulocytes, inflammatory monocytes and some populations of dendritic cells. [8,26,147-149]

Here, we found that large numbers of $CD11b^+$ $Ly6C^{hi}$ $Ly6G^-$ and $CD11b^+$ $Ly6C^{int}$ $Ly6G^+$ cells accumulated and persisted in tissues of mice infected with *S*. Typhimurium. We characterized these cells and found that recruitment of $CD11b^+$ $Ly6C^{hi}$ $Ly6G^-$ but not $CD11b^+$ $Ly6C^{int}$ $Ly6G^+$ cells from the bone marrow depended on chemokine receptor 2 (CCR2). Splenic $CD11b^+$ $Ly6C^{hi}$ $Ly6G^-$ cells differentiated into macrophage-like cells following *ex vivo* culture and could present antigen to both $CD4^+$ and $CD8^+$ T cells *in vitro*. However, robust proliferation of the T cells was observed only when the ability of the $CD11b^+$ $Ly6C^{hi}$ $Ly6G^-$ cells to produce NO was blocked. $CD11b^+$ $Ly6C^{hi}$ $Ly6G^-$ cells recruited in response to *S*. Typhimurium infection could also present antigen to T cells *in vivo*, but increasing the number of these cells by adoptive transfer did not cause a corresponding increase in T cell response. Thus, $CD11b^+$ $Ly6C^{hi}$ $Ly6G^-$ immature myeloid cells provide a balance of immunosuppressive and protective functions in the host response to *S*. Typhimurium. The tipping of this balance may be an important factor influencing the outcome of infection.

2.3 Materials and Methods

Bacteria. *S.* Typhimurium strain IR715 [150], which is a spontaneous, nalidixic acid-resistant derivative of *S.* Typhimurium strain 14028 (American Type Culture Collection), was used as the wild-type strain. Bacteria were grown aerobically for 12-18 h at 37ºC in 3 ml of Luria-Bertani (LB) broth (250 rpm) or on LB agar plates using standard microbiological techniques.

Mice. C57BL/6J and 129X1/SvJ mice (both H-2^b haplotype) were purchased from The Jackson Laboratory. These strains of mice have been used as model hosts to study acute and persistent salmonellosis, respectively.[107] F1 (C57BL/6J x 129X1/SvJ) hybrid mice, which can accept cells from either C57BL/6J or 129X1/SvJ mice, were bred at Stony Brook University, Division of Laboratory Animal Resources. B6.129S4-*Ccr2*^{tm1Ifc}/J (*Ccr2*$^{-/-}$), C57BL/6-Tg(TcraTcrb)1100Mjb/J (OT-I), and B6.Cg-Tg(TcraTcrb)425Cbn/J (OT-II) mice (all H-2^b haplotype) were purchased from The Jackson Laboratory and bred at Stony Brook University, Division of Laboratory Animal Resources. *Ccr2*$^{-/-}$ mice lack chemokine receptor CCR2. OT-I mice are TCR transgenic mice in the C57BL/6J strain background that produce $CD8^+$ T cells specific for amino acid residues 257-264 of chicken egg ovalbumin ($OVA_{257-264}$) bound to class I MHC (H-$2K^b$). OT-II mice are TCR transgenic mice in the C57BL/6J strain background that produce $CD4^+$ T cells specific for amino acid residues 323-339 of chicken egg ovalbumin ($OVA_{323-339}$) bound to class II MHC (I-A^b). The Institutional Animal Care and Use Committee at Stony Brook University approved all procedures and experiments using mice.

Mouse Infections. Mouse infections were performed using naïve, 8- to 12-week-old sex-matched C57BL/6J, 129X1/SvJ, and *Ccr2*$^{-/-}$ mice. Briefly, mice were inoculated intragastrically with *S.* Typhimurium (5×10^7 CFU for C57BL/6J and *Ccr2*$^{-/-}$ mice and 5×10^8 CFU for 129X1/SvJ mice) suspended in 0.1 ml of PBS. To improve the consistency of intragastric infections, food (but not water) was removed 6-8 hours prior to inoculation. Immediately following inoculation, food was provided *ad libitum.* Ten-fold serial dilutions of the inoculum were plated on LB agar to confirm the inoculum titer. At indicated times after inoculation, target organs (i.e. spleen, liver, mesenteric lymph nodes and bone marrow) were harvested and processed to obtain single cell suspensions. At each time point, we controlled for the effect of age by preparing samples from two age-matched mice left uninfected. At the end of the experiment, the results were combined and reported as the uninfected control. Bacterial loads

were determined by lysing the cells with Triton X-100 (0.05%) and plating for CFU on LB agar containing nalidixic acid (50 μg/ml). Mice infected with *S.* Typhimurium were euthanized when moribund, or at the termination of the experiment.

Cell Staining and Analysis by Flow Cytometry. Conjugated monoclonal antibodies and reagents described in this section were purchased from BioLegend unless indicated otherwise. Routinely, cells were stained in the presence of Fc block (anti-mouse CD16/32 antibody; clone 93) using anti-mouse antibodies specific for Gr-1 (clone RB6-8C5), Ly6C (clone HK1.4), Ly6G (clone 1A8), CD11b (clone M1/70), F4/80 (clone CI:A3-1), and I-Ab (clone AF6-120.1). In T cell assays, cells were stained in the presence of Fc block using anti-mouse antibodies specific for CD4 (clone RM4-5), CD8β (clone YTS156.7.7), and CD90.2 (clone 30-H12) to identify T cells; anti-mouse antibodies specific for Vα2 (clone B20.1) and Vβ5.1/5.2 (clone MR9-4; BD Biosciences) to identify the T cell receptor expressed by OT-I and OT-II T cells; and anti-mouse antibody specific for CD69 (clone H1.2F3) to measure T cell activation. Data were acquired and analyzed using a BD FACSCalibur flow cytometer (BD Biosciences) with BD CellQuestPro (BD Biosciences) and FlowJo (Tree Star) software, or a BD FACSAria cell sorter with BD FACSDiva software (BD Biosciences).

$CD11b^+$ $Ly6C^{hi}$ $Ly6G^-$ and $CD11b^+$ $Ly6C^{int}$ $Ly6G^+$ Cell Purification and Analysis. For all experiments that used purified $CD11b^+$ $Ly6C^{hi}$ $Ly6G^-$ and $CD11b^+$ $Ly6C^{int}$ $Ly6G^+$ cells, splenocytes harvested from 129X1/SvJ mice infected with *S.* Typhimurium for 21-28 days were used as a source of $CD11b^+$ $Ly6C^{hi}$ $Ly6G^-$ and $CD11b^+$ $Ly6C^{int}$ $Ly6G^+$ cells. Following treatment of the splenocytes with ACK lysing buffer (0.15 M NH_4Cl, 10 mM $KHCO_3$ and 0.1 mM Na_2EDTA) to lyse red blood cells, magnetic cell separation (MACS) technology (Miltenyi Biotec) was used to enrich for $CD11b^+$ cells. Throughout this process and all other manipulations of the cells, antibiotics (i.e. penicillin, streptomycin and gentamicin) were present to kill all bacteria. Enriched populations of $CD11b^+$ cells were stained in the presence of Fc block using antibodies specific for CD11b, Ly6C, and Ly6G, as described above. A BD FACSAria cell sorter with BD FACSDiva software was used to purify the $CD11b^+$ $Ly6C^{hi}$ $Ly6G^-$ and $CD11b^+$ $Ly6C^{int}$ $Ly6G^+$ cells. In preparation for morphological analysis, 1×10^5 purified $CD11b^+$ $Ly6C^{hi}$ $Ly6G^-$ or $CD11b^+$ $Ly6C^{int}$ $Ly6G^+$ cells were centrifuged onto glass microscope slides that were

subsequently air-dried. The cells were then fixed using methanol, stained using REASTAIN Quick-Diff (Reagena) and visualized by light microscopy.

Nitrite Assay. Purified CD11b^{+} Ly6C^{hi} Ly6G^{-} and CD11b^{+} Ly6C^{int} Ly6G^{+} cells were seeded into flat-bottom 96-well tissue culture plates at 1 x 10^6 cells per well. After 16 hours of incubation at 37ºC in 5% CO_2, we measured the levels of nitrite in supernatant using the Griess Reagent System (Promega).

***Ex Vivo* Culture of CD11b^{+} Ly6C^{hi} Ly6G^{-} and CD11b^{+} Ly6C^{int} Ly6G^{+} Cells.** Purified CD11b^{+} Ly6C^{hi} Ly6G^{-} and CD11b^{+} Ly6C^{int} Ly6G^{+} cells were seeded into 24-well tissue culture plates at 1 x 10^5 cells per well using medium formulated for the *ex vivo* culture of bone marrow-derived macrophages (DMEM with GlutaMAX™-I (Invitrogen) supplemented with 20% heat-inactivated fetal bovine serum (Atlanta Biologicals), 20% L-cell conditioned medium (a source of macrophage colony stimulating factor (M-CSF)), 0.2 M L-Gln, 0.1 M sodium pyruvate, and 1% penicillin/streptomycin). After 7 days of *ex vivo* culture at 37ºC in 5% CO_2, the cells were visualized by light microscopy and analyzed by flow cytometry. As a positive control, bone marrow-derived macrophages were cultured from naïve 129X1/SvJ mice, as described previously.[145,151]

T Cell Enrichment and T Cell Assays. Splenocytes harvested from naïve OT-I, OT-II, and 129X1/SvJ mice were used as a source of T cells. Following treatment of the splenocytes with ACK lysing buffer, MACS technology was used to enrich for CD90.2^{+} T cells. Enriched populations of T cells were suspended in RP-10 medium (RPMI 1640 medium (Invitrogen) supplemented with 10% fetal bovine serum, 0.2 M L-Gln, 0.1 M HEPES, 50 μM 2-ME, and 1% penicillin/streptomycin), labeled with 5 μM CFSE (Invitrogen) and used in T cell assays. CFSE is a cell-permeable fluorescent dye that, once taken up, is retained and distributed evenly among daughter cells with each round of cell division, resulting in a quantum reduction in cell fluorescence that can be measured by flow cytometry.[152]

In assays aimed at measuring antigen-induced T cell proliferation, purified CD11b^{+} Ly6C^{hi} Ly6G^{-} cells were mock-treated or coated with 5 nM $OVA_{257-264}$ or 5 μM $OVA_{323-339}$ peptide (Bio-Synthesis), suspended in RP-10, and seeded into round-bottom 96-well tissue culture plates at 5x10^4 cells per well. Where indicated, the mock-treated or peptide-coated CD11b^{+} Ly6C^{hi}

Ly6G$^-$ cells were fixed with 2% paraformaldehyde (Sigma) and treated with 0.2 M L-Lysine (Sigma), as described previously.[153] CFSE-labeled OT-I (Vα2$^+$ Vβ5$^+$ CD8β^+) or OT-II (Vα2$^+$ Vβ5$^+$ CD4$^+$) T cells were then added to the CD11b$^+$ Ly6C^{hi} Ly6G$^-$ cells at indicated ratios. After 4 days of incubation at 37ºC in 5% CO_2, cells were harvested, stained and analyzed by flow cytometry.

In assays aimed at measuring polyclonal T cell proliferation, CFSE-labeled 129X1/SvJ T cells were suspended in RP-10, seeded into round-bottom 96-well tissue culture plates coated with 3 μg/ml anti-mouse CD3ε antibody (clone 145-2C11; BioLegend) at 5x10^4 cells per well and cultured in the presence of 5 μg/ml anti-mouse CD28 antibody (clone E18; BioLegend). Purified CD11b$^+$ Ly6C^{hi} Ly6G$^-$ cells were then added to the T cells at a 10:1 or 1:1 ratio. Where indicated, the inducible nitric oxide synthase (iNOS) inhibitor 1400W (Sigma) was added to the cultures at a final concentration of 200 μM. After 4 days of incubation at 37ºC in 5% CO_2, cells were harvested, stained and analyzed by flow cytometry.

In assays aimed at measuring antigen-induced T cell activation *in vivo*, the equivalent of 1 x 10^6 splenic $OVA_{257-264}$-specific CD8$^+$ T cells from naïve OT-I mice were adoptively transferred into naïve F1 (C57BL/6J x 129X1/SvJ) mice by intravenous injection. The number of OT-I splenocytes transferred was based on the frequency of Vα2$^+$ Vβ5$^+$ CD8β^+ cells. One day later, recipient mice received 1 x 10^6 or 2 x 10^6 purified, $OVA_{257-264}$ peptide-coated CD11b$^+$ Ly6C^{hi} Ly6G$^-$ cells by intravenous injection. As a positive control, recipient mice received 1 x 10^6 or 2 x 10^6 $OVA_{257-264}$ peptide-coated bone marrow-derived macrophages. As a negative control, recipient mice received 200 μl of PBS. One day after the second adoptive transfer, splenocytes were harvested, stained with anti-mouse antibodies specific for Vα2, Vβ5, CD8β and CD69, and analyzed by flow cytometry.

Statistical Analysis. Statistical analysis was performed using Prism 5.0b (GraphPad Software). Data were analyzed using a two-tailed, paired Student's t-test, one-way analysis of variance (ANOVA) with Bonferroni's multiple comparisons post test or two-way ANOVA with Bonferroni's post test; p values < 0.05 were considered to be statistically significant. Asterisks indicate statistically significant differences (*** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$).

2.4 Results

Large numbers of CD11b$^+$ Gr-1$^+$ cells accumulate in tissues of mice infected with *S.* Typhimurium. To dissect innate immune mechanisms that control spread and growth of *S.* Typhimurium, we characterized cellular responses and determined bacterial loads in spleens and livers of 129X1/SvJ mice inoculated intragastrically with 5 x 10^8 CFU of *S.* Typhimurium. After 7 days of infection, we found more CD11b$^+$ Gr-1$^+$ cells in spleens and livers of mice infected with *S.* Typhimurium than in spleens and livers of mice left uninfected (**Figures 1A and S1A**). After 60 days of infection, a long-term time point used here to assess bacterial persistence (**Figure 1B**), absolute numbers and percentages of CD11b$^+$ Gr-1$^+$ cells in spleen and liver had increased even further (28-fold and 20-fold, respectively) (**Figures 1A and S1A**). Similar results were obtained when we characterized cellular responses in C57BL/6J mice inoculated intragastrically with 5 x 10^7 CFU of *S.* Typhimurium (**Figure S1B**). Thus, large numbers of CD11b$^+$ Gr-1$^+$ cells accumulate in tissues of mice infected with *S.* Typhimurium.

CD11b$^+$ Gr-1$^+$ cells that accumulate in tissues of mice infected with *S.* Typhimurium exhibit phenotypic and morphological heterogeneity. CD11b$^+$ Gr-1$^+$ cells are a heterogeneous population that can be further divided based on expression of Ly6C and Ly6G, two isoforms of Gr-1 that are differentially expressed on the surface of neutrophilic granulocytes, inflammatory monocytes and some populations of dendritic cells [8,26,147-149]. To define the phenotypic and morphological heterogeneity of CD11b$^+$ Gr-1$^+$ cells in the host response to *S.* Typhimurium, we further characterized the CD11b$^+$ Gr-1$^+$ cells present in spleens of 129X1/SvJ mice infected with *S.* Typhimurium for 21-28 days. We found the CD11b$^+$ Gr-1$^+$ cells to be a heterogeneous population that could be divided into CD11b$^+$ Ly6C^{hi} Ly6G$^-$ and CD11b$^+$ Ly6C^{int} Ly6G$^+$ cells (**Figure 2A**). Furthermore, we found that purified CD11b$^+$ Ly6C^{hi} Ly6G$^-$ cells exhibited a mononuclear morphology, whereas purified CD11b$^+$ Ly6C^{int} Ly6G$^+$ cells exhibited a polymorphonuclear or band-shaped nuclear morphology (**Figure 2B**). Thus, CD11b$^+$ Gr-1$^+$ cells in the host response to *S.* Typhimurium are characterized by phenotypic and morphological heterogeneity.

CD11b$^+$ Ly6C^{hi} Ly6G$^-$ and CD11b$^+$ Ly6C^{int} Ly6G$^+$ cells accumulate and persist in tissues of mice infected with *S.* Typhimurium. To determine the kinetics with which CD11b$^+$ Ly6C^{hi} Ly6G$^-$ and CD11b$^+$ Ly6C^{int} Ly6G$^+$ cells respond during infection, we further

characterized cellular responses in 129X1/SvJ mice inoculated with *S*. Typhimurium. After 7 days of infection, we found more CD11b^{+} Ly6C^{hi} Ly6G^{-} and CD11b^{+} Ly6C^{int} Ly6G^{+} cells in spleens and livers of mice infected with *S*. Typhimurium than in spleens and livers of mice left uninfected (**Figures 3A and S2A**). Both absolute numbers and percentages of CD11b^{+} Ly6C^{hi} Ly6G^{-} and CD11b^{+} Ly6C^{int} Ly6G^{+} cells increased dramatically over time (34- and 28-fold increases in absolute numbers in spleen, and 172- and 23-fold increases in absolute numbers in liver, respectively, after 28 days of infection), plateaued at around day 45 after inoculation, and persisted at peak or near peak levels over a period of 90 days (**Figures 3A and S2A**). Similar results were obtained when we characterized CD11b^{+} Ly6C^{hi} Ly6G^{-} and CD11b^{+} Ly6C^{int} Ly6G^{+} cell responses in mesenteric lymph nodes (**Figure S2B**). We recovered substantial numbers of *S*. Typhimurium from spleens and livers of infected mice at every time point (**Figure 3B**), indicating that the response of CD11b^{+} Ly6C^{hi} Ly6G^{-} and CD11b^{+} Ly6C^{int} Ly6G^{+} cells correlated with bacterial persistence. The kinetics with which CD11b^{+} Ly6C^{hi} Ly6G^{-} and CD11b^{+} Ly6C^{int} Ly6G^{+} cells responded in bone marrow were similar to those in spleen and liver (**Figure 3C**). Thus, accumulation and persistence of CD11b^{+} Ly6C^{hi} Ly6G^{-} and CD11b^{+} Ly6C^{int} Ly6G^{+} cells in peripheral tissues of mice infected with *S*. Typhimurium could be the result of increased output from this generative site.

CCR2 is required for the recruitment of CD11b^{+}Ly6C^{hi}Ly6G^{-} cells from the bone marrow to systemic sites of *S*. Typhimurium infection. CCR2 is critical for emigration of monocyte precursors from the bone marrow [26,29]. To define the role of CCR2 in innate immune defense against *S*. Typhimurium, we characterized cellular responses in wild-type (WT) and *Ccr2*$^{-/-}$ C57BL/6J mice inoculated intragastrically with 5 x 10^{7} CFU of *S*. Typhimurium. After 7 days of infection, we observed significant accumulation of CD11b^{+} Ly6C^{hi} Ly6G^{-} cells in spleens of WT but not *Ccr2*$^{-/-}$ mice (**Figure 4A, left panel**). As expected for granulocyte precursors, which do not require CCR2 for emigration from the bone marrow [29], we observed significant and comparable accumulation of CD11b^{+} Ly6C^{int} Ly6G^{+} cells in spleens of both WT and *Ccr2*$^{-/-}$ mice (**Figure 4A, right panel**). We recovered similar numbers of *S*. Typhimurium from spleens of infected WT and *Ccr2*$^{-/-}$ mice (**Figure 4B**), indicating that the lack of accumulation of CD11b^{+} Ly6C^{hi} Ly6G^{-} cells in spleens of infected *Ccr2*$^{-/-}$ mice was not due to a difference in bacterial load. Consistent with a role for CCR2 in emigration of monocyte precursors from the bone marrow [26], we observed significant accumulation of CD11b^{+} Ly6C^{hi}

Ly6G$^-$ cells in bone marrow of infected *Ccr2*$^{-/-}$ but not WT mice (**Figure 4C**). Thus, CCR2 is required for the recruitment of CD11b$^+$Ly6C^{hi}Ly6G$^-$ cells from the bone marrow to systemic sites of *S.* Typhimurium infection.

CD11b$^+$ Ly6C^{hi} Ly6G$^-$ cells purified from spleens of mice infected with *S.* Typhimurium can differentiate into macrophages cells following *ex vivo* culture. Next, we examined the ability of CD11b$^+$ Ly6C^{hi} Ly6G$^-$ and CD11b$^+$ Ly6C^{int} Ly6G$^+$ cells purified from spleens of 129X1/SvJ mice infected with *S.* Typhimurium to differentiate *ex vivo*. After 7 days of *ex vivo* culture in the presence of L-cell conditioned medium, a source of M-CSF, we found that the CD11b$^+$ Ly6C^{hi} Ly6G$^-$ but not CD11b$^+$ Ly6C^{int} Ly6G$^+$ cells had differentiated into adherent cells that exhibited a macrophage-like morphology (**Figure 5A and data not shown**). The differentiated cells expressed increased levels of surface CD11b and F4/80, but had lost expression of surface Ly6C (**Figure 5B**). The differentiated cells also expressed increased levels of surface class II MHC (I-A^b), but the observed difference did not reach statistical significance (**Figure 5B**). Collectively, these results indicate that the CD11b$^+$ Ly6C^{hi} Ly6G$^-$ cells that accumulate in spleens of mice infected with *S.* Typhimurium are immature myeloid cells capable of differentiating into macrophages.

CD11b$^+$ Ly6C^{hi} Ly6G$^-$ cells purified from spleens of mice infected with *S.* Typhimurium can present antigen to both CD4$^+$ and CD8$^+$ T cells. Given that CD11b$^+$ Ly6C^{hi} Ly6G$^-$ cells purified from spleens of mice infected with *S.* Typhimurium could differentiate into macrophage-like cells (**Figure 5**), we characterized the ability of these immature myeloid cells to present antigen to T cells *in vitro*. T cells enriched from spleens of naïve OT-I mice, which produce CD8$^+$ T cells specific for amino acid residues 257-264 of chicken egg ovalbumin ($OVA_{257-264}$) bound to class I MHC (H-2K^b), or OT-II mice, which produce CD4$^+$ T cells specific for amino acid residues 323-339 of chicken egg ovalbumin ($OVA_{323-339}$) bound to class II MHC (I-A^b), were labeled with CFSE and used as responder cells. CD11b$^+$ Ly6C^{hi} Ly6G$^-$ cells purified from spleens of 129X1/SvJ mice infected with *S.* Typhimurium were mock-treated or coated with $OVA_{257-264}$ or $OVA_{323-339}$ peptide, fixed with paraformaldehyde, and used as antigen presenting cells. Responder T cells and antigen presenting cells were mixed at a 5:1 ratio and seeded into tissue culture plates. After 4 days of incubation, we found that T cells cultured with peptide-coated CD11b$^+$ Ly6C^{hi} Ly6G$^-$ cells had proliferated extensively when compared to T

cells cultured with mock-treated CD11b^{+} Ly6C^{hi} Ly6G^{-} cells (**Figure 6A**). However, significant proliferation of the T cells was not observed when live antigen presenting cells were used (**Figure 6B**). Thus, the CD11b^{+} Ly6C^{hi} Ly6G^{-} immature myeloid cells that accumulate and persist in spleens of mice infected with *S.* Typhimurium can present antigen to T cells when fixed, but produce an inhibitor of T cell proliferation when alive.

CD11b^{+} Ly6C^{hi} Ly6G^{-} cells purified from spleens of mice infected with *S.* Typhimurium can inhibit T cell proliferation via a NO-dependent mechanism. Early studies linked NO-producing macrophage precursors to immunosuppression in salmonellosis [110]. Therefore, we examined the ability of CD11b^{+} Ly6C^{hi} Ly6G^{-} and CD11b^{+} Ly6C^{int} Ly6G^{+} cells purified from spleens of mice infected with *S.* Typhimurium to produce NO. After 16 hours of *ex vivo* culture, we found high levels of nitrite in the supernatants of the CD11b^{+} Ly6C^{hi} Ly6G^{-} but not CD11b^{+} Ly6C^{int} Ly6G^{+} cells (**Figure 7A**), indicating that large amounts of NO were produced by the former but not latter population of cells.

To define the impact of NO production by the CD11b^{+} Ly6C^{hi} Ly6G^{-} cells on the ability of T cells to proliferate, we added the selective iNOS inhibitor 1400W to cultures of responder T cells mixed with OVA peptide-coated antigen presenting cells, as described above. After 4 days of incubation, we found that T cells cultured with peptide-coated CD11b^{+} Ly6C^{hi} Ly6G^{-} cells and the selective iNOS inhibitor 1400W had proliferated significantly more than T cells cultured with peptide-coated or mock-treated CD11b^{+} Ly6C^{hi} Ly6G^{-} cells only (**Figure 7B**). Similar results were obtained when we examined the ability of purified CD11b^{+} Ly6C^{hi} Ly6G^{-} cells, most of which are uninfected, to suppress polyclonal T cell proliferation through a bystander effect (**Figures 7C and S3**). Thus, the CD11b^{+} Ly6C^{hi} Ly6G^{-} immature myeloid cells that accumulate and persist in tissues of mice infected with *S.* Typhimurium can inhibit T cell proliferation via a NO-dependent mechanism. Furthermore, these results indicate that the accumulation and persistence of NO-producing CD11b^{+} Ly6C^{hi} Ly6G^{-} immature myeloid cells in peripheral tissues of mice infected with *S.* Typhimurium could lead to a bystander effect responsible for the delay in onset of protective immune responses.

CD11b^{+} Ly6C^{hi} Ly6G^{-} cells recruited in response to *S.* Typhimurium infection can modulate T cell function *in vivo.* Next, we used an adoptive transfer approach to determine the ability of CD11b^{+} Ly6C^{hi} Ly6G^{-} cells recruited in response to *S.* Typhimurium infection to

modulate T cell function *in vivo*. Splenocytes from naïve OT-I mice were used as a source of T cells. We adoptively transferred 1 x 10^6 OT-I T cells into naïve F1 (C57BL/6J x 129X1/SvJ) mice. One day later, recipient mice received 1 x 10^6 or 2 x 10^6 $OVA_{257-264}$ peptide-coated $CD11b^+$ $Ly6C^{hi}$ $Ly6G^-$ cells purified from spleens of 129X1/SvJ mice infected with *S.* Typhimurium. Recipient mice that received PBS or $OVA_{257-264}$ peptide-coated bone marrow-derived macrophages were used as controls. One day after the second adoptive transfer, splenocytes were harvested, stained and analyzed to determine the level of OT-I T cell activation. We found that the percentages of activated OT-I T cells in spleens of mice that received $OVA_{257-264}$ peptide-coated $CD11b^+$ $Ly6C^{hi}$ $Ly6G^-$ cells were significantly higher than the percentage of activated OT-I T cells in spleens of mice that received PBS (**Figure 8**). However, no significant increase in the percentage of activated OT-I T cells was observed in spleens of mice that received 2 x 10^6 instead of 1 x 10^6 $OVA_{257-264}$ peptide-coated $CD11b^+$ $Ly6C^{hi}$ $Ly6G^-$ cells (**Figure 8**). In contrast, we found that a two-fold increase in the number of $OVA_{257-264}$ peptide-coated bone marrow-derived macrophages transferred resulted in a corresponding two-fold increase in the percentage of activated OT-I T cells in spleen (**Figure 8**). Thus, $CD11b^+$ $Ly6C^{hi}$ $Ly6G^-$ cells recruited in response to *S.* Typhimurium infection can present antigen to T cells *in vivo*, but as their numbers in the periphery increase there does not appear to be a corresponding increase in the response of T cells. We interpret these results to suggest that accumulation and persistence of $CD11b^+$ $Ly6C^{hi}$ $Ly6G^-$ cells beyond a certain threshold level may cause immunosuppression.

2.5 Figures

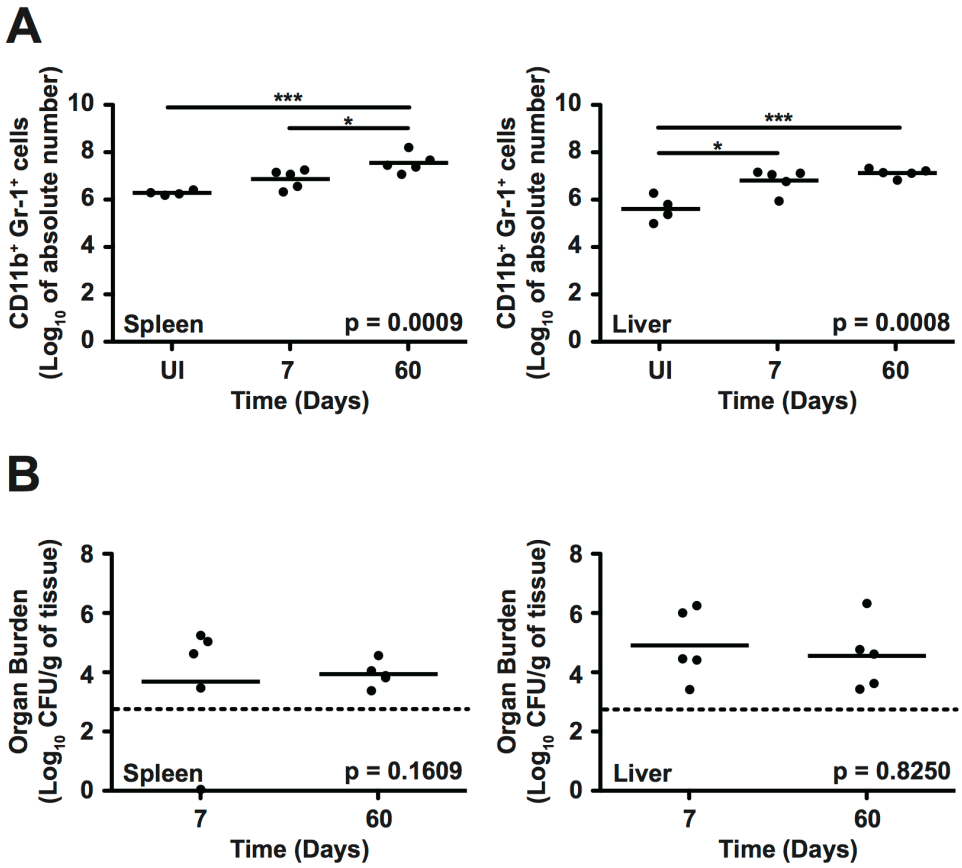


Figure 1. Large numbers of CD11b$^+$ Gr-1$^+$ cells accumulate in tissues of mice infected with *S.* Typhimurium. (*A*) Absolute numbers of CD11b$^+$ Gr-1$^+$ cells in spleens (left) and livers (right) of 129X1/SvJ mice (n = 4-5 per group per time point) left uninfected (UI) or infected for 7 or 60 days with *S.* Typhimurium. (*B*) Corresponding bacterial loads per gram of spleen (left) and liver (right) tissue. Dashed lines denote limit of detection. Data show mean with spread from a single experiment that is representative of two independent experiments. Data were analyzed using a one-way ANOVA with Bonferroni's multiple comparisons post test; p values < 0.05 were considered to be statistically significant. Asterisks indicate statistically significant differences (*** $p < 0.001$, * $p < 0.05$). See also Figure S1.

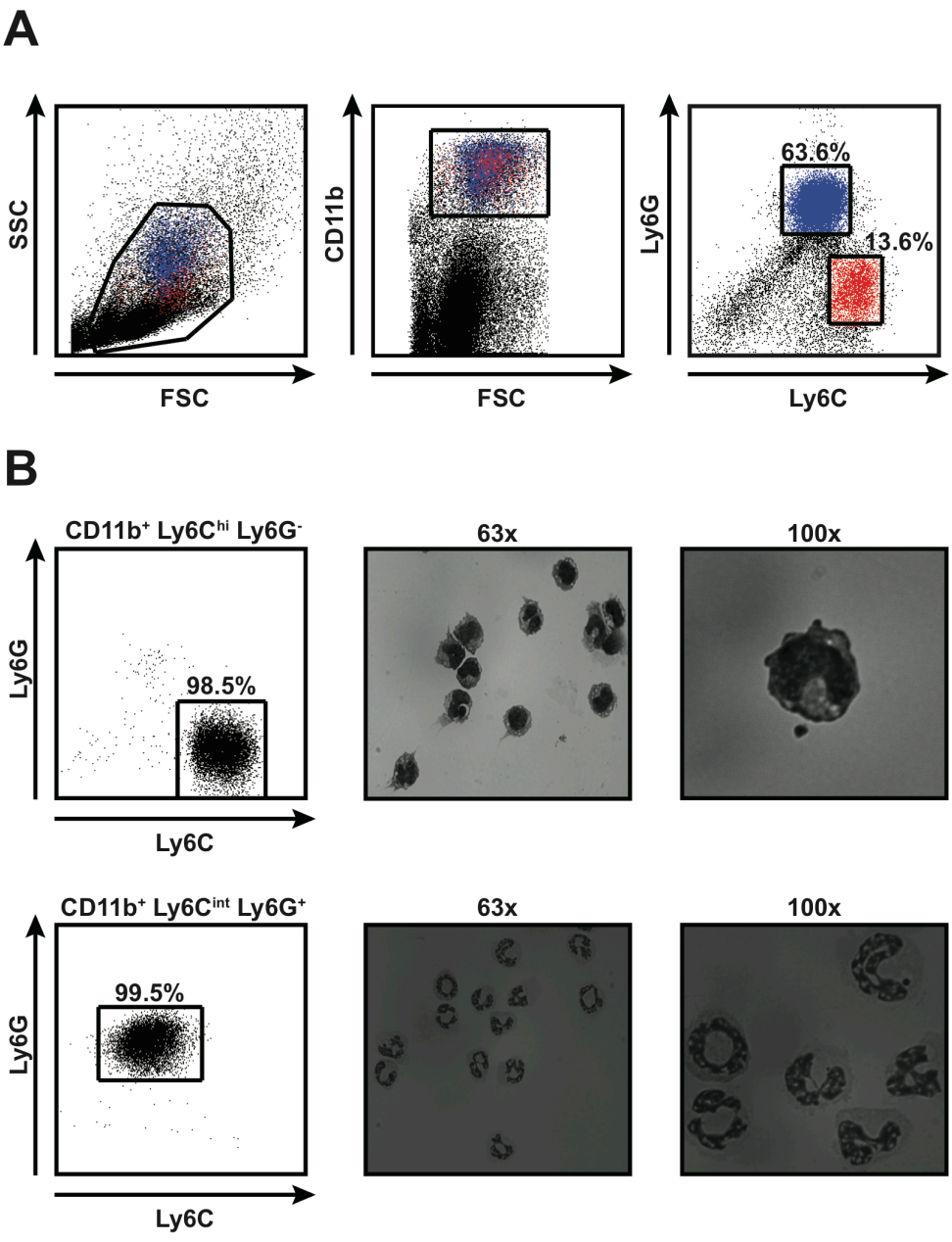

A
SSC
FSC
CD11b
FSC
Ly6G
63.6%
13.6%
Ly6C
B
CD11b+ Ly6Chi Ly6G-
Ly6G
98.5%
Ly6C
63x
100x
CD11b+ Ly6Cint Ly6G+
Ly6G
99.5%
Ly6C
63x
100x

Figure 2. $CD11b^+$ $Gr\text{-}1^+$ cells that accumulate in tissues of mice infected with *S.* Typhimurium exhibit phenotypic and morphological heterogeneity. (*A*) Flow cytometric analysis of splenocytes harvested from 129X1/SvJ mice infected for 28 days with *S.* Typhimurium. Live splenocytes (left) that expressed CD11b (middle) could be divided into $CD11b^+$ $Ly6C^{hi}$ $Ly6G^-$ and $CD11b^+$ $Ly6C^{int}$ $Ly6G^+$ cells (right). Red and blue colors indicate $CD11b^+$ $Ly6C^{hi}$ $Ly6G^-$ and $CD11b^+$ $Ly6C^{int}$ $Ly6G^+$ cells, respectively. Numbers refer to $CD11b^+$ $Ly6C^{hi}$ $Ly6G^-$ and $CD11b^+$ $Ly6C^{int}$ $Ly6G^+$ cells as percentages of live, $CD11b^+$ cells. (*B*) Morphological analysis of $CD11b^+$ $Ly6C^{hi}$ $Ly6G^-$ and $CD11b^+$ $Ly6C^{int}$ $Ly6G^+$ cells purified by FACS. Purity of the cells was consistently greater than 98% (left). Purified cells were stained with REASTAIN Quick-Diff and visualized by light microscopy at 63x (middle) and 100x (right) magnifications. Numbers refer to $CD11b^+$ $Ly6C^{hi}$ $Ly6G^-$ and $CD11b^+$ $Ly6C^{int}$ $Ly6G^+$ cells as percentages of live cells. Data are representative of six independent experiments.

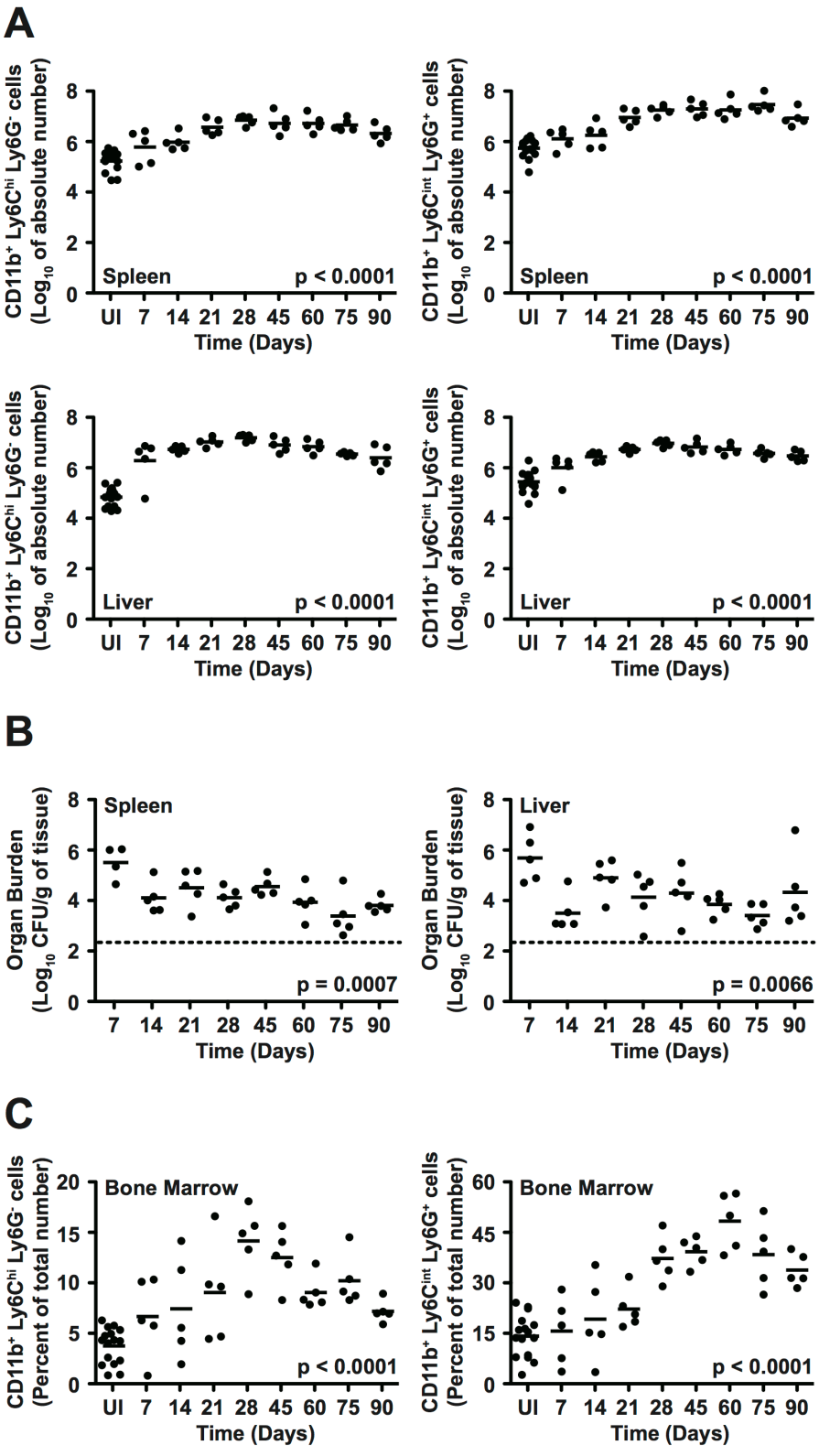

A
CD11b+ Ly6Chi Ly6G- cells (Log10 of absolute number)
Spleen
p < 0.0001
UI 7 14 21 28 45 60 75 90
Time (Days)
CD11b+ Ly6Cint Ly6G+ cells (Log10 of absolute number)
Spleen
p < 0.0001
Liver
p < 0.0001
Liver
p < 0.0001
B
Organ Burden (Log10 CFU/g of tissue)
Spleen
p = 0.0007
Liver
p = 0.0066
C
CD11b+ Ly6Chi Ly6G- cells (Percent of total number)
Bone Marrow
p < 0.0001
CD11b+ Ly6Cint Ly6G+ cells (Percent of total number)
Bone Marrow
p < 0.0001

Figure 3. $CD11b^+$ $Ly6C^{hi}$ $Ly6G^-$ and $CD11b^+$ $Ly6C^{int}$ $Ly6G^+$ cells accumulate and persist in tissues of mice infected with *S.* Typhimurium. (*A*) Absolute numbers of $CD11b^+$ $Ly6C^{hi}$ $Ly6G^-$ cells (left) and $CD11b^+$ $Ly6C^{int}$ $Ly6G^+$ cells (right) in spleens (top) and livers (bottom) of 129X1/SvJ mice (n = 5 per group per time point) left uninfected (UI) or infected for up to 90 days with *S.* Typhimurium. (*B*) Bacterial loads per gram of spleen (left) and liver (right) tissue. (*C*) Percentages of $CD11b^+$ $Ly6C^{hi}$ $Ly6G^-$ cells (left) and $CD11b^+$ $Ly6C^{int}$ $Ly6G^+$ cells (right) in bone marrow. Dashed lines denote limit of detection. Data show mean with spread from a single experiment that is representative of two independent experiments. Data were analyzed using a one-way ANOVA; p values < 0.05 were considered to be statistically significant. See also Figure S2.

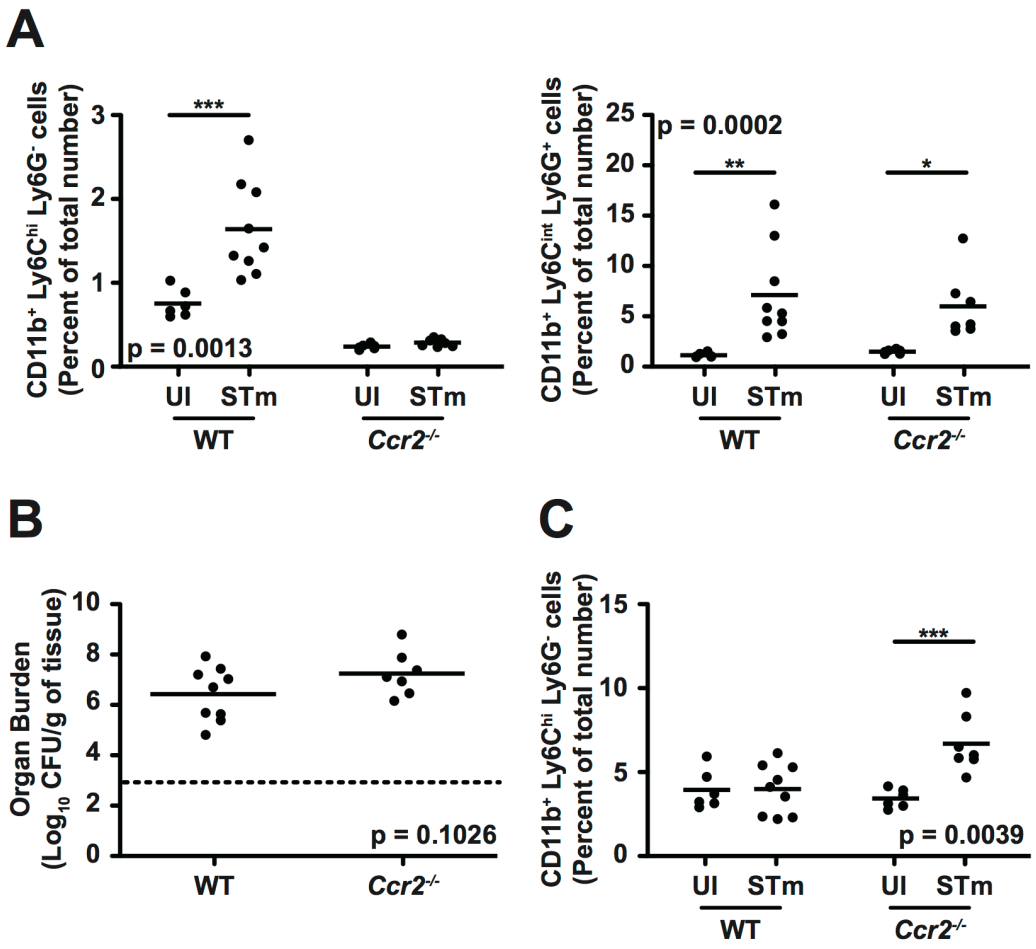
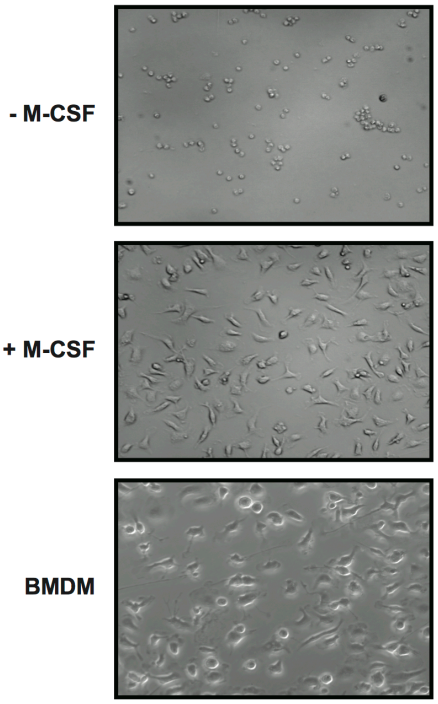


Figure 4. CCR2 is required for the recruitment of CD11b^{+}Ly6C^{hi}Ly6G^{-} cells from the bone marrow to systemic sites of *S.* Typhimurium infection. (*A*) Percentages of CD11b^{+} Ly6C^{hi} Ly6G^{-} cells (left) and CD11b^{+} Ly6C^{int} Ly6G^{+} cells (right) in spleens of WT and *Ccr2*$^{-/-}$ mice (n = 6-9 per group) left uninfected (UI) or infected for 7 days with *S.* Typhimurium (STm). (*B*) Bacterial loads per gram of spleen tissue. (*C*) Percentages of CD11b^{+} Ly6C^{hi} Ly6G^{-} cells in bone marrow. Dashed line denotes limit of detection. Data show mean with spread and are cumulative of two independent experiments. Data were analyzed using a two-way ANOVA with Bonferroni's post test (A and C) or using a two-tailed, paired Student's t-test (B); p values < 0.05 were considered to be statistically significant. Asterisks indicate statistically significant differences (*** p < 0.001, ** p < 0.01, * p < 0.05).

A


- M-CSF
+ M-CSF
BMDM

B

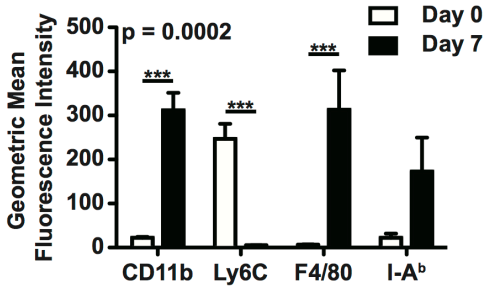

Geometric Mean Fluorescence Intensity
500
400
300
200
100
0
p = 0.0002
***
***
***
Day 0
Day 7
CD11b
Ly6C
F4/80
I-Ab

Figure 5. CD11b^{+} Ly6C^{hi} Ly6G^{-} cells purified from spleens of mice infected with *S.* Typhimurium can differentiate into macrophage-like cells following *ex vivo* culture. (*A*) Light microscopy images of purified CD11b^{+} Ly6C^{hi} Ly6G^{-} cells cultured *ex vivo* for 7 days in the absence (top) or presence (middle) of M-CSF. Cultures of bone marrow-derived macrophages (BMDM; bottom) were used as a control. (*B*) Flow cytometric analysis of purified CD11b^{+} Ly6C^{hi} Ly6G^{-} cells (white bars; Day 0) or purified CD11b^{+} Ly6C^{hi} Ly6G^{-} cells cultured *ex vivo* for 7 days in the presence of M-CSF (black bars; Day 7). Analysis was performed to detect expression of surface CD11b, Ly6C, F4/80 and class II MHC (I-A^{b}). Data are representative of (A), or show mean with SEM from (B), three independent experiments. Data were analyzed using a two-way ANOVA with Bonferroni's post test; a p value < 0.05 was considered to be statistically significant. Asterisks indicate statistically significant differences (*** $p < 0.001$).

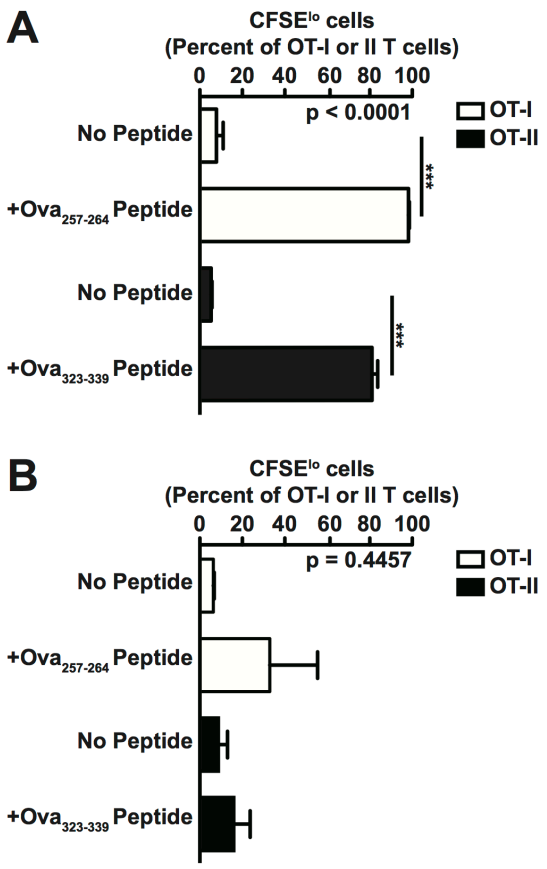


Figure 6. CD11b^{+} Ly6C^{hi} Ly6G^{-} cells purified from spleens of mice infected with *S.* Typhimurium can present antigen to both CD4^{+} and CD8^{+} T cells. (*A and B*) Proliferation of CFSE-labeled OT-I T cells (white bars) or OT-II T cells (black bars) cultured with paraformaldehyde-fixed (A) or live (B), purified CD11b^{+} Ly6C^{hi} Ly6G^{-} cells mock-treated (No Peptide) or coated with $OVA_{257-264}$ or $OVA_{323-339}$ peptide. The ratio of antigen presenting cells to responder cells was 5:1. Data show mean with SEM from three independent experiments. Data were analyzed using a one-way ANOVA with Bonferroni's multiple comparisons post test; p values < 0.05 were considered to be statistically significant. Asterisks indicate statistically significant differences (*** $p < 0.001$).

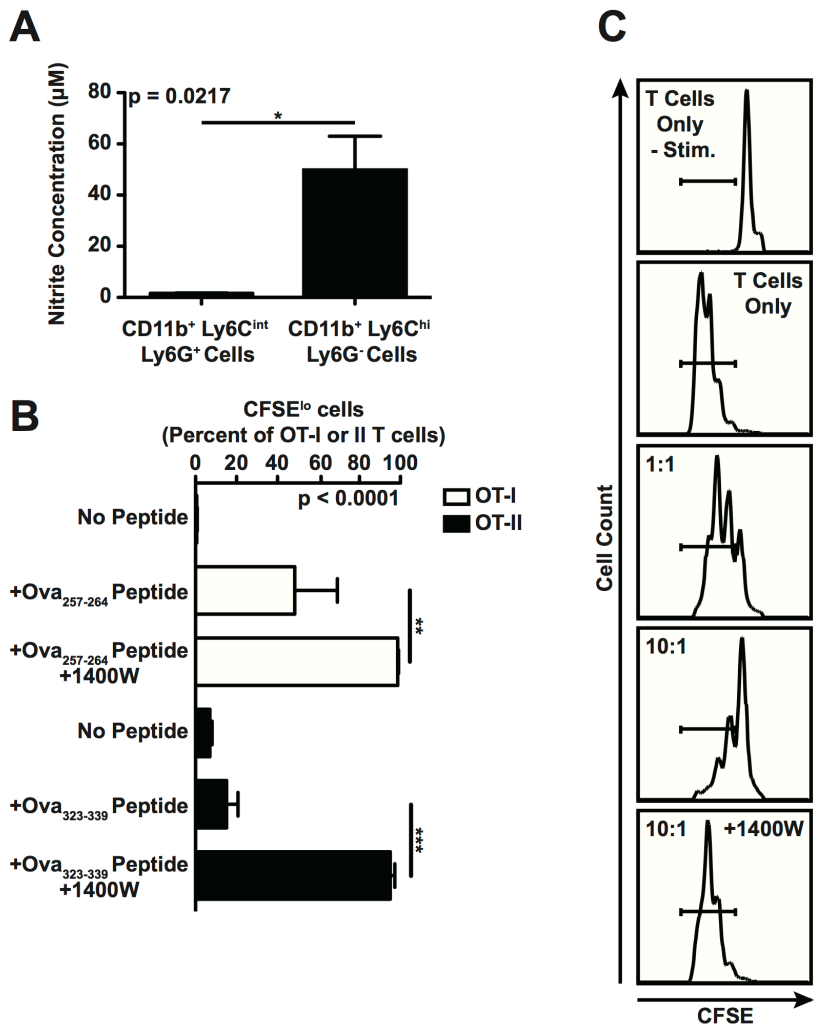

A
Nitrite Concentration (μM)
80
60
40
20
0
p = 0.0217
*
CD11b+ Ly6Cint Ly6G+ Cells
CD11b+ Ly6Chi Ly6G- Cells
B
CFSElo cells
(Percent of OT-I or II T cells)
0 20 40 60 80 100
p < 0.0001
OT-I
OT-II
No Peptide
+Ova257-264 Peptide
+Ova257-264 Peptide +1400W
**
No Peptide
+Ova323-339 Peptide
+Ova323-339 Peptide +1400W
***
C
T Cells Only - Stim.
T Cells Only
1:1
10:1
10:1 +1400W
Cell Count
CFSE

Figure 7. $CD11b^{+}$ $Ly6C^{hi}$ $Ly6G^{-}$ cells purified from spleens of mice infected with *S.* Typhimurium can inhibit T cell proliferation via a NO-dependent mechanism. (*A*) Production of nitrite by purified $CD11b^{+}$ $Ly6C^{hi}$ $Ly6G^{-}$ and $CD11b^{+}$ $Ly6C^{int}$ $Ly6G^{+}$ cells as measured using the Griess Reagent System. (*B*) Proliferation of CFSE-labeled OT-I T cells (white bars) or OT-II T cells (black bars) cultured with purified $CD11b^{+}$ $Ly6C^{hi}$ $Ly6G^{-}$ cells mock-treated (No Peptide) or coated with $OVA_{257-264}$ or $OVA_{323-339}$ peptide. The ratio of antigen presenting cells to responder cells was 10:1. Where indicated, the iNOS inhibitor 1400W was added to the cultures. (*C*) Proliferation of CFSE-labeled 129X1/SvJ T cells cultured in the absence or presence of purified $CD11b^{+}$ $Ly6C^{hi}$ $Ly6G^{-}$ bystander cells. Proliferation of the T cells was induced using anti-mouse CD3ε and anti-mouse CD28 antibody, except where noted (-Stim.). The ratio of bystander cells to responder cells was 1:1 or 10:1. Where indicated, the iNOS inhibitor 1400W was added to the cultures. Data show mean with SEM from (A and B), or are representative of (C), at least three independent experiments. Data were analyzed using a two-tailed, paired Student's t-test (A) or using a one-way ANOVA with Bonferroni's multiple comparisons post test (B); p values < 0.05 were considered to be statistically significant. Asterisks indicate statistically significant differences (*** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$). See also Figure S3.

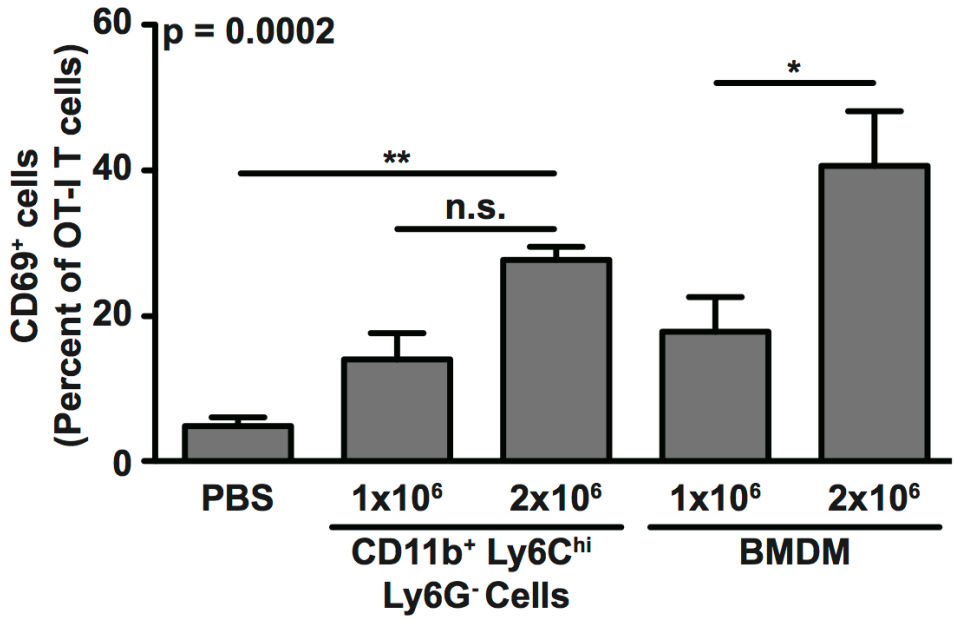


Figure 8. CD11b^{+} Ly6C^{hi} Ly6G^{-} cells recruited in response to *S.* Typhimurium infection can modulate T cell function *in vivo*. Activation of adoptively transferred OT-I T cells in F1 (C57BL/6J x 129X1/SvJ) mice (cumulative total of n = 4 per group) that received $OVA_{257-264}$ peptide-coated CD11b^{+} Ly6C^{hi} Ly6G^{-} cells purified from spleens of 129X1/SvJ mice infected with *S.* Typhimurium. Activation of OT-I T cells was measured by analyzing expression of surface CD69. Recipient mice that received PBS or $OVA_{257-264}$ peptide-coated bone marrow-derived macrophages were used as controls. Cumulative data from three independent experiments show mean with SEM and were analyzed using a one-way ANOVA with Bonferroni's multiple comparisons post test; a p value < 0.05 was considered to be statistically significant. Asterisks indicate statistically significant differences (** $p < 0.01$, * $p < 0.05$; n.s. = not significant).

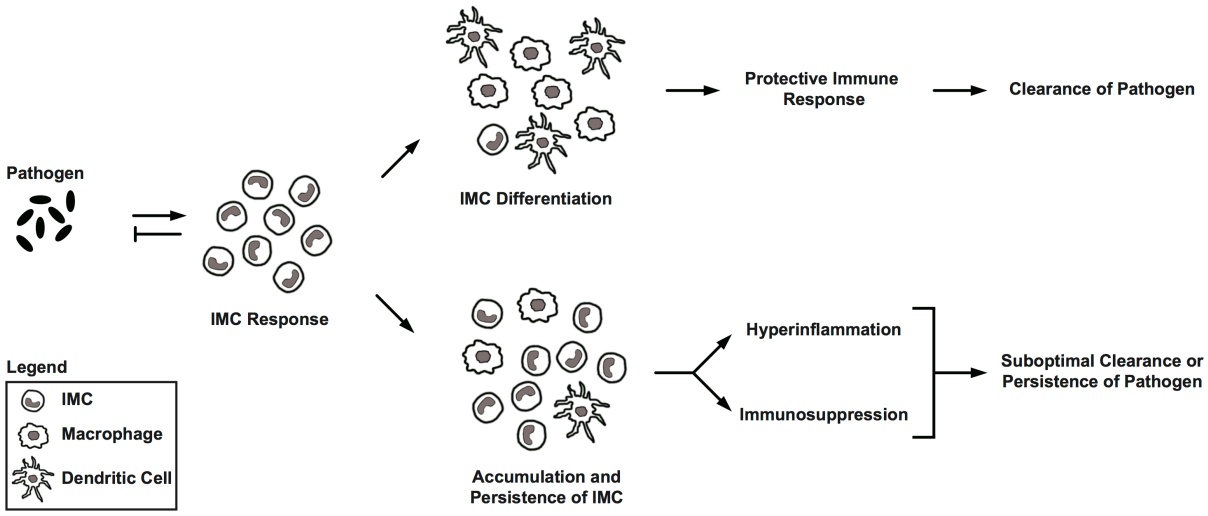


Figure 9. Model of the role of CD11b^{+} Ly6C^{hi} Ly6G^{-} immature myeloid cells in the host response to infection. We propose a model in which NO-producing CD11b^{+} Ly6C^{hi} Ly6G^{-} immature myeloid cells (IMC) provide protective functions in the host response to infection (top), where accumulation and persistence of these cells beyond a certain threshold level may cause collateral effects that prolong or exacerbate disease (bottom). For further explanation, see the text.

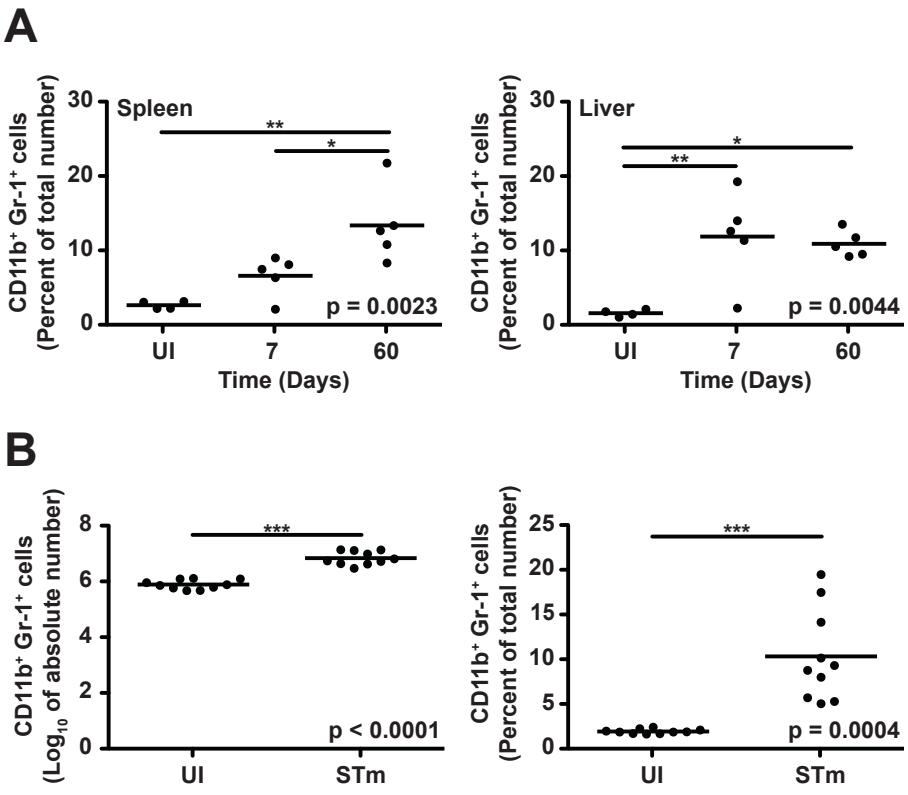


Figure S1, related to Figure 1. (*A*) Percentages of CD11b$^+$ Gr-1$^+$ cells in spleens (left) and livers (right) of 129X1/SvJ mice (n = 4-5 per group per time point) left uninfected (UI) or infected for 7 or 60 days with *S.* Typhimurium. (*B*) Absolute numbers (left) and percentages (right) of CD11b$^+$ Gr-1$^+$ cells in spleens of C57BL/6J mice (n = 10 per group) left UI or infected for 7 days with *S.* Typhimurium (STm). Data show mean with spread from a single experiment that is representative of two independent experiments. Data were analyzed using a one-way ANOVA with Bonferroni's multiple comparisons post test (A) or using a two-tailed, paired Student's t-test (B); p values < 0.05 were considered to be statistically significant. Asterisks indicate statistically significant differences (*** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$).

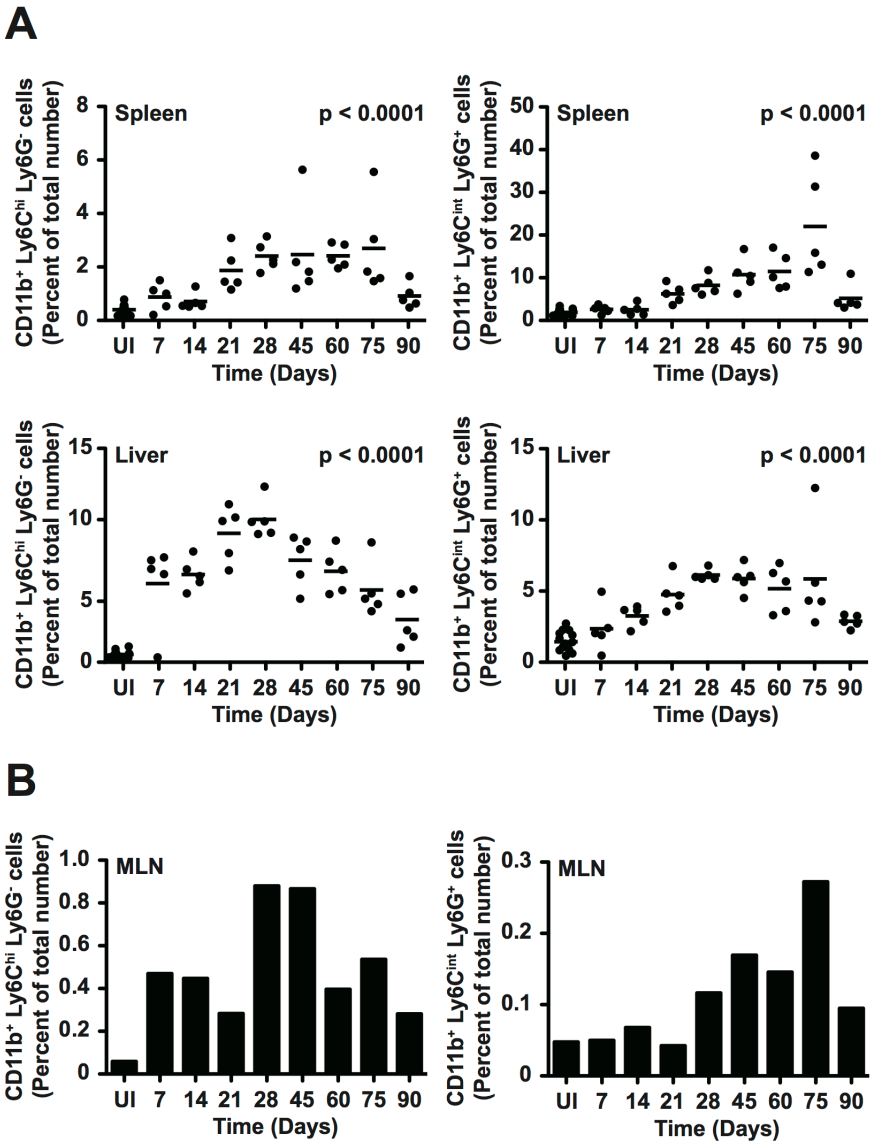

A
Spleen
p < 0.0001
CD11b+ Ly6Chi Ly6G- cells (Percent of total number)
CD11b+ Ly6Cint Ly6G+ cells (Percent of total number)
Liver
UI 7 14 21 28 45 60 75 90
Time (Days)
B
MLN

Figure S2, related to Figure 3. (*A*) Percentages of $CD11b^{+}$ $Ly6C^{hi}$ $Ly6G^{-}$ cells (left) and $CD11b^{+}$ $Ly6C^{int}$ $Ly6G^{+}$ cells (right) in spleens (top) and livers (bottom) of 129X1/SvJ mice (n = 5 per group per time point) left uninfected (UI) or infected for up to 90 days with *S.* Typhimurium. (*B*) Percentages of $CD11b^{+}$ $Ly6C^{hi}$ $Ly6G^{-}$ cells (left) and $CD11b^{+}$ $Ly6C^{int}$ $Ly6G^{+}$ cells (right) in mesenteric lymph nodes of 129X1/SvJ mice (n = 5 per group per time point) left uninfected (UI) or infected for up to 90 days with *S.* Typhimurium. Data show mean with spread (A) or analysis of combined single cell suspensions of mesenteric lymph nodes from five mice per group per time point (B) from a single experiment that is representative of two independent experiments. Data were analyzed using a one-way ANOVA; p values < 0.05 were considered to be statistically significant.

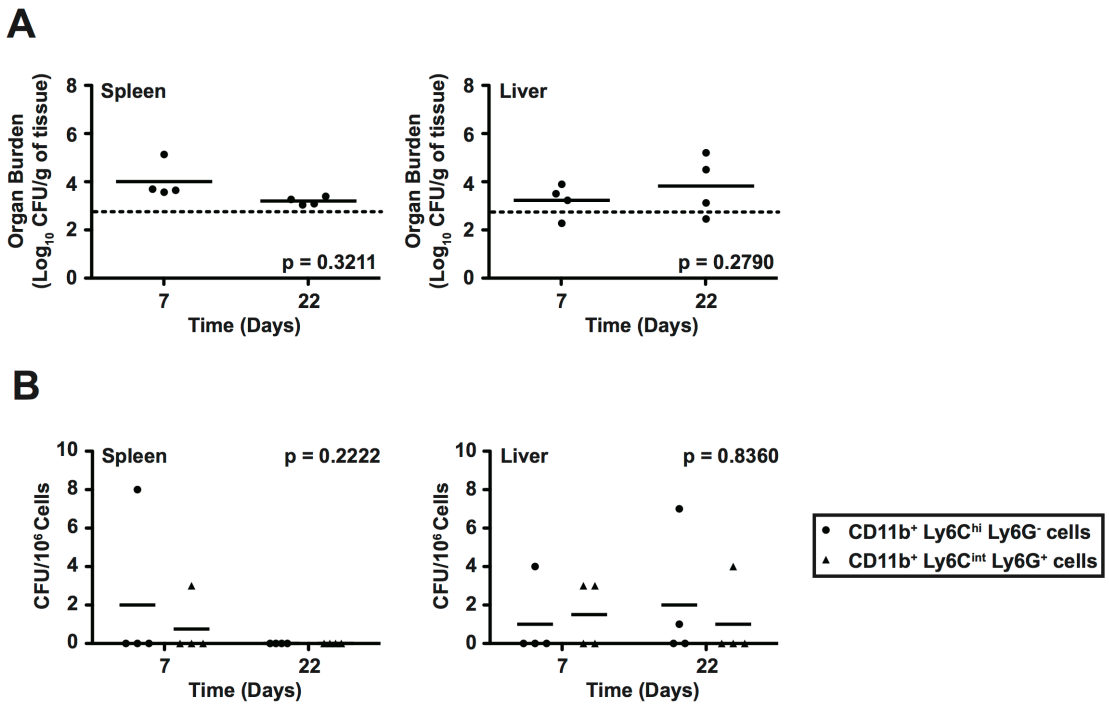


Figure S3, related to Figure 7. (*A*) Bacterial loads per gram of spleen (left) and liver (right) tissue of 129X1/SvJ mice (n = 4 per group per time point) infected with *S.* Typhimurium for 7 days or 22 days. (*B*) Enumeration of viable intracellular *S.* Typhimurium associated with $CD11b^{+}$ $Ly6C^{hi}$ $Ly6G^{-}$ cells (circles) and $CD11b^{+}$ $Ly6C^{int}$ $Ly6G^{+}$ cells (triangles) purified from spleens (left) and livers (right) of 129X1/SvJ mice (n = 4 per group per time point) infected with *S.* Typhimurium for 7 or 22 days. Purified cells were suspended in medium containing 25 μg/ml of gentamicin to kill extracellular bacteria. After 2 hours of incubation at 37ºC in 5% CO_2, the cells were washed using PBS and lysed using 0.1% Triton X-100 to release intracellular bacteria. These bacteria were enumerated by plating onto LB agar. Data show mean with spread from a single experiment and were analyzed using a two-tailed, paired Student's t-test (A) or a two-way ANOVA with Bonferroni's post test (B); p values < 0.05 were considered to be statistically significant.

2.6 Discussion

We have found that large numbers of CD11b^{+} Ly6C^{hi} Ly6G^{-} and CD11b^{+} Ly6C^{int} Ly6G^{+} cells accumulate and persist in tissues of mice infected with *S.* Typhimurium. Furthermore, we have found that CCR2 is critical for the recruitment of CD11b^{+} Ly6C^{hi} Ly6G^{-} cells from the bone marrow to systemic sites of *S.* Typhimurium infection, where these cells may regulate CD4^{+} and CD8^{+} T cell responses via antigen presentation and NO production. Our results provide an understanding of a mechanism by which *S.* Typhimurium may induce acute immunosuppression and delay onset of protective immune responses. An immediate implication of our results is that, while phagocytes are essential for control of *S.* Typhimurium, accumulation and persistence of CD11b^{+} Ly6C^{hi} Ly6G^{-} phagocyte precursors in infected tissues could lead to a buildup of NO, a potent antimicrobial effector molecule that can also cause suppression of T cell function [154-156]. We propose a model in which NO-producing CD11b^{+} Ly6C^{hi} Ly6G^{-} immature myeloid cells provide protective functions in the host response to infection, where accumulation and persistence of these cells beyond a certain threshold level may cause collateral effects that prolong or exacerbate disease (**Figure 9**).

Consistent with a role for CD11b^{+} Ly6C^{hi} Ly6G^{-} cells in *S.* Typhimurium-induced inhibition of T cell responses, immature myeloid cells that have differentiated into inflammatory monocytes have been implicated recently in cytomegalovirus-induced suppression of antiviral CD8^{+} T cell responses [68]. Furthermore, sustained expansion of immature myeloid cells in chronic but not acute infection with lymphocytic choriomeningitis virus has been linked to inhibition of antiviral CD8^{+} T cell responses [69]. Thus, immature myeloid cells provide a balance of protective and immunosuppressive functions in the host response to infection.

The accumulation and persistence of CD11b^{+} Ly6C^{hi} Ly6G^{-} and CD11b^{+} Ly6C^{int} Ly6G^{+} cells in tissues of mice infected with *S.* Typhimurium could be the result of sustained inflammation, hyperinflammation or failure to differentiate into protective cell populations such as macrophages, dendritic cells and granulocytes. CD11b^{+} Gr-1^{+} cells are the predominant population of cells targeted by *S.* Typhimurium type III secretion *in vivo* and contain most of the total number of intracellular bacteria recovered from spleen [146]. However, the majority of splenic immature myeloid cells are not directly infected with *S.* Typhimurium (**Figure S3**) and, therefore, it is unknown if *S.* Typhimurium can directly inhibit differentiation of immature

myeloid cells through a mechanism that would require targeting of these cells by type III secretion.

Suppression of T cell function by CD11b^{+} Ly6C^{hi} Ly6G^{-} cells occurs through the iNOS metabolic pathway and may be exploited by *S*. Typhimurium to avoid clearance. Consistent with this notion, the kinetics of CD11b^{+} Ly6C^{hi} Ly6G^{-} cell accumulation and persistence correspond to the delay in onset and dampening of T cell responses to *S*. Typhimurium, which peak in weeks 3-4 after infection [129,142,145]. Furthermore, early studies linked NO-producing macrophage precursors to *S*. Typhimurium-induced immunosuppression, and protective immunity against *S*. Typhimurium to diminished expansion of CD11b^{+} Gr-1^{+} cells [110,130]. Several recent studies have associated CD11b^{+} Gr-1^{+} cells with functional immunosuppression in the host response to infection [39,42,68,69,131,157,158], and some have implicated NO production by these cells in the suppression of T cell function. Thus, a role for NO-producing immature myeloid cells in mediating immunosuppression may be a common feature of many types of infections, particularly persistent or chronic infections.

The CD11b^{+} Ly6C^{hi} Ly6G^{-} cells that accumulate and persist in tissues of mice infected with *S*. Typhimurium resemble mononuclear myeloid-derived suppressor cells (MDSC), which have been associated with immunosuppression in cancer and, more recently, infection [28,159]. Direct roles of iNOS and Arginase-1 in the suppressive activity of mononuclear MDSC are well established, whereas reactive oxygen species and peroxynitrite have emerged more recently as factors that contribute to the suppressive activity of MDSC [28,159]. Thus, production of NO, which can freely diffuse across membranes, may be only one of the immunomodulatory factors produced by CD11b^{+} Ly6C^{hi} Ly6G^{-} cells in the host response to *S*. Typhimurium.

In conclusion, our work provides an immunologic basis for a host-directed component that can exacerbate *S*. Typhimurium infection by inhibiting T cell responses. Historically, immature myeloid cells are considered essential for the protective host response to infection [29]. Here, we establish that CD11b^{+} Ly6C^{hi} Ly6G^{-} immature myeloid cells provide a balance of protective and immunosuppressive functions in the host response to *S*. Typhimurium. The tipping of this balance may be an important factor influencing the outcome of infection. Thus, CD11b^{+} Ly6C^{hi} Ly6G^{-} immature myeloid cells could prove to be a promising target for the development of innovative, broad-spectrum therapeutic approaches to overcome infection.

2.7 Acknowledgements

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Chapter 3. Essential role for CCR2$^+$ inflammatory monocytes in early and late control of persistent *Salmonella* infection

This chapter is a manuscript in preparation:

J. W. Tam, J. B. Bliska, and A. W. M. van der Velden. Essential role for CCR2$^+$ inflammatory monocytes in early and late control of persistent *Salmonella* infection. Manuscript in Preparation.

3.1 Abstract

Monocytes play a critical role in innate and adaptive immunity. Monocytes originate from progenitors in the bone marrow and traffic via the bloodstream to peripheral tissues, where they can differentiate into protective cell types such as macrophages and dendritic cells. Emigration of Ly6C^{hi} monocytes from the bone marrow is dependent on CC-chemokine receptor 2 (CCR2) and largely mediated by CC-chemokine ligand 2 (CCL2, also referred to as MCP1). We previously showed that large numbers of CD11b$^+$ Ly6C^{hi} Ly6G$^-$ monocytes accumulate and persist in tissues of mice infected with the bacterial pathogen *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium). Here, we report that *Ccr2* is required and *Ccl2* is important for the recruitment of CD11b$^+$ Ly6C^{hi} Ly6G$^-$ monocytes into tissues during persistent *S.* Typhimurium infection of hemizygous C57BL/6J *Nramp1*^{G169} mice, which are phenotypically Nramp1$^+$. We show that *Ccr2*- and *Ccl2*-deficient mice are more susceptible to *S.* Typhimurium infection than wild-type mice, with *Ccr2*-deficient mice being more susceptible to *S.* Typhimurium infection than *Ccl2*-deficient mice. We also show that depletion of CCR2$^+$ cells during the first or third week of *S.* Typhimurium infection increases susceptibility to salmonellosis. These results indicate that CCR2$^+$ cells, including CD11b$^+$ Ly6C^{hi} Ly6G$^-$ monocytes, play an essential role in early and late control of persistent *S.* Typhimurium infection.

3.2 Introduction

Salmonellae are a leading cause of morbidity and mortality in humans worldwide.[87-90] Infections with *Salmonellae* range in severity from self-limiting gastroenteritis to typhoid fever, and can lead to chronic carriage. Non-typhoidal *Salmonellae* such as *S*. Typhimurium are a leading cause of inflammatory enterocolitis and death due to foodborne illness, and a significant cause of invasive bacteremia in immunocompromised hosts. Typhoidal *Salmonellae* such as *Salmonella enterica* serovar Typhi cause systemic infections characterized by bacterial penetration of the intestinal barrier and extraintestinal dissemination to the liver and spleen, where the microorganisms survive and replicate in professional phagocytes. Septic shock and death can occur if systemic infections are left untreated.[89,137]

Much of what is known about the pathogenesis of and host response to *Salmonellae* comes from experimental infection of mice with *S*. Typhimurium, which has served as a useful model for the human disease caused by *Salmonella enterica* serovar Typhi.[105-107] During early stages of infection, macrophages, dendritic cells and neutrophilic granulocytes are critical for controlling spread and growth of *S*. Typhimurium. These cells originate from progenitors in the bone marrow and are produced by the differentiation of immature myeloid cells recruited into infected tissues.[29]

Monocytes represent a heterogeneous population of mononuclear immature myeloid cells that can be divided into circulating blood monocytes and inflammatory monocytes based on differential surface expression of CX_3C-chemokine receptor 1 (CX_3CR1) and CC-chemokine receptor 2 (CCR2), respectively.[29,149] Circulating blood monocytes enter the circulatory system from the bone marrow and, under normal conditions, provide tissues with dendritic cell and macrophage precursors.[29] During infection, homeostatic control of monocyte differentiation is overridden and inflammatory monocytes can expand in bone marrow and appear in tissues.[29] Inflammatory monocytes exhibit important antimicrobial activities and play a critical role in host defense against infection.[26,29] Although the role of inflammatory monocytes in host interactions with pathogens that cause acute infections is well established [26,29], the role of inflammatory monocytes in host interactions with pathogens that cause chronic infections is not well understood.

Using a murine model of persistent salmonellosis, we recently showed that CD11b^{+} Ly6C^{hi} Ly6G^{-} monocytic cells accumulate and remain in tissues of mice infected with *S.* Typhimurium.[136] We also showed that these cells could differentiate into macrophage-like cells following *ex vivo* culture and present antigen to T cells *in vitro*.[136] However, significant proliferation of the T cells was observed only when the ability of the CD11b^{+} Ly6C^{hi} Ly6G^{-} cells to produce nitric oxide was blocked.[136] Although our work indicated that CD11b^{+} Ly6C^{hi} Ly6G^{-} monocytic cells recruited in response to persistent *S.* Typhimurium infection exhibit protective and immunosuppressive properties, the role of these cells in the host response to *S.* Typhimurium infection has remained poorly defined.

Here, we found that *Ccr2* is required and *Ccl2* is important for the recruitment of Ly6C^{hi} Ly6G^{-} CD11b^{+} cells into tissues during persistent salmonellosis. In addition, we found that *Ccr2*- and *Ccl2*-deficient mice are more susceptible to persistent *S.* Typhimurium infection than wild-type mice, with *Ccr2*-deficient mice being more susceptible than *Ccl2*-deficient mice. We also found that depletion of CCR2^{+} cells during the first or third week of *S.* Typhimurium infection increases susceptibility to persistent salmonellosis. These findings indicate that CCR2^{+} cells, including CD11b^{+} Ly6C^{hi} Ly6G^{-} monocytes, play an essential role in early and late control of *S.* Typhimurium infection.

3.3 Materials and Methods

Ethics Statement. All procedures using mice were approved by the Institutional Animal Care and Use Committee at Stony Brook University, and were conducted in accordance with the recommendations outlined in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All procedures using mice were designed to use the fewest number of mice possible but still achieve meaningful results. Euthanasia of mice was performed by inhalation of carbon dioxide, a method consistent with the recommendations of the Panel on Euthanasia of the American Veterinary Medical Association.

Bacterial Strains and Culture Conditions. *S.* Typhimurium strain IR715 [150], which is a spontaneous, nalidixic acid-resistant derivative of *S.* Typhimurium strain 14028 (American Type Culture Collection), was used as the wild-type (WT) strain. Using standard microbiological techniques, bacteria were grown aerobically at 37°C in Luria-Bertani (LB) broth or on LB agar supplemented with nalidixic acid (50 μg/ml).

Mice. C57BL/6J *Nramp1G169* transgenic mice [160], which encode a functional *Nramp1/Slc11a1* gene, are phenotypically Nramp1^{+} and were used as the WT strain. The *Nramp1* gene encodes a divalent metal transporter that enhances host resistance to a number of intracellular pathogens, including *S.* Typhimurium, by limiting essential metal availability within the phagocyte phagosome.[160] A breeder pair of C57BL/6J *Nramp1G169* transgenic mice was generously provided by Dr. Ferric Fang (University of Washington). Homozygous C57BL/6J *Nramp1G169* mice were crossed to C57BL/6J mice (The Jackson Laboratory), which lack a functional *Nramp1/Slc11a1* gene and, therefore, are phenotypically Nramp1^{-}. In addition, homozygous C57BL/6J *Nramp1G169* mice were crossed to B6.129S4-*Ccr2*^{tm1Ifc}/J, B6.129S4-*Ccl*^{2tm1Rol}/J mice (both from The Jackson Laboratory), or *Caspase-1/11*-deficient (*Casp-1/11*$^{-/-}$) mice in the C57BL/6J strain background [161] (a generous gift from Abbott Bioresearch Center). B6.129S4-*Ccr2*^{tm1Ifc}/J mice lack a functional *Ccr2* gene, B6.129S4-*Ccl*^{2tm1Rol}/J mice lack a functional *Ccl2* gene, and both strains of mice lack a functional *Nramp1/Slc11a1* gene. Lastly, homozygous C57BL/6J *Nramp1G169* were crossed to CCR2-DTR transgenic C57BL/6J mice, which express cyan fluorescent protein (CFP) and simian diphtheria toxin receptor (DTR) under control of the CCR2 promoter. [33] A breeder pair of CCR2-DTR transgenic mice, which lack a functional *Nramp1/Slc11a1* gene, was generously provided by Dr. Eric Pamer (Memorial Sloan

Kettering Cancer Center). The resulting hemizygous C57BL/6J *Nramp1G169* mice, *Casp-1/11*$^{-/-}$ C57BL/6J *Nramp1G169* mice, *Ccr2*-deficient (*Ccr2*$^{-/-}$) C57BL/6J *Nramp1*^{G169} mice, *Ccl2*-deficient (*Ccl2*$^{-/-}$) C57BL/6J *Nramp1*^{G169} mice, and CCR2-DTR C57BL/6J *Nramp1*^{G169} mice encoded a functional *Nramp1* allele and, therefore, were phenotypically Nramp1^{+}.

Mouse infections and assays. Mouse infections were performed using naïve, 8-24-week-old sex-matched mice. Briefly, mice were inoculated intravenously with $5x10^3$ colony forming units (CFU) of *S.* Typhimurium strain IR715 suspended in 0.1 ml of PBS, unless indicated otherwise. Ten-fold serial dilutions of the inoculum were plated on LB agar to confirm the inoculum titer. The mice were monitored for survival or sacrificed at various times post-inoculation, after which target organs (i.e. spleen, liver and bone marrow) were harvested and processed for analysis by flow cytometry or organ burden assay. Where indicated, *S.* Typhimurium-infected WT and CCR2-DTR C57BL/6J *Nramp1*^{G169} mice were injected intraperitoneally with PBS or 20 ng/g of diphtheria toxin (DT, List Biological Laboratories). The mice were monitored for survival or sacrificed at various times post-administration of PBS or DT, after which target organs were harvested and processed for analysis by flow cytometry or organ burden assay. Bacterial loads in target organs were determined by lysing cells from a single cell suspension with Triton X-100 (0.05%) and plating for CFU on LB agar containing nalidixic acid (50 μg/ml). Mice infected with *S.* Typhimurium were euthanized when moribund or at the termination of the experiment.

Cell staining and analysis by flow cytometry. All antibodies and reagents described in this section were purchased from BioLegend, unless indicated otherwise. Routinely, cells were stained in the presence of Fc block (anti-mouse CD16/32 antibody; clone 93) using anti-mouse antibodies specific for Ly6C (clone HK1.4), Ly6G (clone 1A8), CD11b (clone M1/70), and F4/80 (clone CI:A3-1). For intracellular cytokine staining, cells were cultured directly *ex vivo* in RP-10 medium (RPMI1640 medium (Invitrogen) supplemented with 10% fetal bovine serum, 0.2 M L-glutamine, 0.1 M HEPES, 50 μM 2-ME, and 1 % penicillin and streptomycin) containing 1x brefeldin A (BioLegend) and 10 μg/ml anti-mouse CD3ε (BioLegend; clone 145-2C11). After 4 hours of incubation at 37°C in 5% CO_2, cells were stained in the presence of Fc block using anti-mouse antibodies specific for CD4 (clone RM4-5), CD8β (clone YTS156.7.7), and CD90.2 (clone 30-H12). The cells were then fixed and permeabilized according to the manufacturer's protocol, and stained in the presence of Fc block using anti-mouse IFN-γ (clone

XMG1.2). Data were acquired and analyzed using a BD FACSCalibur flow cytometer (BD Biosciences) with BD CellQuestPro (BD Biosciences) and FlowJo (Tree Star) software or a BD FACScan flow cytometer (BD Biosciences) with Digital Extra Parameter upgrade (Cytek) and FlowJo Collectors' Edition software (Cytek).

Statistical analysis. Statistical analysis was performed using Prism 5.0b (GraphPad Software). Data were analyzed using a Mann-Whitney t-test, log-rank test, or one-way or two-way ANOVA with Bonferroni's multiple comparisons posttest; p values < 0.05 were considered to be statistically significant. Asterisks indicate statistically significant differences (*** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$).

3.4 Results

Large numbers of CD11b$^+$ Ly6C^{hi} Ly6G$^-$ and CD11b$^+$ Ly6C^{int} Ly6G$^+$ cells accumulate and persist in tissues of C57BL/6J *Nramp1^{G169}* mice infected with *S.* Typhimurium. We previously showed that CD11b$^+$ Ly6C^{hi} Ly6G$^-$ and CD11b$^+$ Ly6C^{int} Ly6G$^+$ cells accumulate and persist in tissues of C57BL/6J and 129X1/SvJ mice infected with *S.* Typhimurium.[136] C57BL/6J mice have been used extensively to study host interactions with *S.* Typhimurium. These mice lack a functional *Nramp1/Slc11a1* gene, which encodes the natural resistance-associated macrophage protein 1 (Nramp1, also referred to as Slc11a1) divalent metal transporter. This transporter enhances host resistance to a number of intracellular pathogens, including *S.* Typhimurium, by limiting essential metal availability within the phagocyte phagosome.[160] C57BL/6J mice are phenotypically Nramp1$^-$ due to a G169D mutation in the *Nramp1/Slc11a1* gene and are highly susceptible to acute salmonellosis. In contrast, 129X1/SvJ mice are phenotypically Nramp1$^+$ and have been used as model hosts to study persistent salmonellosis.[104,136,145]

To dissect innate immune mechanisms that control spread and growth of *S.* Typhimurium during persistent salmonellosis, we needed to use various gene-deficient mice available only in the C57BL/6J strain background. To limit the number of genetic crosses necessary to be able to perform experiments aimed at dissecting innate immune mechanisms that control spread and growth of *S.* Typhimurium during persistent salmonellosis, we used C57BL/6J *Nramp1^{G169}* mice as the WT strain instead of 129X1/SvJ mice. C57BL/6J *Nramp1^{G169}* mice are transgenic C57BL/6J mice that encode a functional *Nramp1/Slc11a1* gene.[160] These mice are phenotypically Nramp1$^+$ and have been used as model hosts to study persistent salmonellosis.[162,163] We infected C57BL/6J *Nramp1^{G169}* mice with *S.* Typhimurium and analyzed the recruitment of CD11b$^+$ Ly6C^{hi} Ly6G$^-$ and CD11b$^+$ Ly6C^{int} Ly6G$^+$ cells into tissues. C57BL/6J *Nramp1^{G169}* mice left uninfected were used as controls. Consistent with our published results[136], we found that large numbers of CD11b$^+$ Ly6C^{hi} Ly6G$^-$ and CD11b$^+$ Ly6C^{int} Ly6G$^+$ cells accumulate and persist in spleens (**Figures 1A and 1B**) and livers (**Figures 1C and 1D**) of C57BL/6J *Nramp1^{G169}* mice infected with *S.* Typhimurium. The response of CD11b$^+$ Ly6C^{hi} Ly6G$^-$ and CD11b$^+$ Ly6C^{int} Ly6G$^+$ cells correlated with bacterial persistence over a period of at least 60 days (**Figures 1E and 1F**). Collectively, these results indicate that C57BL/6J

Nramp1^{G169} mice can be used to dissect innate immune mechanisms that control spread and growth of *S*. Typhimurium during persistent salmonellosis.

Recruitment of CD11b^{+} Ly6C^{hi} Ly6G^{-} cells is normal in *Casp-1/11*$^{-/-}$ C57BL/6J *Nramp1*^{G169} mice infected with *S*. Typhimurium. Caspase-1 and caspase-11 are pro-inflammatory caspases that play a key role in innate immunity.[164,165] Activation of these caspases causes pyroptotic cell death and secretion of IL-1β and IL-18, two important pro-inflammatory cytokines that contribute to innate immune defense against *S*. Typhimurium infection.[164-168] Here, we crossed the C57BL/6J *Nramp1*^{G169} mice to *Casp-1/11*$^{-/-}$ C57BL/6J mice and used the resulting *Casp-1/11*$^{-/-}$ C57BL/6J *Nramp1*^{G169} mice to establish the role of caspase-1 and caspase-11 in the recruitment of CD11b^{+} Ly6C^{hi} Ly6G^{-} cells during persistent salmonellosis. Specifically, we infected WT and *Casp-1/11*$^{-/-}$ C57BL/6J *Nramp1*^{G169} mice with *S*. Typhimurium for 10 or 30 days. WT and *Casp-1/11*$^{-/-}$ C57BL/6J *Nramp1*^{G169} mice left uninfected were used as controls. Consistent with published evidence[168,169], we found significant differences in bacterial loads as determined by organ burden assays (**Figures 2A and 2B**). However, we found no significant differences in the recruitment of CD11b^{+} Ly6C^{hi} Ly6G^{-} cells into spleen (**Figure 2C**) and liver (**Figure 2D**) as determined by flow cytometry. These results suggest that caspase-1 and caspase-11 are not required for the recruitment of CD11b^{+} Ly6C^{hi} Ly6G^{-} and CD11b^{+} Ly6C^{int} Ly6G^{+} cells into tissues during persistent salmonellosis.

***Ccr2* is required for the recruitment of CD11b^{+} Ly6C^{hi} Ly6G^{-} cells into tissues during persistent salmonellosis.** CC-chemokine receptor 2 (CCR2) plays a critical role in the emigration of Ly6C^{hi} monocytes from bone marrow.[26,72] Here, we crossed the C57BL/6J *Nramp1*^{G169} mice to *Ccr2*$^{-/-}$ C57BL/6J mice and used the resulting *Ccr2*$^{-/-}$ C57BL/6J *Nramp1*^{G169} mice to define the role of CCR2 in the host response to persistent salmonellosis. Specifically, we infected WT and *Ccr2*$^{-/-}$ C57BL/6J *Nramp1*^{G169} mice with *S*. Typhimurium and analyzed the recruitment of CD11b^{+} Ly6C^{hi} Ly6G^{-} cells into tissues. WT and *Ccr2*$^{-/-}$ C57BL/6J *Nramp1*^{G169} mice left uninfected were used as controls. After 10 days of infection, we found significant accumulation of CD11b^{+} Ly6C^{hi} Ly6G^{-} cells in spleens of WT but not *Ccr2*$^{-/-}$ C57BL/6J *Nramp1*^{G169} mice (**Figures 3A**). In addition, we found significant accumulation of CD11b^{+} Ly6C^{hi} Ly6G^{-} cells in bone marrow of both WT and *Ccr2*$^{-/-}$ C57BL/6J *Nramp1*^{G169} mice, with relative accumulation being the greatest in *Ccr2*$^{-/-}$ C57BL/6J

Nramp1^{G169} mice (**Figure 3B**). As expected for granulocytes and their precursors, which do not require CCR2 to emigrate from bone marrow[29], we found significant, comparable accumulation of CD11b^{+} Ly6C^{int} Ly6G^{+} cells in spleens of WT and *Ccr2*$^{-/-}$ C57BL/6J *Nramp1*^{G169} mice (**Figure 3C**). Collectively, these results indicate that *Ccr2* is required for the recruitment of CD11b^{+} Ly6C^{hi} Ly6G^{-} cells into tissues during persistent salmonellosis. Furthermore, these results suggest that CD11b^{+} Ly6C^{hi} Ly6G^{-} cells may accumulate in bone marrow of *Ccr2*$^{-/-}$ C57BL/6J *Nramp1*^{G169} mice persistently infected with *S.* Typhimurium.

***Ccl2* is important for the recruitment of CD11b^{+} Ly6C^{hi} Ly6G^{-} cells into tissues during persistent salmonellosis.** CC-chemokine ligand 2 (CCL2) is a CCR2 ligand that plays an important role in the emigration of Ly6C^{hi} monocytes from bone marrow.[26] Here, we crossed the C57BL/6J *Nramp1*^{G169} mice to *Ccl2*$^{-/-}$ C57BL/6J mice and used the resulting *Ccl2*$^{-/-}$ C57BL/6J *Nramp1*^{G169} mice to define the role of CCL2 in the host response to persistent salmonellosis. Specifically, we infected WT and *Ccl2*$^{-/-}$ C57BL/6J *Nramp1*^{G169} mice with *S.* Typhimurium and analyzed the recruitment of CD11b^{+} Ly6C^{hi} Ly6G^{-} cells into tissues. WT and *Ccl2*$^{-/-}$ C57BL/6J *Nramp1*^{G169} mice left uninfected were used as controls. At various times after infection, we found significant accumulation of CD11b^{+} Ly6C^{hi} Ly6G^{-} cells in spleens of both WT and *Ccl2*$^{-/-}$ C57BL/6J *Nramp1*^{G169} mice, with relative accumulation being the smallest in *Ccl2*$^{-/-}$ C57BL/6J *Nramp1*^{G169} mice (**Figure 4A**). In addition, we found significant accumulation of CD11b^{+} Ly6C^{hi} Ly6G^{-} cells in bone marrow of both WT and *Ccl2*$^{-/-}$ C57BL/6J *Nramp1*^{G169} mice, with relative accumulation being the greatest in *Ccl2*$^{-/-}$ C57BL/6J *Nramp1*^{G169} mice (**Figure 4B**). Consistent with our results indicating that *Ccr2* is not required for the emigration of granulocytic cells from bone marrow (Figure 3C), we found significant, comparable accumulation of CD11b^{+} Ly6C^{int} Ly6G^{+} cells in spleens of WT and *Ccl2*$^{-/-}$ C57BL/6J *Nramp1*^{G169} mice (**Figures 4C**). Collectively, these results indicate that *Ccl2* is important for the recruitment of CD11b^{+} Ly6C^{hi} Ly6G^{-} cells into tissues during persistent salmonellosis. Furthermore, these results suggest that CD11b^{+} Ly6C^{hi} Ly6G^{-} cells accumulate in bone marrow of *Ccl2*$^{-/-}$ C57BL/6J *Nramp1*^{G169} mice persistently infected with *S.* Typhimurium.

Increased susceptibility of *Ccr2*$^{-/-}$ and *Ccl2*$^{-/-}$ C57BL/6J *Nramp1*^{G169} mice to *S.* Typhimurium infection. To establish the role of CCR2 and CCL2 in susceptibility to persistent salmonellosis, we infected WT, *Ccr2*$^{-/-}$ and *Ccl2*$^{-/-}$ C57BL/6J *Nramp1*^{G169} mice with *S.*

Typhimurium and performed survival assays. We found that both *Ccr2*$^{-/-}$ and *Ccl2*$^{-/-}$ C57BL/6J *Nramp1*^{G169} mice succumbed to *S.* Typhimurium infection significantly faster than WT C57BL/6J *Nramp1*^{G169} mice (**Figure 5A**). Consistent with our results indicating that CCR2 is more important than CCL2 with respect to the recruitment of CD11b^{+} Ly6C^{hi} Ly6G^{-} cells into tissues during persistent salmonellosis (Figures 3 and 4), we found that *Ccr2*$^{-/-}$ C57BL/6J *Nramp1*^{G169} mice succumbed to *S.* Typhimurium infection significantly faster than *Ccl2*$^{-/-}$ C57BL/6J *Nramp1*^{G169} mice (**Figure 5A**). We next performed organ burden assays and found that, after 1 day of infection, similar numbers of *S.* Typhimurium were recovered from spleens of WT, *Ccr2*$^{-/-}$, and *Ccl2*$^{-/-}$ C57BL/6J *Nramp1*^{G169} mice (**Figure 5B**). However, after 10 or 28 days of infection, significantly higher numbers of *S.* Typhimurium were present in spleens and livers of *Ccr2*$^{-/-}$ and *Ccl2*$^{-/-}$ C57BL/6J *Nramp1*^{G169} mice than in spleens of WT C57BL/6J *Nramp1*^{G169} mice (**Figures 5C and 5D**). Collectively, these results indicate that *Ccr2*$^{-/-}$ and *Ccl2*$^{-/-}$ C57BL/6J *Nramp1*^{G169} mice are more susceptible to *S.* Typhimurium infection than WT C57BL/6J *Nramp1*^{G169} mice, with *Ccr2*$^{-/-}$ C57BL/6J *Nramp1*^{G169} mice being most susceptible to *S.* Typhimurium infection.

CCR2^{+} cells play an essential role in early and late control of persistent *S.* Typhimurium infection. An immediate implication of our results indicating that *Ccr2*$^{-/-}$ C57BL/6J *Nramp1*^{G169} mice exhibit increased susceptibility to *S.* Typhimurium infection (Figure 5) is that CCR2^{+} cells play an important role in the protective host response to persistent salmonellosis. Because recruitment of CD11b^{+} Ly6C^{hi} Ly6G^{-} monocytes and other CCR2^{+} cells in *Ccr2*$^{-/-}$ C57BL/6J *Nramp1*^{G169} mice is defective throughout the course of infection, the role of these cells at different stages during infection could not be established. Therefore, we crossed the C57BL/6J *Nramp1*^{G169} mice to CCR2-DTR C57BL/6J mice[33] and used the resulting CCR2-DTR C57BL/6J *Nramp1*^{G169} mice to define the role of CCR2^{+} cells in early and late control of persistent salmonellosis. CCR2-DTR C57BL/6J mice are transgenic mice that express cyan fluorescent protein (CFP) and simian diphtheria toxin receptor (DTR) under control of the CCR2 promoter. These mice have been used for the identification and diphtheria toxin (DT)-mediated depletion of CCR2^{+} cells, respectively.[33,78,84]

Here, we infected CCR2-DTR C57BL/6J *Nramp1*^{G169} mice with *S.* Typhimurium and analyzed the recruitment of CD11b^{+} Ly6C^{hi} Ly6G^{-} cells into spleen using flow cytometry. WT

C57BL/6J *Nramp1*^{G169} mice (littermates) infected with *S.* Typhimurium were used as controls. After 21 days of infection, we found that the vast majority of splenic CD11b^{+} Ly6C^{hi} Ly6G^{-} cells expressed CFP and, therefore, CCR2 (**Figures 6A and 6B, and data not shown**). As expected, splenic CD11b^{+} Ly6C^{int} Ly6G^{+} and CD11b^{-} cells did not express CFP, and, therefore, not CCR2 (**Figure 6B and data not shown**). After 21 days of *S.* Typhimurium infection, we administered DT or PBS to the mice and, after three days, analyzed the recruitment of CD11b^{+} Ly6C^{hi} Ly6G^{-} and CD11b^{+} Ly6C^{int} Ly6G^{+} cells into spleen using flow cytometry and performed organ burden assays.

We found that the administration of DT but not PBS to *S.* Typhimurium-infected CCR2-DTR C57BL/6J *Nramp1*^{G169} mice caused depletion of splenic and hepatic CD11b^{+} Ly6C^{hi} Ly6G^{-} but not CD11b^{+} Ly6C^{int} Ly6G^{+} cells (**Figures 6C, 6D, S1A, and S1B**). Administration of DT to *S.* Typhimurium-infected CCR2-DTR C57BL/6J *Nramp1*^{G169} mice also caused a reduction in splenohepatomegaly (**Figures S1C and S1D)** and IFN-γ production by T cells (**Figure S1E**). Furthermore, we found significantly higher numbers of *S.* Typhimurium in spleens and livers of DT-treated CCR2-DTR C57BL/6J *Nramp1*^{G169} mice as compared to PBS-treated CCR2-DTR C57BL/6J *Nramp1*^{G169} mice, or DT- or PBS-treated WT mice (**Figures 6E and S1F**). Consistent with published evidence[33], we found that the number of CD11b^{+} Ly6C^{hi} Ly6G^{-} cells returned to near normal levels by day 5 after administration of DT (**Figure 6F**), indicating that DT-mediated depletion of CD11b^{+} Ly6C^{hi} Ly6G^{-} cells was transient. Interestingly, bacterial numbers recovered from spleen continued to rise despite the re-emergence of CD11b^{+} Ly6C^{hi} Ly6G^{-} cells (**Figure 6G**), suggesting that control of persistent *S.* Typhimurium infection, once lost, could not be re-established.

We next infected CCR2-DTR C57BL/6J *Nramp1*^{G169} mice with *S.* Typhimurium, administered DT or PBS on day 5 or 21 after infection, and performed survival assays. Consistent with our results indicating that transient depletion of CCR2^{+} cells in *S.* Typhimurium-infected CCR2-DTR C57BL/6J *Nramp1*^{G169} mice causes an increase in bacterial burden (Figures 6D-F and S1F), we found that *S.* Typhimurium-infected CCR2-DTR C57BL/6J *Nramp1*^{G169} mice treated with DT succumbed significantly faster than those treated with PBS (**Figure 6H**). Unexpectedly, we found that, regardless of the time point at which DT was administered, all *S.* Typhimurium-infected CCR2-DTR C57BL/6J *Nramp1*^{G169} mice died (**Figure 6H**). Collectively,

these results demonstrate that $CCR2^{+}$ cells play an essential role in early and late control of persistent *S*. Typhimurium infection.

3.5 Figures

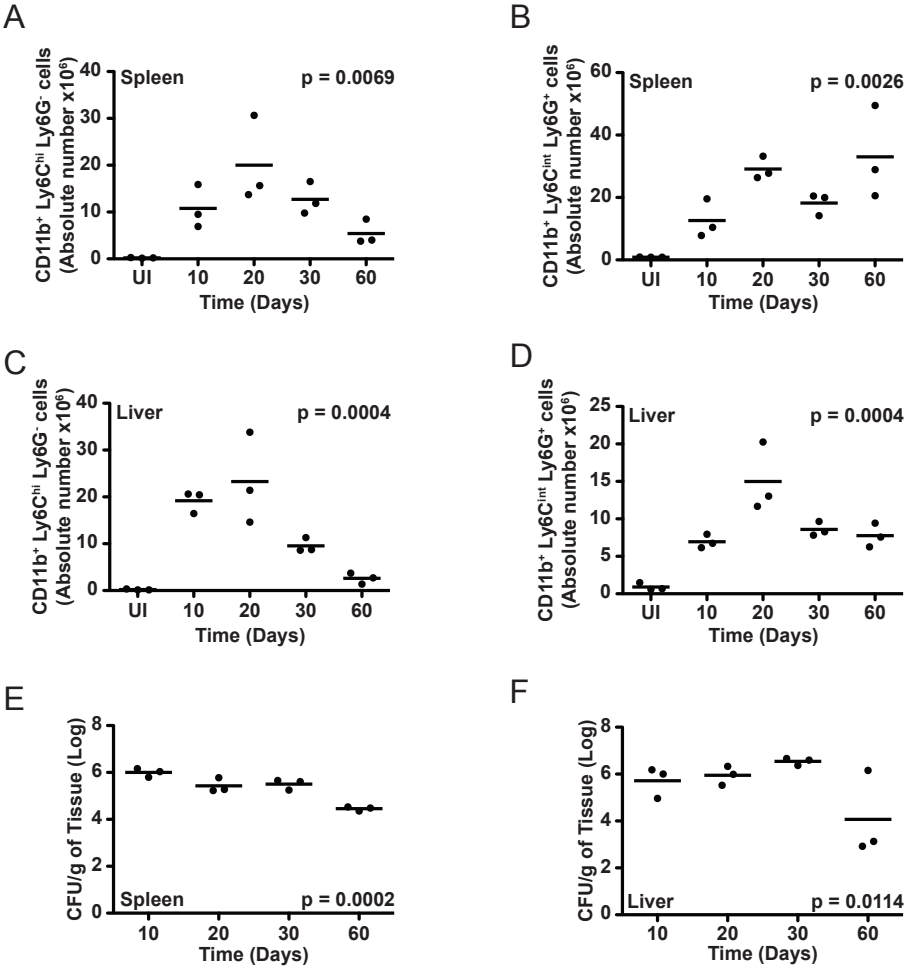


Figure 1. Large numbers of CD11b$^+$ Ly6C^{hi} Ly6G$^-$ and CD11b$^+$ Ly6C^{int} Ly6G$^+$ cells accumulate and persist in tissues of C57BL/6J *Nramp1*^{G169} mice infected with *S.* Typhimurium. (*A-D*) Absolute numbers of CD11b$^+$ Ly6C^{hi} Ly6G$^-$ (A, C) and CD11b$^+$ Ly6C^{int} Ly6G$^+$ cells (B, D) in spleens (A, B) and livers (C, D) of C57BL/6J *Nramp1*^{G169} mice (n = 3 per time point) left uninfected (UI) or infected with *S.* Typhimurium. Mice were inoculated intravenously with 5 x 10^3 CFU of *S.* Typhimurium. At various times post-infection, tissues were harvested and processed for flow cytometric analysis or organ burden assay. **(*E, F*)** Corresponding bacterial loads per gram of spleen (E) and liver (F) tissue. Data show mean with spread from a single experiment that is representative of two independent experiments. Data were analyzed using one-way ANOVA with Bonferroni's multiple comparisons post-test; *p* values < 0.05 were considered to be statistically significant.

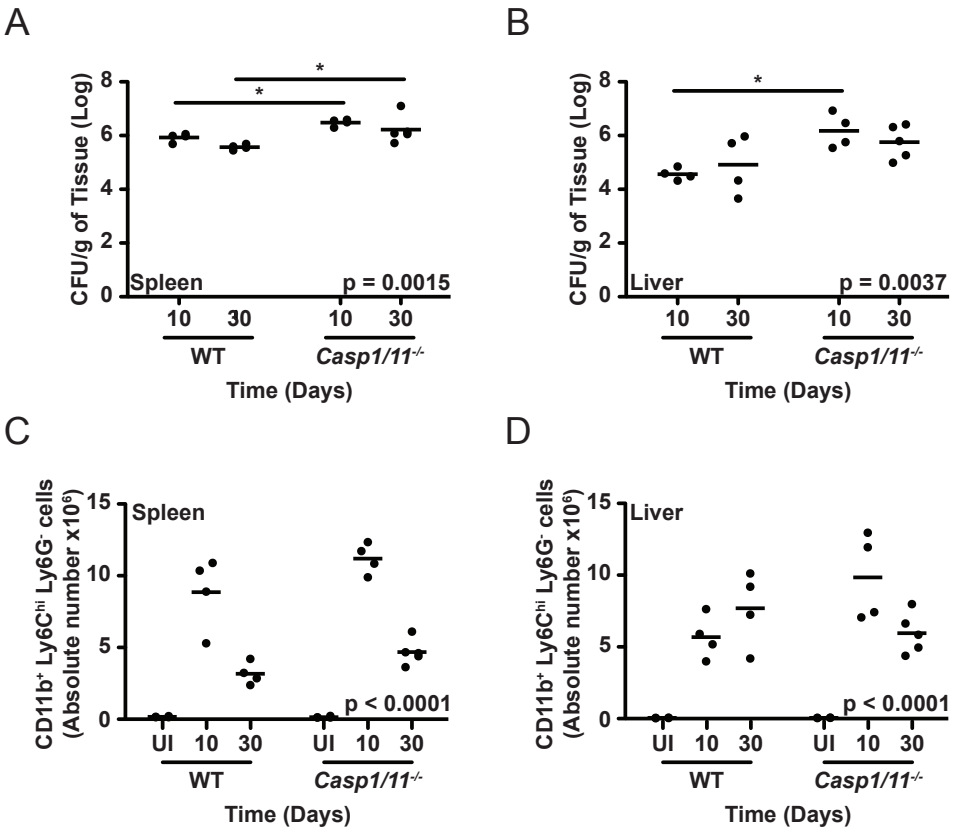


Figure 2. Recruitment of CD11b^{+}Ly6C^{hi}Ly6G^{-} cells is normal in *Casp-1/11*$^{-/-}$ C57BL/6J *Nramp1*^{G169} mice infected with *S.* Typhimurium. (*A, B*) Bacterial loads per gram of spleen (A) and liver (B) tissue harvested from C57BL/6J *Nramp1*^{G169} and *Casp-1/11*$^{-/-}$ C57BL/6J *Nramp1*^{G169} mice infected with *S.* Typhimurium (n = 4-5 per group per time point). Mice were inoculated intravenously with 5 x 10^3 CFU of *S.* Typhimurium. At various times post-infection, tissues were harvested and processed for organ burden assay or flow cytometric analysis. **(*C, D*)** Absolute numbers of CD11b^{+}Ly6C^{hi}Ly6G^{-} cells in spleens (C) and livers (D) of C57BL/6J *Nramp1*^{G169} and *Casp-1/11*$^{-/-}$ C57BL/6J *Nramp1*^{G169} mice (n = 4-5 per group per time point) left uninfected (UI) or infected with *S.* Typhimurium. Data show mean with spread from a single experiment that is representative of at least two independent experiments. Data were analyzed using a two-way ANOVA with Bonferroni's post-test; p values < 0.05 were considered to be statistically significant. Asterisks indicate statistically significant differences (* $p < 0.05$).

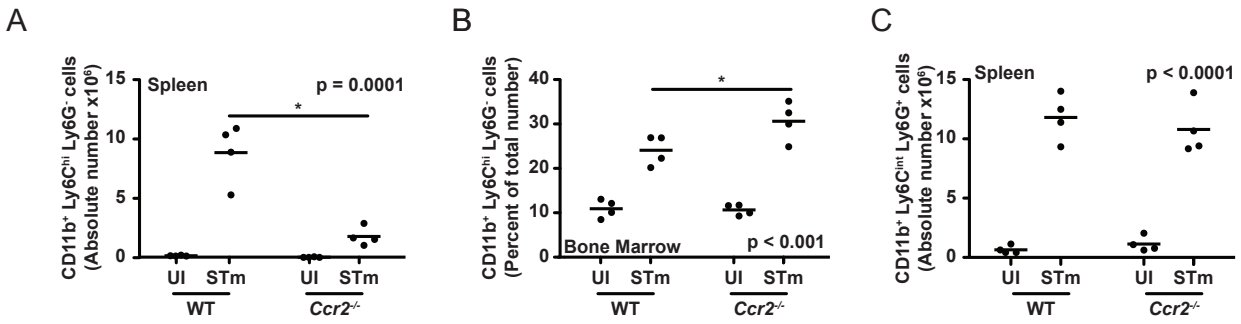


Figure 3. *Ccr2* is required for the recruitment of CD11b^{+} Ly6C^{hi} Ly6G^{-} cells during persistent salmonellosis. (*A*) Absolute numbers of CD11b^{+} Ly6C^{hi} Ly6G^{-} cells in spleens of WT and *Ccr2*$^{-/-}$ C57BL/6J *Nramp1*^{G169} mice (n = 4 per time point) left uninfected (UI) or infected for 10 days with *S*. Typhimurium (STm). Mice were inoculated intravenously with 5 x 10^3 CFU of *S*. Typhimurium. After 10 days of infection, spleens were harvested and processed for flow cytometric analysis. **(*B*)** Corresponding percentages of CD11b^{+} Ly6C^{hi} Ly6G^{-} cells in bone marrow. **(*C*)** Absolute numbers of CD11b^{+} Ly6C^{int} Ly6G^{+} cells in spleen of WT and *Ccr2*$^{-/-}$ C57BL/6J *Nramp1*^{G169} mice (n = 4 per time point) left uninfected (UI) or infected for 10 days with *S*. Typhimurium. Data show mean with spread from a single experiment that is representative of two independent experiments. Data were analyzed using two-way ANOVA with Bonferroni's post-test; *p* values < 0.05 were considered to be statistically significant. Asterisks indicate statistically significant differences (* p < 0.05).

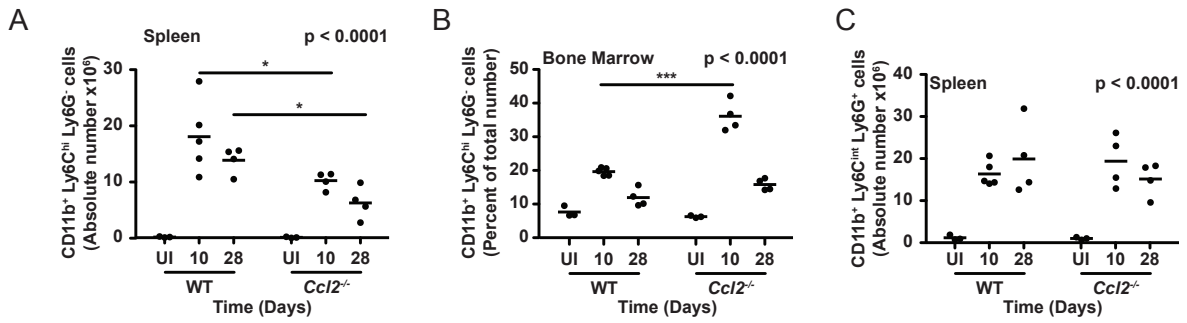


Figure 4. *Ccl2* is important for the recruitment of CD11b^{+} Ly6C^{hi} Ly6G^{-} cells during persistent salmonellosis. (*A*) Absolute numbers of CD11b^{+} Ly6C^{hi} Ly6G^{-} cells in spleens of WT and *Ccl2*$^{-/-}$ C57BL/6J *Nramp1*^{G169} mice (n = 4-5 per time point) left uninfected (UI) or infected for 10 or 28 days with *S.* Typhimurium. Mice were inoculated intravenously with 5 x 10^3 CFU of *S.* Typhimurium. After 10 or 28 days of infection, spleens were harvested and processed for flow cytometric analysis. **(*B*)** Corresponding percentages of CD11b^{+} Ly6C^{hi} Ly6G^{-} cells in bone marrow. **(*C*)** Absolute numbers of CD11b^{+} Ly6C^{int} Ly6G^{+} cells in spleen of WT and *Ccl2*$^{-/-}$ C57BL/6J *Nramp1*^{G169} mice (n = 4 per time point) left uninfected (UI) or infected for 10 or 28 days with *S.* Typhimurium. Data show mean with spread from a single experiment that is representative of two independent experiments. Data were analyzed using two-way ANOVA with Bonferroni's post-test; *p* values < 0.05 were considered to be statistically significant. Asterisks indicate statistically significant differences (*** $p < 0.001$, * $p < 0.05$).

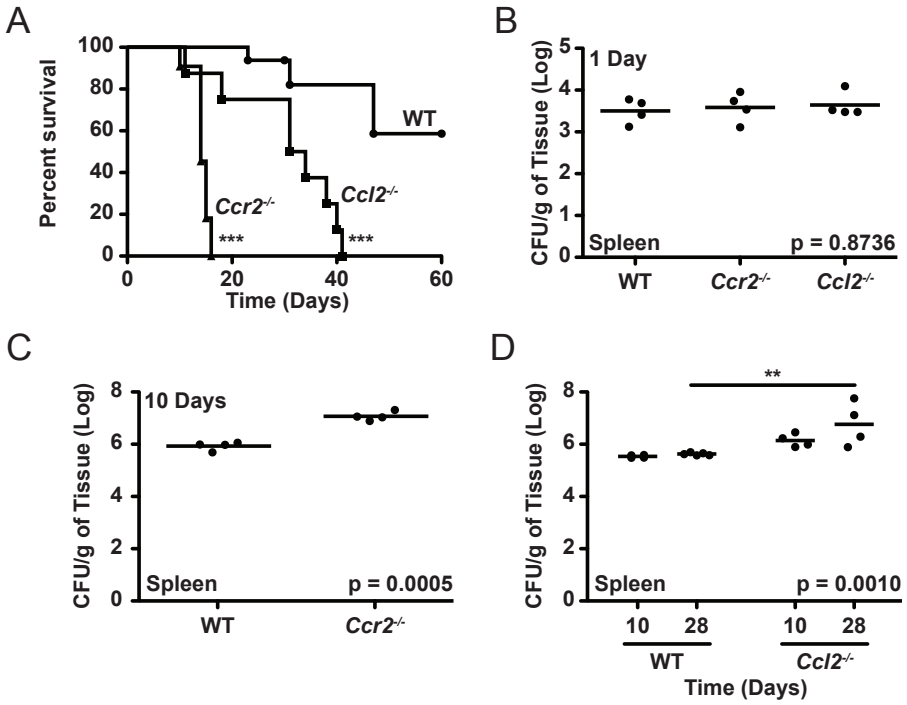
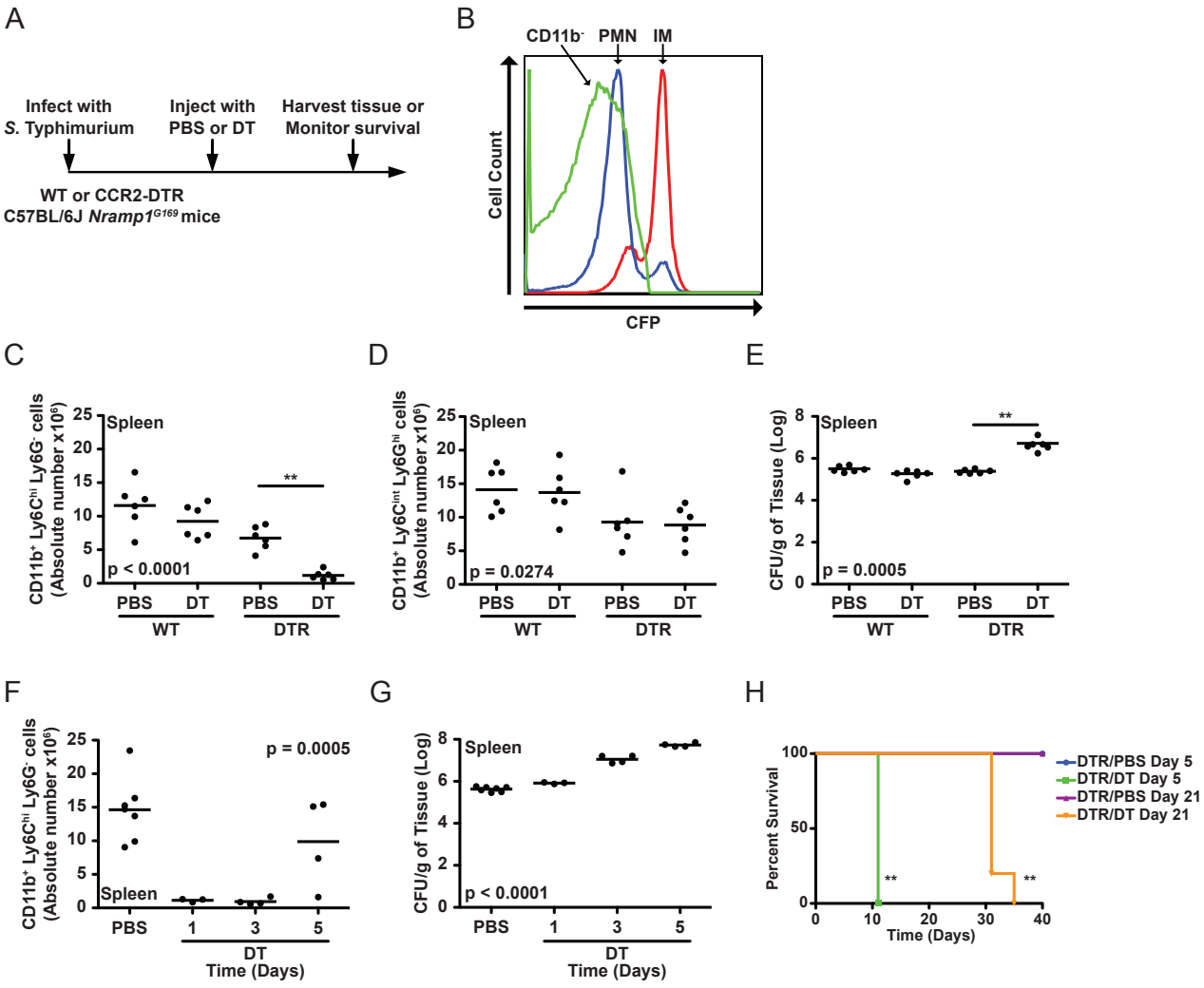


Figure 5. Increased susceptibility of *Ccr2*$^{-/-}$ and *Ccl2*$^{-/-}$ C57BL/6J *Nramp1*^{G169} mice to *S.* Typhimurium infection. (*A*) Survival of WT, *Ccr2*$^{-/-}$ and *Ccl2*$^{-/-}$ C57BL/6J *Nramp1*^{G169} mice (n = 8-16 per group) inoculated intravenously with 5 x 10^3 CFU of *S.* Typhimurium; $p < 0.0001$. **(*B-D*)** Bacterial loads per gram of spleen tissue harvested from WT, *Ccr2*$^{-/-}$ and *Ccl2*$^{-/-}$ C57BL/6J *Nramp1*^{G169} mice infected with *S.* Typhimurium as in (A). On day 1 (B), day 10 (C, D) or 28 (D) post-infection, spleens were harvested and processed for organ burden assay. Data are cumulative from two independent experiments (A) or show mean with spread from a single experiment that is representative of two independent experiments (B-D). Data were analyzed using log-rank test (A), one-way ANOVA with Bonferroni's multiple comparisons post-test (B), Mann-Whitney *t*-test (C) or two-way ANOVA with Bonferroni's post-test (D); *p* values < 0.05 were considered to be statistically significant. Asterisks indicate statistically significant differences (*** $p < 0.001$, ** $p < 0.01$).


A
Infect with
S. Typhimurium
Inject with
PBS or DT
Harvest tissue or
Monitor survival
WT or CCR2-DTR
C57BL/6J Nramp1G169 mice
B
CD11b-
PMN
IM
Cell Count
CFP
C
CD11b+ Ly6Chi Ly6G- cells
(Absolute number x10^6)
Spleen
p < 0.0001
**
PBS
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DTR
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CD11b+ Ly6Cint Ly6Ghi cells
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PBS
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E
CFU/g of Tissue (Log)
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p = 0.0005
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CD11b+ Ly6Chi Ly6G- cells
(Absolute number x10^6)
p = 0.0005
Spleen
PBS
1
3
5
DT
Time (Days)
G
CFU/g of Tissue (Log)
Spleen
p < 0.0001
PBS
1
3
5
DT
Time (Days)
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Percent Survival
**
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Time (Days)
DTR/PBS Day 5
DTR/DT Day 5
DTR/PBS Day 21
DTR/DT Day 21

Figure 6. CCR2$^+$ cells play an essential role in early and late control of *S.* Typhimurium infection. (*A*) Overview of experimental design. WT or CCR2-DTR C57BL/6J *Nramp1*^{G169} mice were inoculated intravenously with 5 x 10^3 CFU of *S.* Typhimurium. CCR2-DTR C57BL/6J *Nramp1*^{G169} mice encode a functional *Nramp1* gene and express cyan fluorescent protein (CFP) and simian diphtheria toxin receptor (DTR) under control of the CCR2 promoter. At various times post-infection, the mice were given PBS or 20 ng/g of diphtheria toxin (DT). The mice were then monitored for survival or sacrificed at various times post-treatment, after which spleens were harvested and processed for analysis by flow cytometry or organ burden assay. **(*B*)** Expression of cyan fluorescent protein (CFP) by CD11b$^+$ Ly6C^{hi} Ly6G$^-$ cells (IM), CD11b$^+$ Ly6C^{int} Ly6G$^+$ cells (PMN) and CD11b$^-$ cells in spleens of CCR2-DTR C57BL/6J *Nramp1*^{G169} mice infected for 21 days with *S.* Typhimurium. **(*C, D*)** Absolute numbers of CD11b$^+$ Ly6C^{hi} Ly6G$^-$ (C) and CD11b$^+$ Ly6C^{int} Ly6G$^+$ cells (D) in spleens of *S.* Typhimurium-infected WT or CCR2-DTR C57BL/6J *Nramp1*^{G169} mice (n = 6 per group) on day 24 post-infection after PBS or DT treatment on day 21 post-infection. **(*E*)** Corresponding bacterial loads per gram of spleen tissue. **(*F*)** Absolute numbers of CD11b$^+$ Ly6C^{hi} Ly6G$^-$ cells in spleens of *S.* Typhimurium-infected WT or CCR2-DTR C57BL/6J *Nramp1*^{G169} mice (n = 3-7 per group) on days 22, 24 or 26 post-infection after PBS or DT treatment on day 21 post-infection. **(*G*)** Corresponding bacterial loads per gram of spleen tissue. **(*H*)** Survival of *S.* Typhimurium-infected WT or CCR2-DTR C57BL/6J *Nramp1*^{G169} mice (n = 5 per group) after PBS or DT treatment on day 5 or 21 post-infection; $p = 0.0022$. Data show a representative flow cytometry plot (B), mean with spread from a single experiment that is representative of two independent experiments (C-E), mean with spread from a single experiment (F, G) or cumulative data from two independent experiments (H). Data were analyzed using one-way ANOVA with Bonferroni's multiple comparisons post-test (C-G) or log-rank test (H); p values < 0.05 were considered to be statistically significant. Asterisks indicate statistically significant differences (** $p < 0.01$).

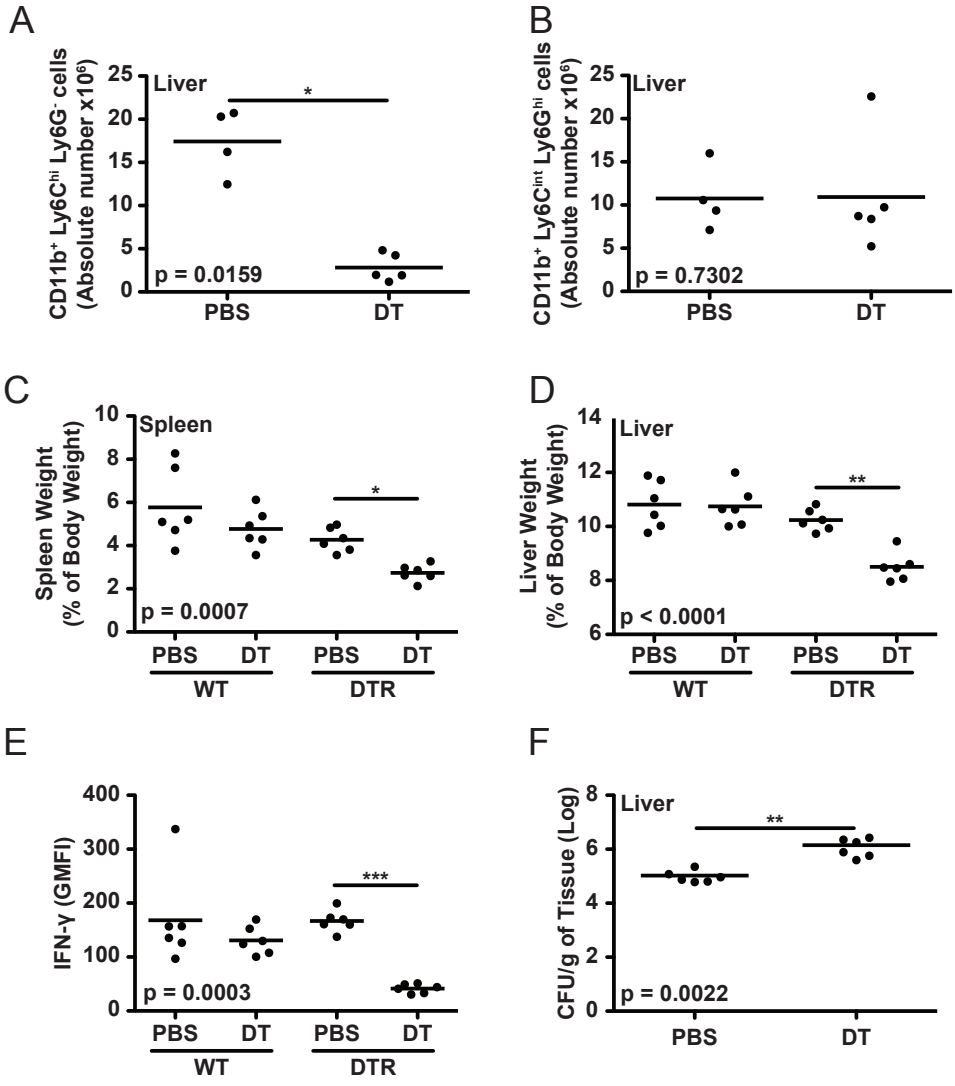

A
CD11b+ Ly6Chi Ly6G- cells (Absolute number x10^6)
Liver
*
p = 0.0159
PBS
DT
B
CD11b+ Ly6Cint Ly6Ghi cells (Absolute number x10^6)
Liver
p = 0.7302
PBS
DT
C
Spleen Weight (% of Body Weight)
Spleen
*
p = 0.0007
PBS
DT
PBS
DT
WT
DTR
D
Liver Weight (% of Body Weight)
Liver
**
p < 0.0001
PBS
DT
PBS
DT
WT
DTR
E
IFN-γ (GMFI)
***
p = 0.0003
PBS
DT
PBS
DT
WT
DTR
F
CFU/g of Tissue (Log)
Liver
**
p = 0.0022
PBS
DT

Figure S1. (*A, B*) Absolute numbers of $CD11b^+$ $Ly6C^{hi}$ $Ly6G^-$ (A) and $CD11b^+$ $Ly6C^{int}$ $Ly6G^+$ cells (B) in livers of *S.* Typhimurium-infected CCR2-DTR C57BL/6J *Nramp1*^{G169} (DTR) mice (n = 4-6 mice per group) on day 24 post-infection after PBS or DT treatment on day 21 post-infection. **(*C, D*)** Spleens (C), and livers (D) were weighed from *S*. Typhimurium-infected WT or CCR2-DTR C57BL/6J *Nramp1*^{G169} mice (n = 6 per group) on day 24 post-infection after PBS or DT treatment on day 21 post-infection. The weight of tissues was normalized to the mouse body weight. **(*E*)** Splenocytes from *S*. Typhimurium-infected WT or CCR2-DTR C57BL/6J *Nramp1*^{G169} mice (n = 6 per group) on day 24 post-infection after PBS or DT treatment on day 21 post-infection were treated *ex vivo* for 4 hours in the presence of brefeldin A and α-CD3ε at 37°C/5% CO_2. The cells were then stained for T cells, fixed, permeabilized, and stained for IFNγ. T cells are defined as $CD90.2^+$ and $CD4^+$ or $CD8^+$. ***(F)*** Corresponding bacterial loads per gram of liver tissue as in (A, B). Data show mean with spread from a single experiment that is representative of at least two independent experiments. Data were analyzed using a Mann-Whitney t-test (A, B, F), or a one-way ANOVA with Bonferroni's multiple comparisons post-test (C, D, E); p values < 0.05 were considered to be statistically significant. Asterisks indicate statistically significant differences (*** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$).

3.6 Discussion

We report that *Ccr2* is required and *Ccl2* is important for the recruitment of $CD11b^+$ $Ly6C^{hi}$ $Ly6G^-$ cells into tissues during persistent salmonellosis. In addition, we report that *Ccr2*$^{-/-}$ and *Ccl2*$^{-/-}$ mice are more susceptible to *S.* Typhimurium infection than WT mice, and that depletion of $CCR2^+$ cells during the first and third week of *S.* Typhimurium infection increases susceptibility to salmonellosis. Collectively, our results indicate that $CCR2^+$ cells, including $CD11b^+$ $Ly6C^{hi}$ $Ly6G^-$ monocytes, play an essential role in early and late control of persistent *S.* Typhimurium infection. Our results advance knowledge of the role of $CD11b^+$ $Ly6C^{hi}$ $Ly6G^-$ monocytes and other $CCR2^+$ cells in the pathogenesis of and host response to *S.* Typhimurium infection.

We propose a model in which $CD11b^+$ $Ly6C^{hi}$ $Ly6G^-$ monocytes and other $CCR2^+$ cells control spread and growth of *S.* Typhimurium during early stages of infection and, in the absence of clearance, contain *S.* Typhimurium during later stages of infection. Although *S.* Typhimurium containment mediated by $CD11b^+$ $Ly6C^{hi}$ $Ly6G^-$ monocytes and other $CCR2^+$ cells is critical for host survival, accumulation and persistence of these cells beyond a certain threshold level may cause collateral effects that prolong or exacerbate disease.

Inflammatory monocytes can produce large amounts of antimicrobial effectors such as TNF-α and nitric oxide [23,26,72,135], which, under certain pathological conditions, can contribute to immunopathology.[78] Numerous studies have shown that TNF-α and nitric oxide are essential for host defense against *S.* Typhimurium infection.[105,106] However, *S.* Typhimurium can detoxify nitric oxide[156], whereas T cells are exquisitely sensitive to the effects of nitric oxide.[28,110,159] Consistent with a role for $CCR2^+$ cells in immunopathology during persistent salmonellosis, we previously published that $CD11b^+$ $Ly6C^{hi}$ $Ly6G^-$ cells recruited into tissues during persistent *S.* Typhimurium infection can inhibit T cell proliferation via a nitric oxide-dependent mechanism.[136] Thus, the collateral damage that may be incurred by $CD11b^+$ $Ly6C^{hi}$ $Ly6G^-$ monocytes and other $CCR2^+$ cells during *S.* Typhimurium containment could well be the price the host must pay to avoid death.

Containment of *S.* Typhimurium mediated by $CD11b^+$ $Ly6C^{hi}$ $Ly6G^-$ monocytes and other $CCR2^+$ cells could come by way of typhoid nodules [103-105], which are leukocyte-rich micro-

abscesses that resemble granulomas formed during *Mycobacterium tuberculosis* infection.[122,170] We previously published that the vast majority of $CD11b^+$ $Ly6C^{hi}$ $Ly6G^-$ monocytes recruited into tissues during persistent salmonellosis do not harbor viable bacteria.[136] Here, we found that transient depletion of these and other $CCR2^+$ cells during late stages of persistent salmonellosis resulted in increased bacterial loads and, ultimately, death. An immediate implication of these results is that, once innate immune control of *S.* Typhimurium mediated by $CD11b^+$ $Ly6C^{hi}$ $Ly6G^-$ monocytes and other $CCR2^+$ cells is lost, it can not be regained. Imaging studies are currently underway to test this hypothesis.

A number of recent studies have shown that inflammatory monocytes can transport pathogens from initial to secondary sites of infection, and can indirectly contribute to the priming of T cells.[33,84] A number of studies have implicated $CD18^+$ phagocytes and CX_3CR1^+ dendritic cells in the extraintestinal dissemination of *S.* Typhimurium.[101,171,172] In addition, several studies have implicated $CCR6^+$ phagocytes in the induction of *S.* Typhimurium-specific T cell responses in the gut.[173,174] However, the contribution of $CD11b^+$ $Ly6C^{hi}$ $Ly6G^-$ monocytes and other $CCR2^+$ cells to these processes is not known. Future studies will focus on establishing the role of $CD11b^+$ $Ly6C^{hi}$ $Ly6G^-$ monocytes and other $CCR2^+$ cells in extraintestinal dissemination of *S.* Typhimurium and induction of protective immune responses in the gut.

3.7 Acknowledgements

We thank Dr. Ferric Fang for providing C57BL/6J *Nramp1*^{G169} mice, and Dr. Eric G. Pamer for providing CCR2-DTR mice.

Chapter 4. Persistent salmonellosis causes pancreatitis in a murine model of infection.

K. E. DelGiorno, **J. W. Tam***, J. C. Hall, G. Thotakura, H. C. Crawford, and A. W. M. van der Velden. Persistent Salmonellosis Causes Pancreatitis in a Murine Model of Infection. *PLoS One* 2014, 9(4):e92807. **DOI: 10.1371/journal.pone.0092807.** [162]

***Co-First Author**

4.1 Abstract

Pancreatitis, a known risk factor for the development of pancreatic ductal adenocarcinoma, is a serious, widespread medical condition usually caused by alcohol abuse or gallstone-mediated ductal obstruction. However, many cases of pancreatitis are of an unknown etiology. Pancreatitis has been linked to bacterial infection, but causality has yet to be established. Here, we found that persistent infection of mice with the bacterial pathogen *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) was sufficient to induce pancreatitis reminiscent of the human disease. Specifically, we found that pancreatitis induced by persistent *S.* Typhimurium infection was characterized by a loss of pancreatic acinar cells, acinar-to-ductal metaplasia, fibrosis and accumulation of inflammatory cells, including $CD11b^+$ $F4/80^+$, $CD11b^+$ $Ly6C^{int}$ $Ly6G^+$ and $CD11b^+$ $Ly6C^{hi}$ $Ly6G^-$ cells. Furthermore, we found that *S.* Typhimurium colonized and persisted in the pancreas, associated with pancreatic acinar cells *in vivo*, and could invade cultured pancreatic acinar cells *in vitro*. Thus, persistent infection of mice with *S.* Typhimurium may serve as a useful model for the study of pancreatitis as it relates to bacterial infection. Increased knowledge of how pathogenic bacteria can cause pancreatitis will provide a more integrated picture of the etiology of the disease and could lead to the development of new therapeutic approaches for treatment and prevention of pancreatitis and pancreatic ductal adenocarcinoma.

4.2 Introduction

Pancreatitis affects over 80,000 Americans every year, and, in its chronic form, is a known risk factor for the development of pancreatic ductal adenocarcinoma (PDA).[175,176] Acute pancreatitis ranges in severity from mild interstitial pancreatitis to a much more severe condition associated with necrosis and concomitant multi-organ failure.[177] Most patients with acute pancreatitis suffer from mild interstitial pancreatitis, but up to 20% of patients suffer from severe pancreatitis, which is often fatal.[178] Under conditions of persistent or repeated insult, acute pancreatitis can progress to chronic pancreatitis, which is an often asymptomatic condition diagnosed only after the development of complications. The incidence of biliary pancreatitis increased by 32% between 1994 and 2001, likely due to the climbing obesity rate and obesity-associated increase in the development of gallstones.[179,180] Other established risk factors for the development of pancreatitis include excessive alcohol consumption, cigarette smoking, and genetic predisposition.[180] Even though 70% of chronic pancreatitis cases are attributed to alcohol abuse, 95% of alcoholics never develop pancreatitis. The remaining chronic pancreatitis cases are considered idiopathic in nature.[175]

The etiology of pancreatitis remains incomplete. A number of studies have linked pancreatitis to bacterial infection, but causality has yet to be established.[181-190] Several case reports have implicated *Salmonellae* as a causative agent of pancreatitis [187,191-201] and two retrospective studies of *Salmonella*-infected individuals have shown that the frequencies of hyperamylasemia and clinical pancreatitis were 50% and ranged from 28 to 62%, respectively.[188,202] Although a prospective study of 30 patients infected with *Salmonella* found no direct evidence for the development of pancreatitis, increased levels of lipase in serum indicated a role for the pancreas in human salmonellosis.[203]

Salmonellae are a leading cause of morbidity and mortality in humans worldwide.[22] Infections with *Salmonellae* range in severity from self-limiting gastroenteritis to typhoid fever and can lead to chronic carriage. Non-typhoidal *Salmonellae* such as *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) are a leading cause of inflammatory enterocolitis and death due to foodborne illness, and are a significant cause of invasive bacteremia in immunocompromised hosts. Typhoidal *Salmonellae* such as *Salmonella enterica* serovar Typhi (*S.* Typhi) cause systemic infections characterized by bacterial penetration of the intestinal barrier and

extraintestinal dissemination to the liver and spleen, where the microorganisms survive and replicate in professional phagocytes.[107,145] Septic shock and death can occur if infections are left untreated.[105] Much of what is known about the pathogenesis of and host response to *Salmonellae* comes from experimental infection of mice with *S.* Typhimurium, which has served as a useful model for the human disease caused by *S.* Typhi.[107]

Here, we report that pancreata of mice persistently infected with *S.* Typhimurium consistently displayed inflammatory, fibrotic and epithelial responses similar to chronic pancreatitis in humans. *S.* Typhimurium colonized the pancreas throughout the course of infection, associated with pancreatic acinar cells *in vivo*, and could directly invade pancreatic acinar cells *in vitro*. Thus, *Salmonella* infection can cause pancreatitis, a known risk factor for the development of PDA.

4.3 Materials and Methods

Ethics Statement. All procedures and experiments using mice were approved by Institutional Animal Care and Use Committees at Stony Brook University or Mayo Clinic, and were conducted in accordance with the recommendations outlined in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All procedures and experiments using mice were designed to use the fewest number of mice possible but still achieve meaningful results. For intravenous inoculations, mice were anesthetized by inhalation of isoflurane to facilitate the procedure and minimize distress. All mice were given food and water ad libitum and were monitored twice daily. Any mice that appeared moribund (e.g. ruffled fur, hunched posture, lack of activity) were euthanized immediately. In any case, euthanasia was performed by inhalation of carbon dioxide, a method consistent with the recommendations of the Panel on Euthanasia of the American Veterinary Medical Association.

Bacteria. *S.* Typhimurium strain IR715, which is a spontaneous, nalidixic acid-resistant derivative of *S.* Typhimurium strain 14028 (American Type Culture Collection), was used as the wild-type strain. Where indicated, isogenic, *invA*-deficient or enhanced GFP-expressing *S.* Typhimurium were used. These strains were generated by bacteriophage P22-mediated transduction, moving the *invA*::*cam* and *rpsM*::*egfp(kan)* mutations described previously [101,116] from isogenic derivatives of *S.* Typhimurium strain 14028 into *S.* Typhimurium strain IR715. Bacteria were grown aerobically for 16-18 h at 37ºC in 3 ml of Luria-Bertani (LB) broth (250 rpm) or on LB agar using standard microbiological techniques.

Mice. C57BL/6J mice (8 to 12 weeks of age), which lack a functional *Nramp1/Slc11a1* locus, were purchased from The Jackson Laboratory and used as the wild-type strain of mouse. The *Nramp1/Slc11a1* locus encodes the natural resistance-associated macrophage protein 1 (Nramp1) (Slc11a1) divalent metal transporter, which enhances host resistance to a number of intracellular pathogens, including *S.* Typhimurium, by limiting essential metal availability within the phagocyte phagosome.[160] C57BL/6J mice are phenotypically Nramp1$^-$ because of a G169D mutation and have been used as model hosts to study acute salmonellosis. A breeder pair of transgenic C57BL/6J *Nramp1*^{G169} mice [160] was generously provided by Dr. Ferric Fang (University of Washington). These mice, which are phenotypically Nramp1$^+$, have been used as model hosts to study persistent salmonellosis [163] and were bred at Stony Brook University,

Division of Laboratory Animal Resources. C57BL/6J *Nramp1*^{G169} mice (8 to 12 weeks of age) bred at Stony Brook University, Division of Laboratory Animal Resources were used for the experiments described in this study.

Mouse Injections. Mouse injections were performed using naïve, 8- to 12-week-old sex-matched C57BL/6J mice. Briefly, mice were injected intraperitoneally with purified *S.* Typhimurium lipopolysaccharide (LPS) (Enzo Life Sciences) (5 mg/kg) every other day for 10 days. One day after the last injection, pancreata were harvested and processed for analysis by histopathology or flow cytometry.

Mouse Infections. Mouse infections were performed using naïve, 8- to 12-week-old sex-matched C57BL/6J *Nramp1*^{G169} mice. Briefly, mice were inoculated intravenously with 5 x 10^3 colony forming units (CFU) of *S.* Typhimurium strain IR715 suspended in 0.1 ml of PBS, unless indicated otherwise. Ten-fold serial dilutions of the inoculum were plated on LB agar to confirm the inoculum titer. At indicated times after inoculation, target organs (i.e. pancreas, liver, and spleen) were harvested and processed for analysis by histopathology, flow cytometry or organ burden assay. Bacterial loads were determined by lysing cells from a single cell suspension with Triton X-100 (0.05%) and plating for CFU on LB agar containing nalidixic acid (50 μg/ml). Mice infected with *S.* Typhimurium were euthanized when moribund or at the termination of the experiment.

Histological Staining and Quantitation. Pancreatic tissues were fixed overnight in 4% paraformaldehyde, dehydrated and paraffin embedded. Routinely, tissue sections were stained using hematoxylin and eosin (H&E) for overall tissue structure, cytokeratin 19 (Abcam, Cambridge, MA) for metaplasia, and Picrosirius Red Stain Kit (Polysciences) for fibrosis. Immunohistochemistry (IHC) was performed as described previously.[204] Briefly, tissue sections were stained using anti-mouse antibodies specific for collagen I, F4/80 or Ly6B.2 (all from AbD Serotec, Kidlington, UK). Stained tissue sections were examined and photographed using an Olympus BX41 light microscope (Olympus). Inflammatory cell infiltration was quantified by examining 5 representative slides (eight 20x fields per slide) per mouse (n = 4 per group) using ImageScope v11.1.2.752 software (Aperio, Vista, CA). F4/80 and collagen staining was quantified by determining the percentage of positive pixels per field, whereas Ly6B.2 staining was quantified by using an algorithm that calculated positive cell nuclei.

Fluorescence Microscopy. Pancreatic tissues were fixed for three hours in 4% paraformaldehyde, washed three times (5 minutes per wash) with PBS (0.1M) and floated overnight in 30% sucrose. Tissues were then incubated for 30 minutes in a 1:1 mixture of 30% sucrose and optimal cutting temperature compound (OCT), embedded in OCT and frozen at -80ºC. Tissue sections of 7 μm each were produced, permeabilized with 0.1% Triton X-100 in 10 mM PBS and blocked with 5% normal donkey serum and 1% BSA in 10 mM PBS for 1 hour at room temperature. Tissue sections were then stained with Alexa Fluor 594 phalloidin (Invitrogen) in 10 mM PBS supplemented with 1% BSA and 0.1% Triton X-100 for 1 hour at room temperature, washed three times with 0.1% Triton X-100 in PBS and rinsed with deionized water. Slides were mounted in VECTASHIELD mounting medium with DAPI (Vector Laboratories). Stained tissue sections were examined and photographed using a Zeiss 510LS Meta confocal microscope (Carl Zeiss MicroImaging).

Cell Staining and Analysis by Flow Cytometry. Conjugated monoclonal antibodies and reagents described in this section were purchased from BioLegend. Routinely, cells were stained in the presence of Fc block (anti-mouse CD16/32 antibody; clone 93) using anti-mouse antibodies specific for CD11b (clone M1/70), F4/80 (clone CI:A3-1), Ly6C (clone HK1.4) and Ly6G (clone 1A8). Data were acquired and analyzed using a BD FACSCalibur flow cytometer (BD Biosciences) with BD CellQuestPro (BD Biosciences) and FlowJo (Tree Star) software or a BD FACScan flow cytometer (BD Biosciences) with Digital Extra Parameter upgrade (Cytek) and FlowJo Collectors' Edition software (Cytek).

Acinar Cell Culture and Infection Assay. The murine acinar cell line 266-6 (American Type Culture Collection) was maintained in DMEM supplemented with 10% fetal bovine serum (Atlanta Biologicals) and sodium pyruvate (1mM), and incubated at 37ºC in 5% CO_2. Acinar cell infections were performed using a standard gentamicin protection assay.[145,205] Briefly, acinar cells were suspended in medium lacking antibiotics and seeded at 5 x 10^5 cells per ml per well into a 24-well tissue culture plate. After overnight incubation, the medium was replaced with 0.5 ml of fresh medium and the cells were infected with bacteria at a multiplicity of infection of 50. Upon addition of bacteria, the plate was centrifuged for 5 minutes at 1,000 rpm to facilitate bacterial contact with the acinar cells. After 20 minutes of incubation, the wells were washed three times with 1 ml of PBS to remove non-cell-associated bacteria, and fresh medium

supplemented with gentamicin (25 μg/ml) was added to each well to kill all extracellular bacteria. This was referred to as the 0 hour time point. After 1 hour of incubation, the wells were washed three times with 1 ml of PBS and the acinar cells were lysed using 0.5 ml of Triton X-100 (0.1%) to release intracellular bacteria. These bacteria were enumerated by plating onto LB agar. In experiments where the acinar cells were infected with GFP-expressing bacteria, the cells were harvested and analyzed by flow cytometry.

Statistical Analysis. Statistical analysis was performed using Prism 5.0b (GraphPad Software). Data were analyzed using a two-tailed, paired Student's t-test, or one-way analysis of variance (ANOVA) with Bonferroni's multiple comparisons posttest; p values < 0.05 were considered to be statistically significant. Asterisks indicate statistically significant differences (*** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$).

4.4 Results

***S.* Typhimurium LPS induces pancreatic inflammation.** Bacterial infection, including that caused by *Salmonellae*, has been implicated in human cases of pancreatitis.[187,191-201] Consistent with this notion, *Escherichia coli* LPS exacerbates pancreatitis in alcohol and caerulein-induced animal models, hypothetically through its direct effects on pancreatic acinar cells.[206-208] Yet, *Escherichia coli* LPS alone fails to induce the desmoplastic reaction or obvious acinar cell stresses that are hallmarks of pancreatitis.[209] It is generally known that administration of LPS induces a systemic inflammatory response that affects many organs. To determine how the pancreas may be affected by *S.* Typhimurium LPS-induced systemic inflammation, C57BL/6J mice were injected intraperitoneally with *S.* Typhimurium LPS every other day for 10 days. One day after the last injection, pancreata were harvested and processed for analysis by histopathology. Consistent with previous studies [209], H&E staining revealed little tissue damage or associated edema (**Figure 1A**). However, IHC detection of F4/80 and Ly6B.2 revealed a uniform, pancreas-wide distribution of $F4/80^+$ macrophages (**Figures 1A and 1B**) with very few $Ly6B.2^+$ neutrophils (data not shown). Flow cytometric analysis confirmed the presence of large numbers of $F4/80^+$ cells in pancreata of LPS-treated mice as compared to mock-treated mice (**Figure 1C**). These cells also expressed surface CD11b (**Figures 1C and 1D**). The $CD11b^+$ cells present in pancreata of mice treated with LPS were a heterogeneous population that consisted mostly of $CD11b^+$ $F4/80^+$ macrophages (**Figures 1C and 1D**), but also included $CD11b^+$ $Ly6C^{hi}$ $Ly6G^-$ inflammatory monocytes and $CD11b^+$ $Ly6C^{int}$ $Ly6G^+$ neutrophilic granulocytes (**Figures 1E and 1F**). Thus, *S.* Typhimurium LPS induces pancreatic inflammation, but fails to induce the reactive epithelial and fibrotic responses that are characteristic of pancreatitis.

***S.* Typhimurium infection induces pancreatitis.** As *S.* Typhimurium LPS alone was insufficient to induce pancreatitis, we sought to determine the ability of *S.* Typhimurium infection to induce a pancreatitis phenotype more similar to the human disease condition. Therefore, C57BL/6J *Nramp1*^{G169} mice were inoculated intravenously with 5×10^3 CFU of *S.* Typhimurium. After 10 days of infection, an early time point used to assess establishment of salmonellosis, histopathologic analysis showed evidence of mild pancreatic edema, but no cytokeratin 19-positive epithelial metaplasia or significant fibrosis (**Figure 2A**). In addition, IHC for F4/80 and Ly6B.2 revealed the presence of significant numbers of focally pooled

macrophages and neutrophils, respectively, in pancreata of mice infected with *S.* Typhimurium as compared to mice left uninfected (**Figures 2B and 2C**). Consistent with these results, we found significantly more $CD11b^+$ $F4/80^+$ macrophages (**Figures 2D and 2E**), $CD11b^+$ $Ly6C^{hi}$ $Ly6G^-$ inflammatory monocytes and $CD11b^+$ $Ly6C^{int}$ $Ly6G^+$ neutrophilic granulocytes (**Figures 2F and 2G**) in pancreata of mice infected with *S.* Typhimurium than in pancreata of mice left uninfected. Thus, *S.* Typhimurium infection induces significant pancreatic inflammation without ductal metaplasia or fibrosis, a phenotype that is similar to acute pancreatitis in humans.

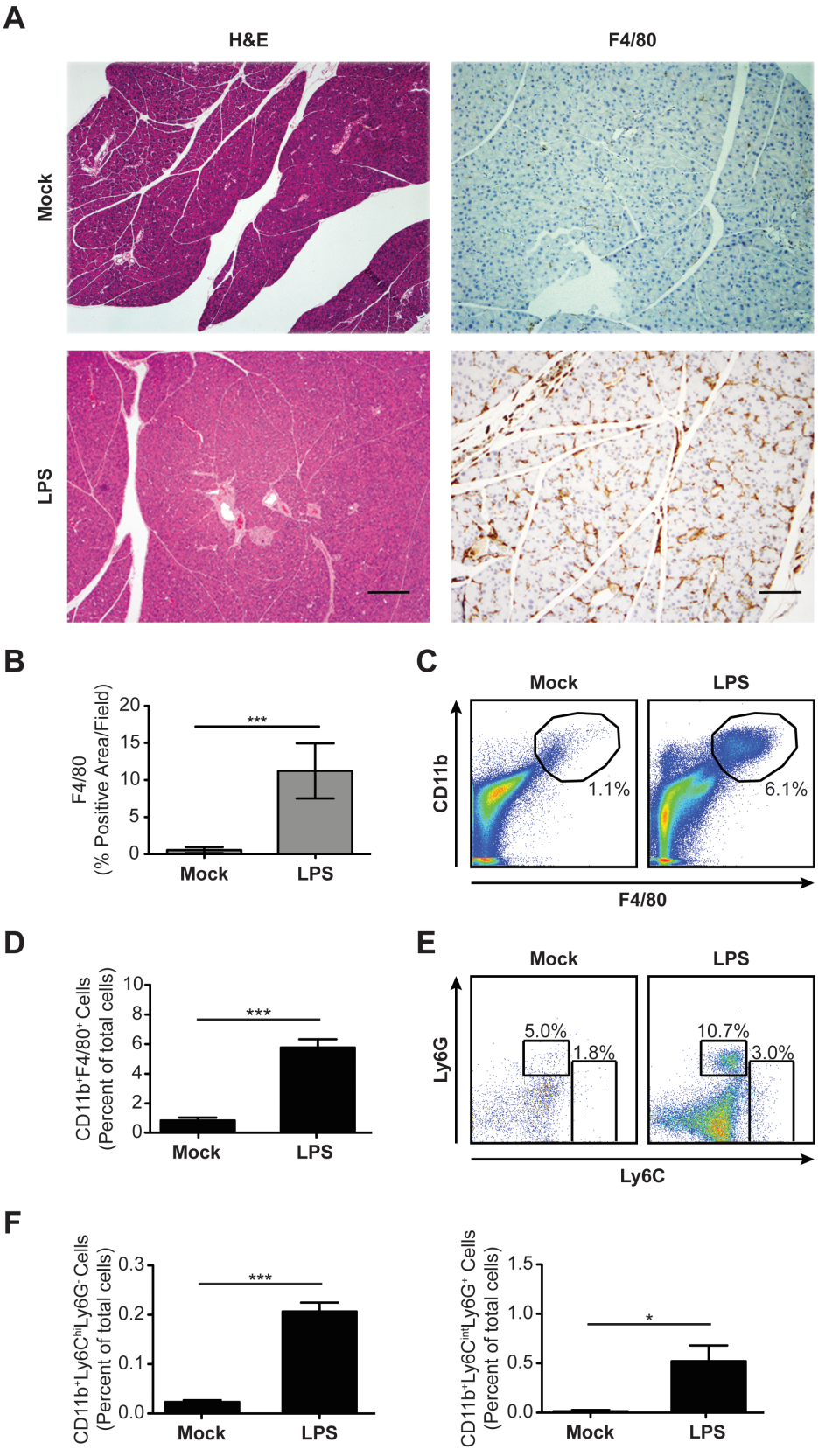
***S.* Typhimurium colonizes and persists in the pancreas, and can invade pancreatic acinar cells.** A recent study reported on the detection of *Salmonella* Enteritidis genomic DNA in pancreata of mice infected with *Salmonella* Enteritidis [210], suggesting that *Salmonellae* may colonize the pancreas. To characterize the ability of *S.* Typhimurium to colonize and persist in pancreas, we determined bacterial loads in pancreata of C57BL/6 $Nramp1^{G169}$ mice inoculated intravenously with 5×10^3 CFU of *S.* Typhimurium. After 10 days of infection, we recovered substantial numbers of *S.* Typhimurium from pancreata of infected mice (**Figure 3A**). These numbers persisted over a period of 60 days (**Figure 3A**) and were similar to the numbers of *S.* Typhimurium recovered from liver and spleen (**Figures 3B and 3C**). To visualize *S.* Typhimurium in the pancreas, we inoculated C57BL/6 $Nramp1^{G169}$ mice intravenously with 5×10^3 CFU of *S.* Typhimurium expressing GFP. After 10 days of infection, we found *S.* Typhimurium associated with acinar cells throughout the pancreas (**Figure 3D**), suggesting that *S.* Typhimurium may directly infect these cells *in vivo*.

To characterize the ability of *S.* Typhimurium to invade pancreatic acinar cells, we infected cultured murine pancreatic acinar cells (line 266-6) with wild-type or *invA*-deficient *S.* Typhimurium at a multiplicity of infection of 50. The *invA* gene encodes an essential structural component of the *Salmonella* Pathogenicity Island (SPI)-1-encoded Type Three Secretion System (TTSS), which is required for invasion of non-phagocytic cells [211]. After 1 hour of infection, we recovered substantial numbers of intracellular bacteria (**Figure 3E**), indicating that *S.* Typhimurium had invaded the acinar cells. We recovered significantly fewer intracellular *invA*-deficient *S.* Typhimurium than wild-type *S.* Typhimurium, indicating that efficient invasion of the acinar cells was dependent on the SPI-1-encoded TTSS (**Figure 3E**). Similar results were obtained when we analyzed by flow cytometry GFP fluorescence of acinar cells infected with

wild-type or *invA*-deficient *S.* Typhimurium expressing GFP (**Figures 3F and 3G**). Collectively, these results indicate that *S.* Typhimurium colonize and persist in pancreas, and that the bacterial burden in the pancreas may be due, at least in part, to direct infection of acinar cells.

Pancreatitis progresses with persistent *S.* Typhimurium infection. Given that *S.* Typhimurium induced a pancreatitis-like phenotype during early stages of infection (Figure 2) and colonized and persisted in the pancreas over a period of 60 days (Figure 3), we next examined pancreata of C57BL/6J *Nramp1*^{G169} mice persistently infected with *S.* Typhimurium. Unlike the mild response found after 10 days of *S.* Typhimurium infection (Figure 2), we found significant pancreatic damage after 60 days of infection, as indicated by large areas of acinar cell loss made evident by H&E staining (**Figure 4A**). These areas were marked by cytokeratin 19-positive acinar to ductal metaplasia (ADM) and a dramatic desmoplastic response highlighted by substantial collagen deposition (**Figures 4A and 4B**). In addition, the relatively mild inflammatory response induced during early stages of *S.* Typhimurium infection had been replaced by large swaths of F4/80^{+} macrophages and Ly6B.2^{+} neutrophils occupying the damaged areas of the pancreas (**Figures 4C and 4D**). Consistent with these results, we found significantly more CD11b^{+} cells, including CD11b^{+} F4/80^{+} cells (**Figures 4E and 4F**) and CD11b^{+} Ly6C^{int} Ly6G^{+} cells (**Figures 4G and H, right panel**) in pancreata of mice persistently infected with *S.* Typhimurium than in pancreata of mice left uninfected. A similar trend was observed for CD11b^{+} Ly6C^{hi} Ly6G^{-} cells (**Figures 4G and 4H, left panel**), but differences did not reach statistical significance. Similar results were obtained when we examined pancreata of 129X1/SvJ mice persistently infected with *S.* Typhimurium administered intragastrically (data not shown). Collectively, the robust inflammatory response and reactive epithelial and fibrotic responses in the pancreas indicate that persistent *S.* Typhimurium infection induces a progressive condition that is highly similar to chronic pancreatitis in humans.

4.5 Figures


A
H&E
F4/80
Mock
LPS
B
F4/80
(% Positive Area/Field)
20
15
10
5
0
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Mock
LPS
C
Mock
LPS
CD11b
1.1%
6.1%
F4/80
D
CD11b+F4/80+ Cells
(Percent of total cells)
10
8
6
4
2
0
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Mock
LPS
E
Mock
LPS
Ly6G
5.0%
1.8%
10.7%
3.0%
Ly6C
F
CD11b+Ly6CintLy6G- Cells
(Percent of total cells)
0.3
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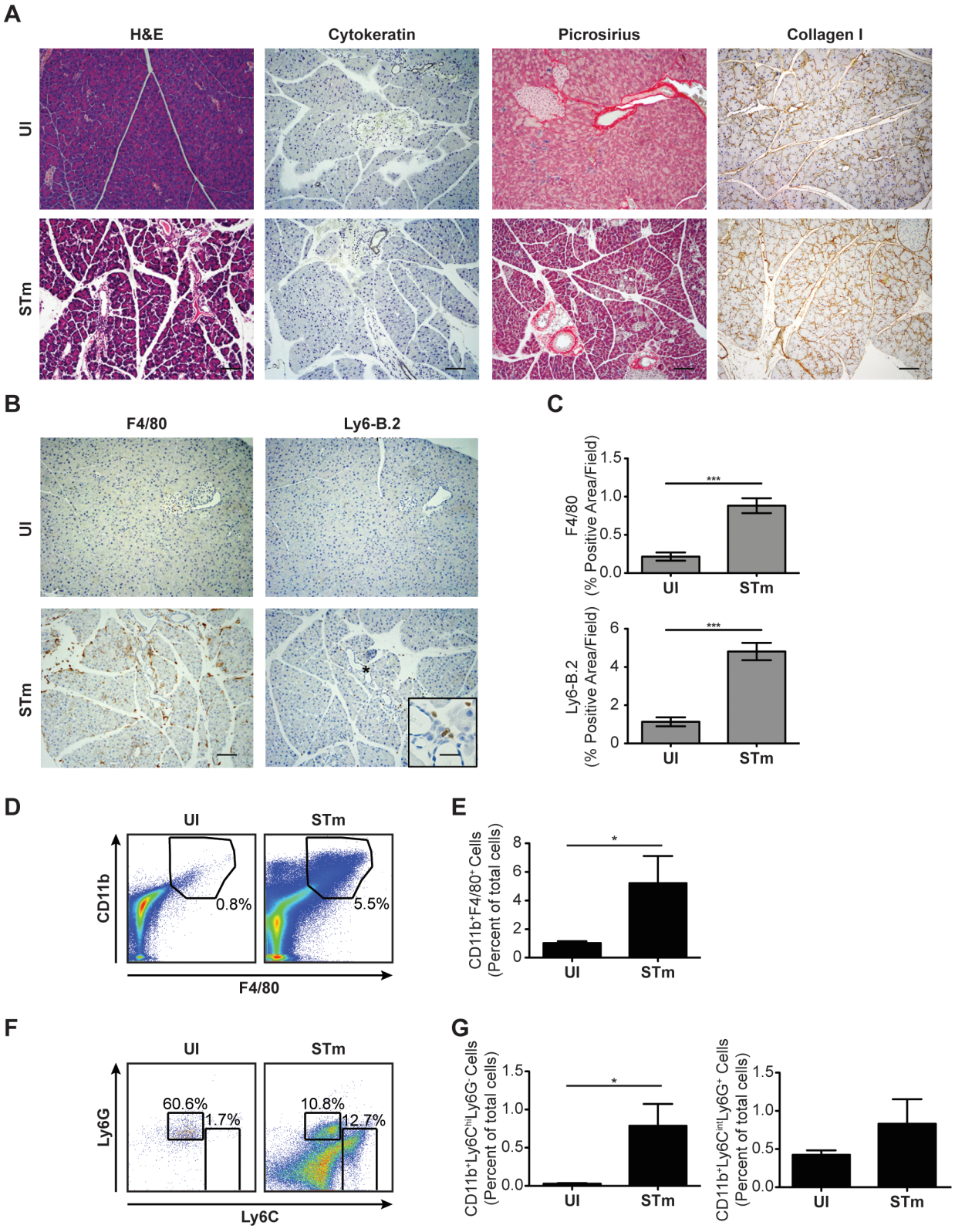
Mock
LPS
CD11b+Ly6CintLy6G+ Cells
(Percent of total cells)
1.5
1.0
0.5
0.0
*
Mock
LPS

Figure 1. *S.* Typhimurium LPS induces pancreatic inflammation. (*A*) Histological analysis of pancreatic tissue sections from mock-treated or LPS-treated C57BL/6J mice (n = 4 per group). Tissue sections were stained using H&E or subjected to IHC using antibodies specific for F4/80. Scale bars for H&E = 200 μm and for IHC = 100 μm. (*B*) Quantitation of IHC data shown in (A). (*C and D*) Expression of surface F4/80 and CD11b by cells harvested from pancreata of mock-treated or LPS-treated C57BL/6J mice (n = 4 per group) as measured using flow cytometry. Numbers in (C) refer to $CD11b^+$ $F4/80^+$ cells as percentages of the total numbers of cells. (*E and F*) Expression of surface Ly6C and Ly6G by $CD11b^+$ cells present in pancreata of mock-treated or LPS-treated C57BL/6J mice (n = 4 per group) as measured using flow cytometry. Numbers in (E) refer to $CD11b^+$ $Ly6C^{hi}$ $Ly6G^-$ and $CD11b^+$ $Ly6C^{int}$ $Ly6G^+$ cells as percentages of the total numbers of $CD11b^+$ cells. Data are representative of (A, C, and E), or show mean with SEM from (B, D and F), two independent experiments. Data were analyzed using a two-tailed, paired Student's t-test; p values < 0.05 were considered to be statistically significant. Asterisks indicate statistically significant differences (*** $p < 0.001$, * $p < 0.05$).


A
H&E
Cytokeratin
Picrosirius
Collagen I
UI
STm
B
F4/80
Ly6-B.2
UI
STm
C
F4/80 (% Positive Area/Field)
1.5
1.0
0.5
0.0
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UI
STm
Ly6-B.2 (% Positive Area/Field)
6
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2
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UI
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CD11b
0.8%
5.5%
F4/80
E
CD11b+F4/80+ Cells (Percent of total cells)
8
6
4
2
0
*
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60.6%
1.7%
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CD11b+Ly6CintLy6G+ Cells (Percent of total cells)
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UI
STm

Figure 2. *S*. Typhimurium infection induces pancreatitis. (*A and B*) Histological analysis of pancreatic tissue sections from C57BL/6J *Nramp1*^{G169} mice (n = 3-4 per group) left uninfected or infected for 10 days with *S*. Typhimurium (STm). Tissue sections were stained using H&E, cytokeratin 19, or Picrosirius Red Stain Kit. In addition, tissue sections were subjected to IHC using antibodies specific for collagen I (A) or F4/80 or Ly6B.2 (B). Scale bars for H&E = 200 μm and for IHC = 100 μm. (*C*) Quantitation of IHC data shown in (B). (*D and E*) Expression of surface F4/80 and CD11b by cells harvested from pancreata of C57BL/6J *Nramp1*^{G169} mice (n = 3-4 per group) left uninfected or infected for 10 days with STm as measured using flow cytometry. Numbers in (D) refer to CD11b^{+} F4/80^{+} cells as percentages of the total numbers of cells. (*F and G*) Expression of surface Ly6C and Ly6G by CD11b^{+} cells present in pancreata of C57BL/6J *Nramp1*^{G169} mice (n = 3-4 per group) left uninfected or infected for 10 days with STm as measured using flow cytometry. Numbers in (F) refer to CD11b^{+} Ly6C^{hi} Ly6G^{-} and CD11b^{+} Ly6C^{int} Ly6G^{+} cells as percentages of the total numbers of CD11b^{+} cells. Data are representative of (A, B, D and F), or show mean with SEM from (C, E and G), two independent experiments. Data were analyzed using a two-tailed, paired Student's t-test; p values < 0.05 were considered to be statistically significant. Asterisks indicate statistically significant differences (*** $p < 0.001$, * $p < 0.05$).

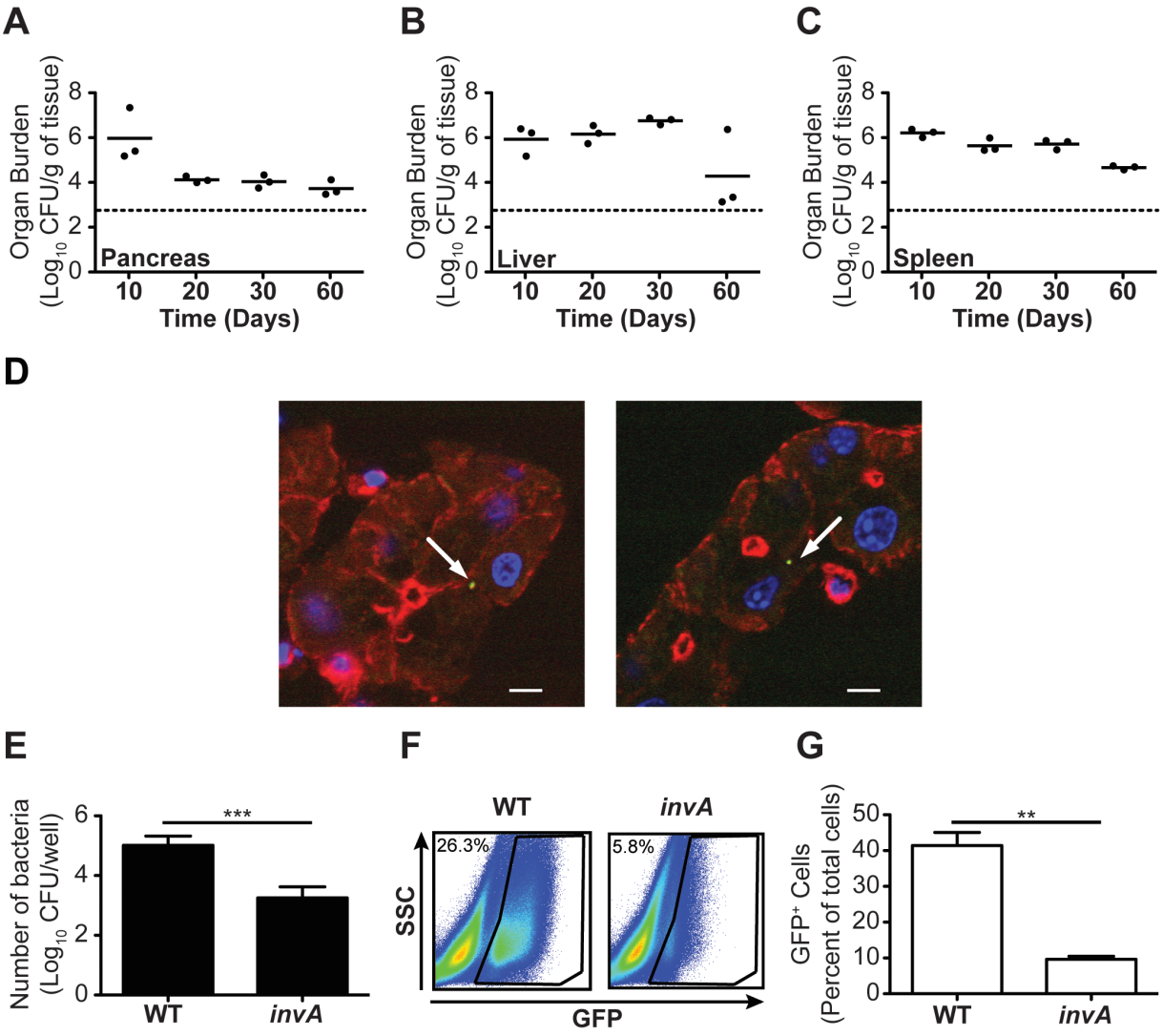
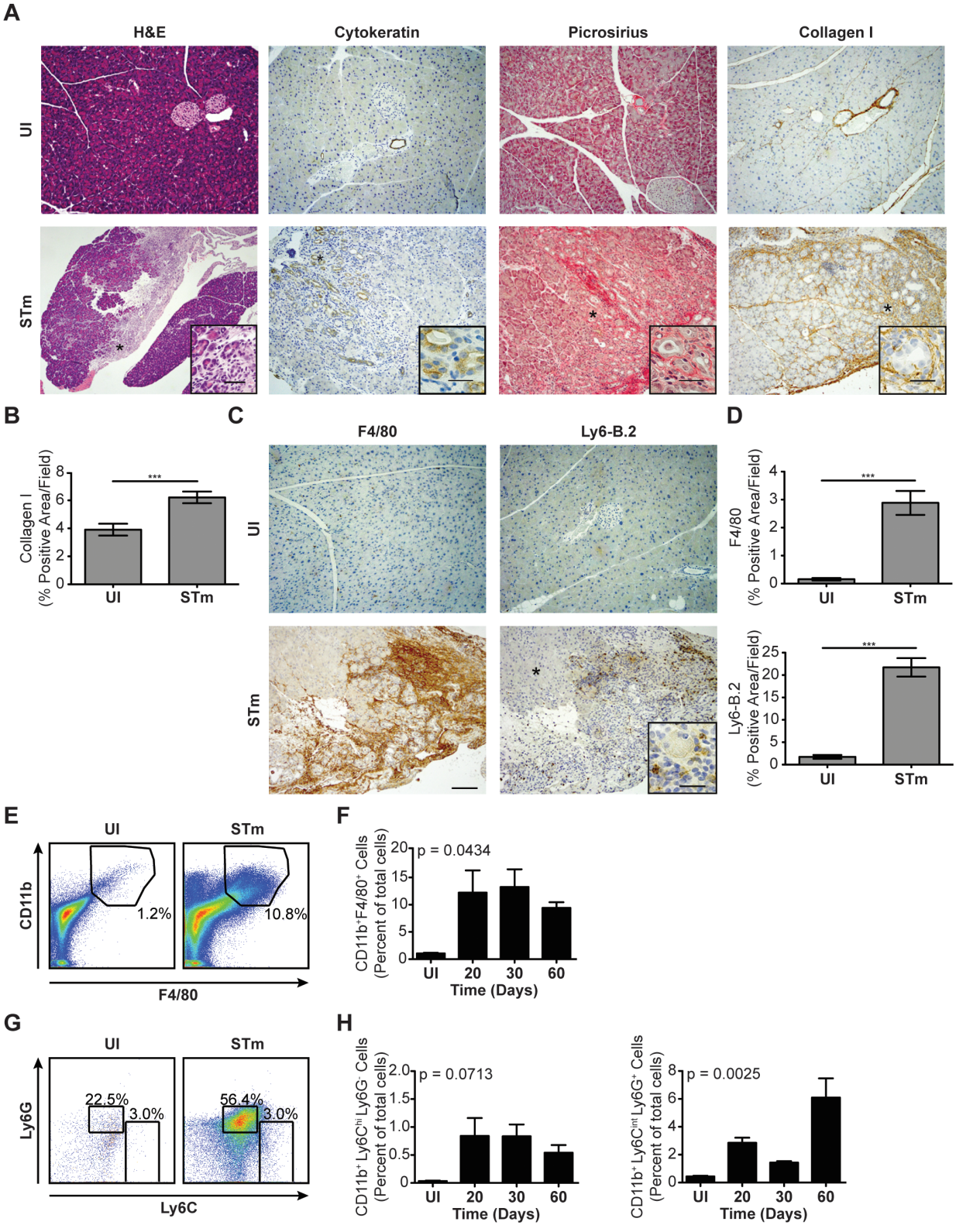

A
B
C
Organ Burden
(Log10 CFU/g of tissue)
Pancreas
Liver
Spleen
Time (Days)
10
20
30
60
D
E
F
G
Number of bacteria
(Log10 CFU/well)
***
WT
invA
SSC
GFP
26.3%
5.8%
GFP+ Cells
(Percent of total cells)
**

Figure 3. *S.* Typhimurium colonize and persist in the pancreas, associate with pancreatic acinar cells *in vivo*, and can invade pancreatic acinar cells *in vitro*. (*A-C*) Bacterial loads per gram of pancreas (A), liver (B) and spleen (C) tissue harvested from C57BL/6J *Nramp1*^{G169} mice (n = 3 per group) at indicated times after infection with *S.* Typhimurium. (*D*) Representative confocal images of pancreatic tissue sections harvested from C57BL/6J *Nramp1*^{G169} mice (n = 3 per group) infected with *S.* Typhimurium expressing GFP. Tissue sections were stained with Alexa Fluor 594 phalloidin (red) and DAPI (blue). Arrows point to GFP-expressing *S.* Typhimurium. (*E*) Invasion of cultured pancreatic acinar cells (line 266-6) by wild-type or *invA*-deficient *S.* Typhimurium as measured by gentamicin protection assay. (*F and G*) Detection of GFP associated with cultured pancreatic acinar cells (line 266-6) infected with wild-type or *invA*-deficient *S.* Typhimurium expressing GFP. Data shown in (A-D) show mean with spread from (A-C), or are representative of (D), two independent experiments. Data shown in (E-G) show mean with SEM from (E and G), or are representative of (F), four independent experiments. Data in (E and G) were analyzed using a two-tailed, paired Student's t-test; p values < 0.05 were considered to be statistically significant. Asterisks indicate statistically significant differences (*** $p < 0.001$, ** $p < 0.01$).


A
H&E
Cytokeratin
Picrosirius
Collagen I
UI
STm
B
Collagen I
(% Positive Area/Field)
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UI
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F4/80
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D
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(% Positive Area/Field)
Ly6-B.2
(% Positive Area/Field)
E
CD11b
F4/80
1.2%
10.8%
F
CD11b+F4/80+ Cells
(Percent of total cells)
p = 0.0434
Time (Days)
G
Ly6G
Ly6C
22.5%
3.0%
56.4%
3.0%
H
CD11b+ Ly6Chi Ly6G- Cells
(Percent of total cells)
p = 0.0713
CD11b+ Ly6Cint Ly6G+ Cells
(Percent of total cells)
p = 0.0025

Figure 4. Pancreatitis progresses with persistent *S*. Typhimurium infection. (*A and C*) Histological analysis of pancreatic tissue sections from C57BL/6J *Nramp1*^{G169} mice (n = 3-4 per group) left uninfected or infected for 60 days with *S*. Typhimurium (STm). Tissue sections were stained using H&E, cytokeratin 19, or Picrosirius Red Stain Kit. In addition, tissue sections were subjected to IHC using antibodies specific for collagen I (A) or F4/80 or Ly6B.2 (C). Scale bars for H&E = 200 μm and for IHC = 100 μm. (*B and D*) Quantitation of IHC data shown in (A and C). (*E and F*) Expression of surface F4/80 and CD11b by cells harvested from pancreata of C57BL/6J *Nramp1*^{G169} mice (n = 3-4 per group) left uninfected or infected for 60 days with STm as measured using flow cytometry. Numbers in (E) refer to CD11b^{+} F4/80^{+} cells as percentages of the total numbers of cells. (*G and H*) Expression of surface Ly6C and Ly6G by CD11b^{+} cells present in pancreata of C57BL/6J *Nramp1*^{G169} mice (n = 3-4 per group) left uninfected or infected for 60 days with STm as measured using flow cytometry. Numbers in (G) refer to CD11b^{+} Ly6C^{hi} Ly6G^{-} and CD11b^{+} Ly6C^{int} Ly6G^{+} cells as percentages of the total numbers of CD11b^{+} cells. Data are representative of (A, C, E and G), or show mean with SEM from (B, D, F and H), two independent experiments. Data were analyzed using a two-tailed, paired Student's t-test (B and D) or a one-way ANOVA (F and H); p values < 0.05 were considered to be statistically significant. Asterisks indicate statistically significant differences (*** $p < 0.001$).

4.6 Discussion

The etiology of pancreatitis, a known risk factor for the development of PDA, is most commonly associated with alcohol abuse and gallstone-mediated ductal obstruction [175], though frequently the specific cause is unknown. Bacterial infection has frequently been associated with pancreatitis, but is usually considered a consequence of the disease rather than a contributor to the disease. In fact, infection of the pancreas is the primary cause of pancreatitis-associated death.[212] Here, we found that persistent salmonellosis could cause pancreatitis in a murine model of infection. Specifically, we found that pancreatitis induced by persistent *S.* Typhimurium infection was characterized by a loss of pancreatic acinar cells, acinar to ductal metaplasia, fibrosis, and accumulation of inflammatory cells (Figures 2 and 4). Furthermore, we found that *S.* Typhimurium colonized and persisted in the pancreas, associated with pancreatic acinar cells *in vivo*, and could invade cultured pancreatic acinar cells *in vitro* (Figure 3). An immediate implication of our results is that persistent, chronic or repeated infections with *Salmonellae* could lead to the development of pancreatitis.

In humans, *S.* Typhi is the major cause of persistent or chronic salmonellosis. It is estimated that 3-5% of patients infected with *S.* Typhi become chronic carriers. Chronic infections with *S.* Typhi can persist for decades and are often asymptomatic, which makes the identification of chronic carriers difficult. The chronic carrier state has been associated with pre-existing hepatobiliary disease such as the presence of gallstones.[213] Chronic carriers have an increased risk of developing hepatobiliary and pancreatic carcinomas [214-219], which links persistent salmonellosis to gastrointestinal cancer. It is generally known that *Salmonellae* can colonize the liver and form biofilms on gallstones in the gallbladder, indicating that direct colonization of these sites may be a mechanism for chronic inflammation, tissue disturbance, and the promotion of a pro-tumorigenic microenvironment.[220] Similarly, our results indicate that direct colonization of the pancreas by *Salmonellae* can cause pancreatic inflammation and tissue injury that is characterized by, but not limited to, metaplasia, a known precursor for neoplastic transformation.[221]

To the best of our knowledge, this report is the first to document that an important long-term consequence of persistent salmonellosis may be induction of pancreatitis, a known risk factor for the development of PDA. The similarities between said long-term consequence of persistent

salmonellosis and the long-term consequences of chronic infections with *Helicobacter pylori*, a bacterial pathogen that has been identified as one of the primary instigators of intestinal metaplasia in the stomach, are striking. Gastric mucosal tissue injury caused by chronic *H. pylori* infection leads to intestinal metaplasia, which is believed to result from the differentiation of gastric stem cells towards cells of an intestinal phenotype.[222] This intestinal metaplasia carries a significantly increased risk of developing gastric cancer, the second most common cancer globally.[223,224] Analogously, pancreatic metaplasia due to persistent salmonellosis likely carries an increased risk of developing PDA.

Central to the development of PDA are activating oncogenic *K-ras* mutations.[225] Although *K-ras* mutations alone may not cause PDA, *K-ras* mutations in conjunction with pancreatitis have been shown to induce progression of pancreatic cancer.[226] In addition to tissue injury, we found that pancreatitis induced by *S.* Typhimurium was characterized by a robust inflammatory response. This response consisted of an influx of macrophages, $CD11b^{+}$ $Ly6C^{hi}$ $Ly6G^{-}$ inflammatory monocytes and $CD11b^{+}$ $Ly6C^{int}$ $Ly6G^{+}$ neutrophilic granulocytes (Figures 2 and 4). Recent studies have shown that macrophages, through the secretion of cytokines, can induce pancreatic metaplasia.[227] Furthermore, the $CD11b^{+}$ $Ly6C^{hi}$ $Ly6G^{-}$ and $CD11b^{+}$ $Ly6C^{int}$ $Ly6G^{+}$ cells that accumulate and persist in tissues of mice infected with *S.* Typhimurium resemble myeloid-derived suppressor cells, which have been associated with immunosuppression in cancer and, more recently, infection.[28,159] We propose a model where persistent salmonellosis induces pancreatic inflammation and tissue injury that may promote the development of metaplasia, which, in conjunction with an activating *K-ras* mutation, may lead to the development of PDA.

In conclusion, we have shown that persistent salmonellosis causes pancreatitis in a murine model of infection. This model recapitulates the complexity of the human disease and, therefore, may be useful for the study of pancreatitis as it relates to bacterial infection. Increased knowledge of how pathogenic bacteria can cause pancreatitis will provide a more integrated picture of the etiology of the disease and could lead to the development of new therapeutic approaches for treatment and prevention of pancreatitis and PDA.

4.7 Acknowledgements

We thank Brandy Edenfield for performing IHC analysis, Dr. Ferric Fang for providing C57BL/6J *Nramp1*^{G169} mice, Dr. Susanne Lindgren for providing *invA*-deficient *S.* Typhimurium and Dr. Raphael Valdivia for providing GFP-expressing *S.* Typhimurium.

Chapter 5. Discussion.

Myeloid cells and their protective role in the host response to infection was first described over 130 years ago in the seminal works of Élie Metchnikoff, the father of natural immunity. Scientists today are still seeking to fill in the details left behind by his formidable body of work. Much of his work and ideas are still applicable to modern immunology, but, with major advancements in science, it has become clear that myeloid cells play a much more complex role during infection than previously thought. This dissertation focuses on innate monocytic cells and their role in the host response to *Salmonella* infection - a topic that Metchnikoff worked on over 100 years ago.

Immature myeloid cells have received significant attention over the last 20 years. The role of these cells in cancer has been extensively studied. Because these IMCs were found to be a major contributor to the immunosuppression of T cells in cancer, they were described as myeloid-derived suppressor cells.[27,28] However, at the same time, IMCs were shown to be an essential contributor to protection against a wide range of pathogens and could contribute to inflammation-mediated tissue damage.[26,29] Emerging data from different laboratories has suggested that in certain types of infections, IMCs could also act like cancer-induced MDSCs insofar as they could suppress T cells.[68,69] These apparent conflicting protective and pathological functions were likely because of the heterogeneity and plasticity of these cells as well as the context in which they were induced. Technical advancements have allowed for a more precise dissection of the different subsets of immature myeloid cells. Furthermore, advancements in our understanding of IMC biology has allowed for more rigorous *in vivo* studies to determine their role in health and disease. Using the latest techniques and knowledge of IMC biology, we studied the role of $CD11b^{+}Ly6C^{hi}Ly6G^{-}$ monocytes in the host response to *Salmonella* infection.

Salmonella is an important human pathogen that has been studied extensively. However, despite over 100 years of research, the host pathogen interaction during infection is not well understood. On the one hand, *Salmonella* genetics and its behavior *in vitro* are well understood, but the inability to produce a vaccine that confers life long protection highlights the dearth of knowledge about *Salmonella* pathogenesis. Many studies have focused on murine models of acute *Salmonella* Typhimurium infection and have found that *Salmonella* could induce acute immunosuppression.[105,106,108-110] However, an important and often overlooked aspect of

Salmonella infection and epidemiology is its ability to cause chronic infection. It is only recently that mouse models have emerged to study this aspect of the disease.[103,104] An underlying requirement for chronicity is the ability for the pathogen to avoid clearance by the host immune system. For example, in a recent study that I co-authored, Kullas *et al.* showed that L-asparaginase II, a conserved metabolic gene, produced by *Salmonella* Typhimurium inhibits T cell responses and mediates virulence.[145]

To dissect innate immune mechanisms that control the spread and growth of *Salmonella*, we characterized the myeloid cell response using a murine model of persistent salmonellosis. In this dissertation, I present evidence indicating that $CD11b^{+}Ly6C^{hi}Ly6G^{-}$ immature myeloid cells recruited in response to *Salmonella* Typhimurium infection exhibit protective and immunosuppressive properties.[136] Furthermore, I present evidence indicating that $CCR2^{+}$ cells, including $CD11b^{+}Ly6C^{hi}Ly6G^{-}$ cells, are essential in the early and late control of *Salmonella* infection. We propose a model in which $CD11b^{+}$ $Ly6C^{hi}$ $Ly6G^{-}$ monocytes provide protective functions in the host response to infection, where accumulation and persistence of these cells beyond a certain threshold level may cause collateral effects that prolong or exacerbate disease.

Consistent with the literature reporting the recruitment of $CD11b^{+}Ly6C^{hi}Ly6G^{-}$ cells during infection with a wide range of pathogens, we found that *S.* Typhimurium infection induced the recruitment of $CD11b^{+}Ly6C^{hi}Ly6G^{-}$ cells. In a murine model of acute salmonellosis, we found that mice rapidly succumbed to *S.* Typhimurium infection as the IMC response peaks.[136] In contrast, in a study I was involved in, Rasmussen *et al.* found that, during *Francisella tularensis* infection, the IMC response rapidly peaked and then declined as the infection resolved and the pathogen was cleared.[158] These responses were likely the result of innate immune detection of PAMPs. Indeed, it has been reported that the recruitment of $CD11b^{+}Ly6C^{hi}Ly6G^{-}$ monocytes could be induced by TLR ligands, such as LPS.[75] We have also shown that *Salmonella* LPS alone could induce recruitment of these cells to the pancreas[162], and spleen (Tam and van der Velden, unpublished). However, it is surprising that persistent *Salmonella* infection resulted in a sustained IMC response. In order to better understand the role of $CD11b^{+}Ly6C^{hi}Ly6G^{-}$ cells persistently infected *S.* Typhimurium mice, we characterized the cells *ex vivo*.

Characterization of the IMCs recruited during persistent *Salmonella* infection revealed that the $CD11b^{+}Ly6C^{hi}Ly6G^{-}$ monocytes exhibited protective properties. We found that these cells

could differentiate into macrophages and present antigen to T cells *ex vivo*.[136] Although the ability for $CD11b^{+}Ly6C^{hi}Ly6G^{-}$ cells to differentiate into protective cell types such as macrophages and dendritic cells is well established, the role for IMCs in antigen presentation and induction of T cell responses is unclear. Several studies have shown that depletion of immature monocytes during *Aspergillus, Mycobacterium, and Listeria* infection could blunt T cell responses.[33,78,84] This reduction in T cell responses was likely because immature monocytes recruited during *Aspergillus* and *Mycobacterium* infection could transport the pathogens to lymph nodes, but it appeared that these cells do not directly present antigen and prime T cells. Instead, it was suggested that antigens were transferred to dendritic cells by an unknown mechanism, which could then present the antigen and prime T cells.[33,84] In collaboration with the Bliska Laboratory, we showed that $CCR2^{+}CD11b^{+}Ly6C^{hi}Ly6G^{-}$ cells recruited during *Yersinia* infection could display antigen (Yue Zhang, Jason W. Tam, Patricio Mena, Adrianus W. M. van der Velden, and James B. Bliska. CCR2-expressing inflammatory dendritic cells and translocation of YopE are required for a massive CD8+ T cell response during *Yersinia* infection, manuscript in revision). Furthermore, in this study, we found that the large T cell response that developed against a *Yersinia* protein during infection was abrogated in $Ccr2^{-/-}$ mice. In order to better understand the role of $CD11b^{+}Ly6C^{hi}Ly6G^{-}$ cells during infection, we examined host factors, such as CCR2, that drive IMC recruitment and ultimately how recruitment of these cells affects the outcome of infection.

Consistent with the literature on the role for protection of $CD11b^{+}Ly6C^{hi}Ly6G^{-}$ cells in a variety of infections[26,29], we found that the CCR2/CCL2 signaling axis was important for the recruitment of these cells to sites of infection during *Salmonella* infection and was important for control of *Salmonella* infection (Jason W. Tam, James B. Bliska, and Adrianus W. M. van der Velden. Essential role for $CCR2^{+}$ inflammatory monocytes in the early and late control of *Salmonella* infection, manuscript in preparation; also see Chapter 3). However, in a recent study, Grainger *et al.* found that the protective effect of this signaling axis during *Toxoplasma* infection was not due to control of the pathogen but suppression of an over active neutrophil response.[50] Because CCR2 and CCL2 deficiency affects the recruitment of these cells, we determined the effect of depletion of these cells after recruitment. We found that transitory depletion of CCR2 expressing cells resulted in mortality of the mice (Chapter 3). Since during the early stages of infection innate immune control of the pathogen is paramount, it was not surprising that early

depletion of CCR2 expressing cells results in mortality. Indeed, several studies have shown that depletion of CCR2 expressing cells resulted in an increase in susceptibility to pathogens such as *Listeria monocytogenes* and *Mycobacterium tuberculosis*.[26,29,84]

Although our studies and those of others have shown that $CD11b^{+}Ly6C^{hi}Ly6G^{-}$ cells are protective during infection, there is evidence that these cells may have pathological properties during infection. In our work, although we found that $CD11b^{+}Ly6C^{hi}Ly6G^{-}$ cells recruited during *Salmonella* infection could present antigen, it was most efficient when nitric oxide production was inhibited.[136] This result suggested that these cells exhibit immunosuppressive properties and may contribute to the delayed onset of adaptive immunity observed during *Salmonella* infection. Moreover, in infections with certain viral pathogens, immature monocytes were found to inhibit antigen specific antiviral T cell responses.[68,69] An obvious reason for these seemingly dual and perhaps opposing roles was that these studies used different pathogens that have evolved distinct mechanisms for their pathogenesis. However, it is possible that IMCs may function differently in infections that cause acute and chronic illness. Indeed, at least one study has suggested that IMCs induced during the chronic phase of infection have an important immunosuppressive role that was not observed during acute infection.[69]

The majority of studies on IMCs in infection have focused on acute models. In the handful of studies that examine the role of these cells during chronic infection, the effect of IMCs on the outcome of infection is not clear. We found that the persistent recruitment and accumulation of $CD11b^{+}Ly6C^{hi}Ly6G^{-}$ cells we observed during chronic *Salmonella* infection was critical in protection. It was surprising that CCL2 deficient mice succumbed to infection during later stages of infection. Unlike in CCR2 deficient mice, there were substantial, but reduced, numbers of $CD11b^{+}Ly6C^{hi}Ly6G^{-}$ cells in CCL2 deficient mice throughout the infection (Chapter 3). These results would suggest that robust and sustained recruitment of cells by CCL2 is not required during early stages of infection but is required for the later stages. Consistent with this notion, we also found that depletion of CCR2 expressing cells during the later stages of infection resulted in mortality of the mice. Although this may not be especially surprising, no studies to the best of our knowledge have examined later stage depletion of CCR2 expressing cells during chronic infection. Overall, although $CD11b^{+}Ly6C^{hi}Ly6G^{-}$ cells are protective during chronic infection, it is unclear if they could also contribute to the chronicity of infection. It is intriguing to speculate

that these cells may have a similar immunosuppressive capability as the so called MDSCs observed in cancer as both cases fundamentally can be characterized as a type of chronic inflammatory disease.

Although the role of IMCs in chronic bacterial infection has remained largely unstudied, recent studies have examined mature macrophages, specifically alternatively activated macrophages, and their role in chronic infections. In a murine model of *Salmonella* Typhimurium and *Brucella abortus* infection, it was found that these pathogens resided in alternatively activated macrophages during the chronic phase of infection and were a major contributor to persistence.[114,228] Thus, it is intriguing to speculate that IMCs are the precursors to these cells. It is possible that these pathogens modulate the host environment or the cells directly to promote the generation of alternatively activated macrophages that ultimately allow for their persistence.

Generally, the consequences of chronic *Salmonella* infection for the host are not well studied. In a study that I co-first authored, DelGiorno *et al.* showed that persistent salmonellosis caused pancreatitis in a murine model of infection.[*162*] Interestingly, we also found elevated numbers of IMCs as well as macrophages in *Salmonella* infected pancreata.[*162*] Another study also reported pancreatitis associated with chronic *Salmonella* infection.[229] Because chronic pancreatitis is a significant risk factor for pancreatic ductal adenocarcinoma, these studies suggested that the chronic or recurrent inflammation induced by *Salmonella* infection might contribute to the induction of pancreatic cancer. It is intriguing to speculate that the IMC response induced during chronic infection may contribute to tumorigenesis insofar as the IMCs may be recruited to an emerging tumor and may become the so called MDSCs. Indeed, a recent study showed that bacterial infection could promote recruitment of tumor-promoting myeloid cells to gastric tumors. The myeloid cell recruitment could be abrogated with neutralizing antibodies against CCL2 and resulted in tumor regression.[230]

Cumulatively, our studies and those of others have led us to propose the following model on the role of IMCs during infection. In response to microbial insults, the host immune system mobilizes innate myeloid immune cells, such as $CD11b^{+}Ly6C^{hi}Ly6G^{-}$ cells. These cells produce large amounts of anti-microbial effectors that can control the spread and proliferation of the invading microbe. Some of these effectors, such as nitric oxide, can also inhibit T and B cell

responses. However it may be inconsequential, especially during early stages of infection, because adaptive immune responses are likely dispensable during the innate phase of immunity. Rather, this inhibition may prevent potentially damaging autoimmune responses. Under normal circumstances, the $CD11b^{+}Ly6C^{hi}Ly6G^{-}$ cells mature into protective cell types such as dendritic cells that can then direct the adaptive immune response against the microbe.

However, we speculate that during infection with certain pathogens such as *Salmonella,* the normal evolution of the immune response that ultimately clears the infection is altered. Upon infection, pattern recognition receptors recognize bacterial-derived molecules that can rapidly trigger CCR2-dependent recruitment and accumulation of $CD11b^{+}Ly6C^{hi}Ly6G^{-}$ cells in the spleen and liver. The rapid recruitment and the potentially unregulated antimicrobial molecules produced by $CD11b^{+}Ly6C^{hi}Ly6G^{-}$ cells, such as nitric oxide, serves to limit pathogen growth. However, because *Salmonella* is known to express proteins that can disarm these molecules, *Salmonella* can survive in the host despite the accumulation of these cells and their associated antimicrobial effectors.

We speculate that *Salmonella* has multiple potential mechanisms that may sustain the recruitment of $CD11b^{+}Ly6C^{hi}Ly6G^{-}$ cells. *Salmonella* can induce a type of cell death known as pyroptosis in several innate myeloid cell types that releases cytokines which can recruit $CD11b^{+}Ly6C^{hi}Ly6G^{-}$ cells to sites of infection.[116,117] *Salmonella* can also suppress the differentiation of myeloid cells.[231] There is also evidence that *Salmonella* may target and inject virulence factors into these cells[146]; this may influence how these cells behave. Together, these factors may help sustain the large numbers of $CD11b^{+}Ly6C^{hi}Ly6G^{-}$ cells while limiting the number of mature myeloid cells that ultimately activates the adaptive immune system.

We speculate that as a consequence of a sustained, large number of $CD11b^{+}Ly6C^{hi}Ly6G^{-}$ cells, the molecules they produce, especially nitric oxide, can create a prolonged state of immunosuppression. This results in decreased T cell responsiveness. Together with the killing of mature myeloid cells, *Salmonella* creates an environment that is not conducive for a T cell response to develop against the pathogen and this contributes to the ability for *Salmonella* to persist in the host. Over time, T cells against *Salmonella* are eventually activated and thus the host eventually mounts a T cell response. This response likely indirectly leads to a decrease in the $CD11b^{+}Ly6C^{hi}Ly6G^{-}$ cells and overall bacterial burden to undetectable levels.

$CD11b^{+}Ly6C^{hi}Ly6G^{-}$ cells are then required during the subclinical phase to contain the remaining *Salmonella* and prevent widespread reinfection of the host.

This model would suggest that, for the host, there is a tradeoff that leads to suboptimal induction of the adaptive immune system but allows for containment of the pathogen and survival of the host. This may be a host intrinsic response to pathogens that the host is unable to rapidly clear where the host takes the strategy of waging a war of attrition. Alternatively, this may be a pathogen strategy to promote persistence. Ultimately, it is likely a composite where the host and pathogen reach a form of homeostasis. The findings presented in this dissertation have important implications in the host pathogen interaction during persistent infections. Ultimately could lead to novel therapeutics to combat currently intractable or difficult to treat chronic infections, such as tuberculosis, a major chronic infection of humans worldwide.[232]

Chapter 6. Future directions.

The role of IMCs during infection, especially during chronic and subclinical phases, is not well understood. In this dissertation, we have focused on a subset of immature monocytes in the host response to *Salmonella* infection. The major finding of this dissertation is that immature monocytes play an important role in protecting the host against chronic *Salmonella* infection. However, many important questions remain to be addressed.

The most pressing question in our studies is how the distribution of *Salmonella* in tissues is affected by a defect in recruitment or depletion of $CD11b^{+}Ly6C^{hi}Ly6G^{-}$ cells during infection. We are currently performing experiments to examine, by histopathology, the spleen, liver, and bone marrow of wildtype, *Ccr2*$^{-/-}$, and *Ccl2*$^{-/-}$ mice chronically infected with *Salmonella*. We are also examining tissues from CCR2-DTR-CFP mice that have been depleted of CCR2 expressing cells during later stages of *Salmonella* infection. Furthermore, using immunohistochemistry, we will determine the spatial distribution of *Salmonella* and $CD11b^{+}Ly6C^{hi}Ly6G^{-}$ cells in these infected mice. Future studies would also examine the other ligands of CCR2, namely CCL7 and CCL12. *Ccl7*$^{-/-}$ mice are commercially available and has been shown to play a role in the recruitment of $CD11b^{+}Ly6C^{hi}Ly6G^{-}$ cells during *Listeria monocytogenes* infection.[71]

Although we found that $CD11b^{+}Ly6C^{hi}Ly6G^{-}$ cells mediate protection against chronic *Salmonella* infection, it is unclear what functions of these cells are important. It would be interesting to perform RNA-seq experiments with $CD11b^{+}Ly6C^{hi}Ly6G^{-}$ cells from day 10, 30 and 180 days post *Salmonella* infection to determine the functional differences that may exist between the acute, chronic, and subclinical stages of infection. We have examined the serum cytokine profile of *Salmonella* infected mice that were depleted of CCR2 expressing cells but further studies validating the findings and examining the functional consequence of these changes are required.

In order to expand our understanding on the role of CCR2 expressing cells during persistent *Salmonella* infection, we took a mixed bone marrow chimera approach to modulate the numbers of CCR2 expressing cells that are depleted. We found that modulating the numbers of depleted CCR2 expressing cells could affect the outcome of infection. Preliminary data suggested that there was enhanced survival of mice as the number of CCR2 expressing cells that were depleted

was decreased (Tam and van der Velden, unpublished). To expand these findings, it would be interesting to take a pharmacological approach to modulate the recruitment of $CD11b^{+}Ly6C^{hi}Ly6G^{-}$ cells; the CCR2 agonist, RS102895, and the peroxisome proliferator-activated receptor gamma agonist, pioglitazone, have been used to modulate the recruitment of immature monocytes.[62,233] Moreover, an important experiment would be to use a mixed bone marrow chimera approach to examine the functional consequences of iNOS, and TNF-α expression in these cells during chronic *Salmonella* infection. Another approach would be to generate CCR2-cre mice and cross them to floxed iNOS or TNF-α mice.

In the CCR2 depletion experiments we used a single dose of diphtheria toxin at either time point and found that it was sufficient to cause mortality. Because the depletion was transitory where the cells were recruited back before we observed mortality (Tam and van der Velden, unpublished), it suggested that a perturbation of the persistent IMC response could lead to loss of control of *Salmonella* infection. Indeed, transitory depletion of CCR2 expressing cells one day before infection also led to mortality (Tam and van der Velden, unpublished) and thus suggested that a delay in the response could also be fatal. Interestingly, when *Salmonella* infection reached a subclinical phase where *Salmonella* could no longer be detected in the stool or blood, transitory depletion of CCR2 expressing cells was fatal (Tam and van der Velden, unpublished). This suggested that CCR2 expressing cells were important beyond primary infection and perhaps suppressed growth of the dormant *Salmonella* during the subclinical phase. Further study would characterize the requirement of CCR2 expressing cells at the onset and subclinical phase of infection.

An important aspect not addressed in this dissertation is the fate of $CD11b^{+}Ly6C^{hi}Ly6G^{-}$ cells recruited in response to *Salmonella* infection. Using an adoptive transfer approach with bone marrow cells from uninfected CCR2-GFP mice, we obtained preliminary data suggesting that $CD11b^{+}Ly6C^{hi}Ly6G^{-}$ cells could proliferate and differentiate into $CD11c^{+}$ cells during *Salmonella* infection. Future experiments would be needed to confirm this finding and determine the role these differentiated cells play during infection. However, it is unclear if this occurred in the periphery or at the site of infection because these cells could presumably traffic to the bone marrow. Future studies would also use $CD11b^{+}Ly6C^{hi}Ly6G^{-}$ cells from CCR2 deficient mice to address this question. It would also be of interest to determine if these cells can differentiate into

alternatively activated macrophages since they have been shown to be the primary cell in which *Salmonella* resides in during the chronic stage of infection.[114] A straightforward approach would be to stain adoptively transferred $CD11b^{+}Ly6C^{hi}Ly6G^{-}$ cells for markers of alternatively activated macrophages. Another approach could be to infect these cells *ex vivo* and adoptively transfer them into infected mice. The goal would be to determine if these *Salmonella* infected cells could be actively manipulated by the pathogen to differentiate into alternatively activated macrophages.

In addition to host factors, we also examined *Salmonella* factors that drive the IMC response. We examined genes with known effects on *Salmonella* virulence in mice and found that genes involved in *Salmonella* virulence, pathogen defense against antimicrobials and, surprisingly, metabolism have an effect on the IMC response. The metabolic gene and pathway we focused on were *aroA* and the chrosimate pathway. The *aroA* gene was examined because *aroA* mutants are well-characterized attenuated strains that have been used as vaccines. It is thought that *aroA* mutants are attenuated because these strains cannot produce p-amino-benzoic acid and 2,3-dihydroxybenzoate, both of which are not found in mammals.[234] We reasoned that vaccine strains might not induce a strong IMC response and thus protective adaptive immunity could form, which is the case for *aroA* mutants. Despite intense study and its use as a vaccine, the mechanisms that underlie the ability for *aroA* mutants to induce potent adaptive immune responses are unclear. Several studies have determined that these mutations could have many effects on *Salmonella.* For example, an *aroA* mutant was shown to have defects in their cell wall[235] and to be defective in the formation of biofilms.[236] In our work, we found the *aroA* mutants failed to induce a robust IMC response. Intriguingly, after examining the metabolic pathways that are downstream of *aroA*, we unexpectedly found that a defect in the ubiquinol-8 pathway also failed to induce a robust IMC response. This suggested that this pathway was a contributor to the inability for the *aroA* mutant to induce a robust IMC response. Future studies would further dissect this pathway. We have generated several mutants in this pathway but deletion of the major genes in this pathway have proven challenging. It is possible that these genes led to an accumulation of a toxic intermediate. Regardless, it is possible that the ubquinol-8 pathway produces a vita-PAMP[237] that is sensed by a host factor, which in turn triggers the IMC response. A potential candidate for this sensor is the aryl hydrocarbon receptor.[238]

Therefore, it would be of interest to study the IMC response in an aryl hydrocarbon receptor knockout mouse.

$CD11b^{+}Ly6C^{hi}Ly6G^{-}$ cells that require CCR2 for their recruitment are only a subset of MN cells. The other major subset is CX_3CR1^{+} monocytes.[29,239] Thus, it would be interesting to study the role of these cells during chronic infection. During acute *Salmonella* infection, it has been shown that these cells play a role in dissemination and protection[171], but their role in chronic *Salmonella* infection is unstudied. Thus, we would use CX_3CR1 and CX_3CL1 knockout, CX_3CR1-GFP, and CX_3CR1-DTR mice [240] to study their role during chronic infection.

Although this dissertation examined the kinetics of $CD11b^{+}Ly6C^{int}Ly6G^{+}$ PMN IMCs recruitment, we did not study the role of these cells during chronic *Salmonella* infection. A straightforward approach to study the role of these cells would be to use antibody mediated-depletion with anti-Ly6G during both the acute and chronic stage of infection. After depletion, we would determine its effect on susceptibility to infection and its impact on adaptive and innate immune function. It would also be interesting to study the molecular mechanisms that drive recruitment of these cells and its effect during acute and chronic *Salmonella* infection. We would examine available knockout mice that may influence recruitment of PMN IMCs. For example, CXCR2, and CXCL2 are involved in recruiting PMN cells out of the bone marrow and CXCR4 is involved in preventing the emigration of PMNs from the bone marrow.[30] Thus, we would use CXCR2, and CXCL2 knockout mice and determine the susceptibility of these mice to chronic infection. CXCR4 knockouts are lethal and thus, we would use a floxed CXCR4 mouse crossed onto a MRP8-cre mouse; the MRP8 promoter would allow for CXCR4 to be knocked out of PMN cells.

An important aspect of *Salmonella* pathogenesis that is not addressed in this dissertation is the role of $CD11b^{+}Ly6C^{hi}Ly6G^{-}$ cells in the gastrointestinal tract because our studies use a mouse model of systemic typhoid infection. To address this, we have been recently using a model of *Salmonella* mediated colitis to study the role of $CD11b^{+}Ly6C^{hi}Ly6G^{-}$ cells in the gut. Preliminary data suggested that these cells were present in the gut during infection. Furthermore, we found that the recruitment of $CD11b^{+}Ly6C^{hi}Ly6G^{-}$ cells to the gut was dependent on CCR2, but surprisingly found that recruitment of these cells to the gut did not affect colonization by *Salmonella* (Tam, Koenig, Bäumler, and van der Velden, unpublished). Current and future work

will focus on the contribution of $CD11b^{+}Ly6C^{hi}Ly6G^{-}$ cells to the inflammation in the colon induced during *Salmonella* infection.

Perhaps the most interesting studies would involve a combination of the host and pathogen factors that are examined and proposed in this dissertation. By studying both aspects, we can more fully understand how the host pathogen interaction evolves and how certain infections lead to clearance while others lead to persistence. Since many chronic and subclinical infections are important reservoirs for pathogens, these studies could contribute to eliminating this reservoir and solve a major public health problem. Ultimately, a better understanding of the IMC response during infection could lead to novel strategies in the treatment diseases.

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