INNATE IMMUNITY TO LISTERIA MONOCYTOGENES INFECTION:
REGULATION OF DENDRITIC CELL AND NEUTROPHIL RESPONSES BY MYD88
AND THE TYPE I IFN RECEPTOR

By

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A Dissertation Submitted to the Graduate Faculty of
WAKE FOREST UNIVERSITY GRADUATE SCHOOL OF ARTS AND SCIENCES
In Partial Fulfillment of the Requirements
For the Degree of
DOCTOR OF PHILOSOPHY
In the Molecular Genetics and Genomics Program
August 2010
Winston-Salem, North Carolina

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ACKNOWLEDGEMENTS

There are a lot of people that I would like to thank, both professionally and personally. First, I would like to thank my mentor Dr. Beth Hiltbold. When I first joined her lab as a technician she taught me not only how to perform immunology experiments, but also supported me and my quest of pursuing a graduate education. For believing that I was up to this task, even when others did not, for this I will always be grateful! I have had the opportunity to learn many things firsthand about running a lab and the details that go with this responsibility. These experiences as well as my academic training under her guidance have helped me become the scientist I am today. I would also like to thank my committee members for being a part of this process and helping me become a better scientist.

Special thanks also go out to other people that have been part of our lab. To Dr. Marlena Westcott for always lending an ear when I needed it. Whether talking about science, my data, or even life in general, she was there to give her opinion and always asked “What is the question?” and this has helped me focus the way I approach my work. Thank you! I would also like to thank Dr. Curtis Henry for always being willing to drop whatever he was doing to help someone out. I have never met a person as gracious and helpful as you. From our discussions about data, immunology and life, I have learned a lot from you and am a better person for knowing you. To Dr. Latoya Mitchell, thank you for always listening and being there when I needed it….even if it was by phone! I would also like to thank Anne Cook for being a good lab mate.

I would also like to thank my undergraduate advisor, Dr. Walter Goodman for taking the time and caring enough about teaching. Your enthusiasm has been an inspiration. I would also like to thank my undergraduate research advisor, Dr. Que Lan for introducing me to what scientific research is all about. You opened the door that began my scientific career. For that experience I will always be grateful.

On a personal level, I would first and foremost like to thank my parents Wally and Barb Brzoza, for instilling in me that the sky is the limit and that I can do anything I set my mind to. I hope I have made you proud. I would also like to thank my sister Kim Brzoza for listening to me complain without having a clue what I was talking about! It is nice to get an outside perspective. I would also like to thank my in-laws Mike and Betty Lewis for their continued support and encouragement throughout the years. Additionally, I would like to thank all of the friends I have made over the years, many of which have come and now moved on to real jobs. Misery loves company! I wish you all continued success in life.

Next, I would like to thank my four legged companion Winston, for always being there to cheer me up when I needed it! For always being by my side and keeping me company while I was writing, even until the wee hours of the
morning. Finally, last but not least, I would like to thank my husband, Dr. Eric Lewis for his continued love, support, and encouragement. Thank you for understanding about the late days and long nights while I was doing experiments. I don’t think I could have made it through this without you. It has been a tough couple of years, with two PhD thesis’ and all of the other things we have gone through. However, I think we came out on the other side stronger for it. I love you and your belief in me has made me strive to be a better person.

To anyone else that has helped me along the way, other Molecular Genetics program members (Dr. Lively and Deborah), other Microbiology and Immunology department members, and anyone else I may have forgotten, thank you!
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LIST OF ABBREVIATIONS

7-AAD, 7-Amino-Actinomycin D

APC, Antigen Presenting Cell

BAL, Bronchial Alveolar Lavage

BHI, Brain Heart Infusion

CD40, Cluster of Differentiation factor 40

CFSE, Carboxyfluorescein Succinimidyl Ester

CFU, Colony Forming Units

CTL, Cytolytic T cell

DC, Dendritic Cell

DHR, Dihydrorhodamine

DNA, Deoxyribonucleic Acid

ELISA, Enzyme Linked Immunosorbant Assay

FCS, Fetal Calf Serum

Flt3L, Fms-like Tyrosine 3 Kinase Ligand

G-CSF, Granulocyte-Colony Stimulating Factor

GM-CSF, Granulocyte Macrophage-Colony Stimulating Factor

h, Hours

Hly, Hemolysin

hpi, hours post infection

IFN, Interferon

IFNAR, Type I IFN receptor

IL-1α/β, Interleukin 1α or β

IL-12, Interleukin-12

IL-12p40, Interleukin 12, p40 subunit
IL-6, Interleukin 6
iNOS, Inducible Nitric Oxide Synthase
i.p., Intraperitoneal
i.v., Intravenous
LLO, Listeriolysin O
*Lm, Listeria monocytogenes*
LPS, Lipopolysaccharide
LTA, Lipoteichoic Acid
MDA-5, Melanoma Differentiation Association Antigen-5
MFI, Mean Fluorescence Intensity
MHC, Major Histocompatibility Complex
MOI, Multiplicity of Infection
µg, Microgram
µl, Microliter
µM, Micromolar
mg, Milligram
ml, Milliliter
mM, Millimolar
MyD88, Myeloid Differentiation Factor 88
NF-κB, Nuclear Factor-Kappa B
ng, Nanogram
NK cell, Natural Killer cell
NO, Nitric Oxide
NOD, Nucleotide-binding Oligomerization Domain
NT, Untreated
OVA, Ovalbumin
PALS, Periarteriolar Lymphoid Sheath
PAM3CSK4, Pam3Cys-Ser-Lys4
PAMP, Pathogen Associated Molecular Pattern
PE, Phycoerythrin
PKR, ds-RNA-Dependent Protein Kinase
PMA, Phorbol Myristate Acetate
Poly I:C, Polyinosinic:Polycytidylic Acid
PRR, Pattern Recognition Receptor
RIG-I, Retinoic Acid Inducible Protein-I
RNA, Ribonucleic Acid
RNI, Reactive Nitrogen Intermediates
ROI, Reactive Oxygen Intermediates
ROS, Reactive Oxygen Species
SLE, Systemic Lupus Erythematosus
Tip DC, TNF/iNOS Producing DC
TLR, Toll-like Receptor
TNF-α, Tumor Necrosis Factor-alpha
TRAIL, TNF-Related Apoptosis Inducing Ligand
WT, Wild-type
ABSTRACT

Kristina L. Brzoza-Lewis

Innate Immunity to *Listeria monocytogenes* Infection: Regulation of Dendritic Cell and Neutrophil Responses by MyD88 and the Type I IFN Receptor

Dissertation under the direction of

Elizabeth Hiltbold Schwartz, Ph.D., Assistant Professor
of Microbiology and Immunology

*Listeria monocytogenes* (*Lm*) is an intracellular bacterial pathogen that is of consequence to pregnant women and immunocompromised individuals. Exploiting a niche, *Lm* gains access to the host cell cytosol via the pore forming toxin Listeriolysin O (LLO) and infects a variety of cell types including dendritic cells (DC). Our laboratory and others have shown that optimal DC maturation occurs when *Listeria* gain access to the host cytosol, and this correlates with protective immunity.

Live *Lm* interact with cell surface/endocytic pattern recognition receptors (PRR), and with cytosolic PRR. We sought to determine the contribution of MyD88, a signaling mediator involved in pathogen recognition via surface/endocytic receptors, and the type I IFN receptor (IFNAR), a signaling mediator used as a surrogate for cytosolic responses, on DC maturation induced by *Listeria*. Using bone marrow-derived DC generated from MyD88-/- and IFNAR-/- mice, we determined that signaling through MyD88 was important for the secretion of pro-inflammatory cytokines such as IL-12, IL-6, and TNF-α.
However, the expression of costimulatory molecules CD40 and CD86 utilized signaling through IFNAR. In a systemic infection model, however, costimulatory molecule expression by splenic DC was both MyD88 and IFNAR-independent. These findings highlight the importance of both MyD88 and type I IFN signaling pathways on Lm-induced DC maturation, as well as the complexity of pathogen-induced signaling in DC.

Mice lacking IFNAR, and thus type I IFN signaling, are more resistant to Listeria infection relative to WT mice. The resistance phenotype of IFNAR-/- mice has been in part attributed to reduced lymphocyte apoptosis and enhanced TNF-α production. Using an innate cell recruitment model, we demonstrate enhanced recruitment of neutrophils, and reduced recruitment of monocytes in IFNAR-/- compared to WT mice following Lm infection. Studies have shown that IFNAR-/- mice exhibit increased production of cytokines and chemokines compared to WT mice. Thus, we hypothesized that increased production of CXC chemokines, important for neutrophil recruitment, could result in the increased neutrophil presence observed in IFNAR-/- mice. We therefore treated IFNAR-/- mice with the CXCR2 inhibitor antileukinate, and following infection, observed reduced neutrophil recruitment into the spleen and this correlated with increased bacterial burdens. Therefore, these studies demonstrate that type I IFN signaling inhibits the immune response to Listeria through the negative regulation of CXC chemokine production, and suggests an additional mechanism for enhanced resistance of IFNAR-/- mice to Listeria.
These studies highlight important differences in the regulation of two essential components of the DC maturation process, costimulatory molecule and pro-inflammatory cytokine production, following Lm infection. In addition, we have demonstrated an additional mechanism by which IFNAR-/- mice resist Listeria infection. Specifically, a more robust neutrophil presence was observed in IFNAR-/- mice relative to WT mice, and this appears to be a function of enhanced chemokine-mediated recruitment of these cells to infection sites. Thus, these studies have implications on vaccine development and also highlight potential targets for therapeutic intervention against secondary bacterial pneumonia.
CHAPTER I

INTRODUCTION

The innate immune system is essential for the control of invading bacterial pathogens and facilitates the generation of protective immunity. Numerous cell types such as neutrophils, NK cells, monocytes, and dendritic cells (DC) have specialized functions that aid in the defense against invading bacteria [1]. When host cells are infected with intracellular bacteria, such as Listeria monocytogenes (Lm), they replicate inside the cells and therefore avoid recognition by traditional innate responses such as neutrophils, complement and antibodies [2]. As a result, clearance of Listeria is mediated by CD8+ T cells [3]. Thus, the immune response has evolved to respond to various bacterial pathogens through different mechanisms based on the niche to which the bacteria are adapted.

Following bacterial infection, there are several cells of the innate immune system that have specialized functions to help eradicate the infection. Neutrophils are the first line of defense against invading bacteria and keep bacterial replication in check until monocytes are recruited to the site of infection and the adaptive response can be generated. In response to IL-12 produced by infected macrophages, NK cells provide additional help in the form of cytokine production that differentiates monocytes and activates bactericidal activity [1, 4]. Finally, DC are capable of acquiring bacterial antigens and maturing in response to bacterial stimuli. Once in a mature state, DC have the capacity to prime T cell
responses [5]. Thus, the innate immune response is composed of several cell types that work in concert to control bacterial replication, acquire antigens and ultimately lead to the generation of protective immunity.

While it is clear that DC are essential for the generation of CD8+ T cell responses and therefore clearance of *Lm*, the mechanisms governing DC maturation in response to intact bacterium are less well defined. I will highlight what is known about the regulation of innate immunity to *Listeria* and where gaps in the knowledge remain. Work in this thesis is focused on understanding how innate immune responses to *Listeria* are regulated. We have determined that the process of DC maturation is differentially regulated with *Listeria*-induced cytokine responses utilizing signaling through the adapter molecule MyD88, and costimulatory molecule expression signaling through the type I IFN receptor (IFNAR). Additionally, we have concluded that type I IFN negatively regulates chemokine production and therefore inhibits neutrophil recruitment. These studies implicate enhanced neutrophil recruitment as an additional mechanism leading to the resistance of mice lacking type I IFN signaling to *Listeria* infection.

**Listeria monocytogenes- an intracellular pathogen**

*Listeria monocytogenes* (*Lm*) is a Gram-positive bacterium that is commonly associated with food borne illnesses [6, 7]. *Listeria* has an intracellular life cycle that confers a survival advantage to the bacterium (Figure 1). First, *Listeria* are taken up by a cell into vacuoles by phagocytosis [8]. Once present in vacuoles, a pH dependent, pore forming toxin, listeriolysin O (LLO) disrupts the phagosomal membrane and allows entry of *Listeria* into the host cell
cytoplasm. LLO is essential for bacterial entry into the cytosol as mutants that lack this molecule are retained in the phagosome and do not grow [9]. A tightly coordinated set of virulence factors are then produced that facilitate bacterial movement within the cytosol and spread to neighboring cells [2, 10]. The intracellular life cycle that *Lm* employs during infection of mammalian hosts prevents recognition by complement and antibody responses of the immune system [2]. Because *Listeria* enters the cytosol, the bacterium itself or proteins it secretes such as LLO, have the ability to be processed and presented in the context of MHC class I. Therefore, clearance and protective immunity to this pathogen are primarily mediated by CD8+ T cells [11, 12]. Thus, the intracellular life cycle of *Listeria* involves its potential interaction with several membrane-bound and/or cytosolic pattern recognition receptors and thus provides many opportunities for the investigation of bacterial sensing by host cells.

**Detection of and response to phagosomally retained *Listeria***

The innate immune system can detect invading pathogens through a set of specialized receptors. An intact bacterium such as *Listeria* contains many “pathogen associated molecular patterns” (PAMPs) than can be recognized by various pattern recognition receptors (PRR) on host cells such as DC and macrophages. These receptors are encoded in the germ line DNA and are conserved in species from animals to plants and can be expressed on the cell surface, in intracellular compartments, or in the cell cytoplasm [13]. PRR recognize conserved molecules on microbes that are imperative for survival such as lipopolysaccharide (LPS) and lipoteichoic acid (LTA) [13]. Toll-like receptors
Figure 1. The Intracellular Life Cycle of *Listeria monocytogenes*. The intracellular life cycle of *Listeria monocytogenes* involves the uptake of the bacterium by a host cell into the endocytic pathway (1). *Listeria* escape from the primary vacuole with the help of a pH-dependent pore forming toxin listeriolysin O (LLO) (2). Upon entry into the cytosol, the bacteria replicate and coat themselves in host cell actin (3). The actin is polarized into a comet like tail which the bacteria use to move around in the host cell cytosol and propel themselves into a neighboring cells, thereby avoiding both complement and antibody responses (4). Once in a neighboring cell (5), *Listeria* is able to escape from the secondary vacuole utilizing LLO (6), and the process repeats again.
The Intracellular Lifecycle of *Listeria*

Adapted from Tilney and Portnoy 1998
(TLR) are a group of membrane-bound receptors that can be found on the surface of cells or within the endocytic pathway (Figure 2). Those TLR on the surface (TLR 1, 2, 4, 5, and 6) have recognition specificities for bacterial products including LPS, flagellin, LTA or others. However, the TLR localized within phagosomes (TLR 3, 7, and 9) tend to recognize nucleotide ligands characteristic of partially degraded viruses and bacteria [14]. Recognition of ligands by their specific TLRs initiates a signaling cascade that recruits the adapter molecule MyD88 to the receptor complex. Ultimately, this signaling cascade leads to the activation of NF-κB and the transcription of target genes such as those encoding for cytokine production and other inflammatory mediators [15].

MyD88 is an adapter molecule utilized in TLR and IL-1/18R signaling. A majority of TLRs utilize MyD88 either alone or in combination with other proximal adapters to transduce signals that ultimately lead to the production of inflammatory cytokines and induction of bactericidal activity. Genes known to be regulated by MyD88 include but are not limited to IL-1α/β, IL-6, TNF-α, IL-12, and IFN-γ [16-19]. Thus, the absence of MyD88 ablates signaling through most TLR and as a result, MyD88-deficient mice are susceptible to bacterial pathogens, including Listeria [20]. MyD88-/− mice infected with Listeria fail to produce cytokines such as IL-12, IFN-γ, or TNF-α in response to infection and succumb to infection within 3 days [16]. Interestingly, if MyD88-deficient mice are infected with a mutant strain of Listeria that has the ability to access the cytosol and therefore induce DC maturation but not spread to neighboring cells, these mice can survive a high dose challenge and clear the bacteria within 7 days.
Figure 2. *Listeria’s Path to Induce DC maturation.* When *Listeria* comes into contact with a host cell, such as dendritic cell (DC), it can interact with pattern recognition receptors (PRR) on the cell surface including Toll-like receptors (TLR) (1). Once *Lm* is taken up into a vacuole, it also has the potential to interact with phagosome-localized TLR (2). With the help of the pH-dependent, pore forming toxin LLO, *Listeria* can gain access to the host cytosol and interact with a currently unknown cytosolic receptor(s) which leads to the production of copious amounts of IFN-β (3). IFN-β then binds to the Type I IFN receptor (IFNAR) on the cell surface either in an autocrine or paracrine manner, and induces the transcription of IFN-inducible genes (4).
Listeria’s path to induce DC maturation

1. Lm
2. Phagosomal TLR’s
3. Cytoplasmic Receptors
4. IFN-β/α Receptor
Additionally, MyD88-/- mice are able to generate *Listeria* specific CD8+ T cell responses that were equivalent to controls [21]. Therefore, these results suggest that if MyD88-/- mice can survive the initial immune response, they are capable of priming adaptive immune responses that are protective against a wild-type *Listeria* challenge. Thus, MyD88 likely does not play a role in naïve T cell priming or the generation of protective immune responses to *Listeria*.

**Detection and response to cytosolic *Listeria***

*Listeria* utilizes the pH-dependent toxin LLO to gain access to the host cell cytosol. Once in the cytosol, *Listeria* interacts with a currently unidentified receptor and induces the production of copious amounts of IFN-β [22]. IFN-β can act in an autocrine or paracrine manner binding to the type I IFN receptor (IFNAR) and resulting in the transcription of IFN-inducible genes [18, 23] (Figure 2). Type I IFN has been shown to mature DC [24, 25] and given the significant amounts of IFN-β produced in response to *Listeria*, it is likely that a large component of *Listeria*-induced DC maturation is the result of a bystander effect of IFN signaling on neighboring cells. Thus, type I IFN likely plays an important role in the immune response against *Listeria* due to the ability of *Listeria* to gain access to the host cell cytosol, like a virus, and thus trigger the production of IFN-inducible genes. Clearance of *Listeria* is mediated by CD8+ T cell effector functions [3]. While type I IFN can indirectly influence naïve antigen specific T cell priming by modulating DC maturation, type I IFN has also been shown to augment T cell priming directly by acting as the cytokine signal required for CD8+
T cell activation [22, 26]. Therefore, type I IFN plays an important role in the immune response against *Listeria* due to its ability to modulate DC maturation and augment CD8+ T cell priming.

Type I IFN enhances the immune response to *Listeria* with effects on DC maturation and T cell priming [22, 24, 26], however, type I IFN also has detrimental effects on *Listeria* specific immunity. Type I IFN signaling has been shown to sensitize macrophages [27] and lymphocytes to apoptosis [28, 29]. This is of consequence since both macrophages and lymphocytes are important for the control of a *Listeria* infection. Mice that lack IFNAR, and therefore type I IFN signaling, have heightened resistance to *Listeria* infection compared with WT mice [28-30]. Studies have demonstrated that WT mice had increased bacterial burdens compared to IFNAR-/- mice at 48 and 72 hours post infection (prior to generation of the T cell response) [28-30]. Additionally, an increase in cells expressing CD11b on the cell surface and producing TNF-α was also observed [30]. The enhancement of a CD11b+ population of cells suggested to us that this cell population may be a factor contributing to the resistance phenotype observed in IFNAR-/- mice. Therefore, we hypothesize that type I IFN signaling negatively regulates innate immune cell function in clearance of *Lm*. Further demonstration of the validity of our hypothesis is that type I IFNs have been shown to alter the expression of chemokines that are essential for innate immune cell recruitment, specifically neutrophils [31, 32].

Other IFN-inducible genes that are important to the immune response include pro-apoptotic genes such as dsRNA-dependent protein kinase (PKR),
TNF-related apoptosis inducing ligand (TRAIL), and Daxx. Type I IFN increases the sensitivity of viral infected cells to apoptosis, a mechanism that is suggested to limit viral spread [18, 23, 33]. These apoptotic cells can also be taken up by antigen presenting cells and cross presented to naïve T cells. Additionally, CD40 has been shown to be an IFN-inducible gene and the expression of this molecule on the surface of DC is important for priming T cell responses [25]. Thus, IFN-inducible genes can help prevent pathogen spread as well as provide antigenic cargo and the costimulatory molecules necessary for the generation of adaptive immune responses.

**Innate immune responses against *Listeria***

While the adaptive immune response is capable of clearing a *Listeria* infection and generating protective immunity, there are several innate immune cell types that perform vital tasks to prevent the pathogenic insult from multiplying rapidly and aid in the development of an adaptive immune response. Many of these innate immune cell types such as: neutrophils, NK cells, and monocytes are critical to the host at early timepoints post infection due to their bactericidal properties, and their production of cytokines that activate and/or recruit other cell types to the infectious foci. DC are also important due to their ability to present bacterial-derived antigens leading to the generation of adaptive immunity.

**Neutrophils:** The innate immune system is comprised of numerous cell types. One specific cell type, neutrophils, are essential for the protection of a host against both bacteria and fungal infections. However, while neutrophils are important for the control of infections, there is also a consequence of neutrophil
infiltration and activation, namely the potential for tissue damage and subsequent pathologic conditions. Therefore, it is important to keep the neutrophil population regulated and only recruited when an infectious insult occurs [34-36]. Neutrophil homeostasis is a process that is tightly regulated at the levels of granulopoiesis, recruitment, and subsequent removal of these cells in order to prevent pathological effects [34]. A majority of the mature neutrophils reside in the bone marrow but can be rapidly mobilized when the correct signals are present. A variety of chemotactic factors can rapidly mobilize the egress of neutrophils out of the bone marrow including: leukotriene B4, complement components, as well as CXC chemokines including CXCL1 (KC) and CXCL2 (MIP-2) [36]. However, the type of signal received has a dramatic impact on the kinetics of neutrophil mobilization. For example, the cytokine G-CSF elicits peak neutrophil mobilization within 4-6 hours, while IL-8 has been shown to recruit large numbers of neutrophils out of the bone marrow within a few minutes [34].

Under homeostatic conditions, neutrophils are retained in the bone marrow via the SDF-1α/CXCR4 axis [37-39]. However, under conditions where the cytokine G-CSF is produced, for example in response to pathogens, neutrophils exit the bone marrow and enter the bloodstream [40]. The effect of G-CSF is indirect as it induces the downregulation of CXCR4 on the surface of the neutrophils and therefore they no longer receive the retention signal [34, 36, 40, 41]. Once the neutrophils are in the blood, they can be recruited into sites of infection by sensing the chemokines CXCL1 and/or CXCL2. These chemokines bind to the CXCR2 receptor on the neutrophil surface. Additionally, CXCL1 and
CXCL2 are able to directly recruit neutrophils out of the bone marrow [34, 36, 38, 42, 43]. Thus, cytokines and/or chemokines play an important role in both the retention of neutrophils in the bone marrow as well as the recruitment of neutrophils out of the bone marrow and into tissues in response to infection. However, the regulation of chemokines and PMN recruitment is not fully understood.

Once at the site of infection, neutrophils are able to kill bacteria through the production of reactive oxygen intermediates (ROI) by the NADPH oxidase complex [44-46] and reactive nitrogen intermediates (RNI) produced by iNOS [47-51]. Demonstration of the importance of ROI in *Listeria* killing has been shown as enhanced susceptibility of mice that lack either gp91phox or p47phox, components of the NADPH oxidase complex [52, 53]. Antimicrobial proteins and peptides are also an effective means of killing bacteria. Additionally, neutrophils also have granules that contain many other cytotoxic compounds including: defensins, lysozyme and myeloperoxidase [45, 54]. Thus, neutrophils are a cell type specialized for killing invading microbes.

While neutrophils are not essential in response to slow replicating bacterial pathogens such as *Mycobacterium* [55], they are however, vital in response to numerous other bacterial pathogens, including but limited to: *Salmonella typhimurium, Yersinia enterocolitica,* and *Listeria monocytogenes* [56]. The importance of neutrophils during a *Listeria* infection was demonstrated by studies that selectively depleted neutrophils with the RB6-8C5 monoclonal antibody. When neutrophils were removed prior to, or at early timepoints post
infection, an increase in bacterial burdens was observed [57] with the contribution of neutrophils in controlling bacterial burdens detected at least four days post infection [58]. Interestingly, neutrophils play a more significant role in managing bacterial replication in the liver than the spleen [59]. The significance of neutrophils in the immune response to *Listeria* is highlighted by the fact that mice succumb to infection within four days post infection in the absence of neutrophils [55, 60].

Neutrophils not only play a vital role in the innate immune response to *Listeria* by controlling bacterial replication, they also influence adaptive immune responses due to their ability to recruit and activate DC [49, 61]. With neutrophils and DC present in the same microenvironment, neutrophils are capable of influencing the behavior of DC. Neutrophil produced TNF-α has been shown to mature DC, as measured by the upregulation of costimulatory molecules and production of the cytokines IL-12p40 and TNF-α [61]. Additionally, studies have shown that neutrophils and DC physically interact and this contact also results in DC maturation [62, 63]. It has also been shown that ROI are capable of maturing DC as demonstrated by the upregulation of costimulatory molecules and production of proinflammatory cytokines [64-66]. Therefore, neutrophils are also capable of modulating adaptive immune responses due to their ability to influence DC maturation.

**NK cells:** Natural killer (NK) cells are an innate immune cell type that is essential for the control of *Listeria* at early time points post infection [67, 68]. NK cells are an early source of IFN-γ that leads to the activation of macrophages and
resulting microbicidal activities that control bacterial burdens [1, 69, 70].

Interestingly, a complex cascade of events results in the NK cell production of IFN-γ. Firstly, infected macrophages produce both IL-12 and TNF-α. These cytokines then act synergistically to drive the production of IFN-γ by NK cells [69, 71-73]. A more recently described cytokine, IL-18, in combination with IL-12 produced by macrophages has also been shown to elicit IFN-γ production by NK cells [74-76]. While it has been shown that NK cells are important in the context of a Listeria infection, it has also been observed that mice that fail to respond to IFN-γ either due to the lack of a functional receptor, or functional cytokine had increased susceptibility to Listeria infection [77, 78]. Therefore, these results highlight the importance of IFN-γ in the immune response against Listeria infection.

**Monocytes:** Monocytes are an additional innate immune cell type that is important in the control of a Listeria infection. Circulating monocytes are a precursor cell that under homeostatic conditions can repopulate either tissue macrophages or DC [79, 80]. However, in response to microbial stimuli, bone marrow monocytes as well as circulating monocytes upregulate the chemokine receptor, CCR2 and can be recruited to sites of infection by monocyte chemoattractant protein-1 (MCP-1), the ligand for CCR2 [4].

Under inflammatory conditions, monocyte precursors can differentiate into specialized DC populations that are important for pathogen clearance, including TNF/iNOS producing (Tip) DC [81, 82]. In response to Listeria, an influx of monocytes and macrophages is observed at infectious foci with maximal
recruitment detected at 72 hours post infection. This recruitment timepoint is in
closest to the rapid influx of neutrophils which can appear at sites of infection in
minutes to hours depending on the stimulus [34, 83]. However, maximal
neutrophil recruitment has been observed at 48 hours post in the spleen
following *Listeria* infection [84], or in the bronchial alveolar lavage (BAL) fluid
after *Streptococcus pneumoniae* infection [85]. Thus, under inflammatory
conditions, monocytes are capable of being recruited to sites of infection in a
MCP-1/CCR2–dependent manner and can differentiate into numerous cell types
that are important for the control of bacterial growth.

The contribution of cells of the innate immune system in response to
*Listeria* has been the topic of this introduction; however, an additional cell type,
TNF/iNOS producing DC (Tip DC) warrants further discussion. Tip DC are a
relatively new subset of DC that has recently been defined as a key contributor in
the innate response against *Listeria* [19, 81]. While Tip DC express intermediate
levels of CD11c (a DC marker) and CD11b (a leukocyte marker) on their surface,
they upregulate costimulatory molecules and MHC class II following infection with
*Listeria* [81]. However, Tip DC are not essential for priming T cells and it is
unlikely that they serve as reservoirs of bacterial replication [81]. Thus, Tip DC
do not behave as conventional DC.

Interestingly, once monocytes have been recruited into the spleen, they
surround an infected DC and are in turn encircled by NK cells. In this cluster, the
NK cell produced IFN-γ differentiates monocytes into Tip DC and results in the
production of large amounts of TNF-α and nitric oxide that kills the bacteria [86].
Tip DC are absent from CCR2-/- mice (the receptor for MCP-1) [81] and these mice are highly susceptible to *Listeria* infection [87], highlighting the important contribution of Tip DC and their products TNF-α and nitric oxide on the control of *Listeria* infection.

Following recruitment of monocytes to sites of infection, they are capable of controlling bacterial growth through several bactericidal mechanisms. Monocytes can kill bacteria by the production of reactive oxygen intermediates (ROI) produced by the NADPH oxidase complex [88-92] or reactive nitrogen intermediates (RNI) generated by iNOS [93-96]. RNI have been shown to be important in the killing of *Listeria* as mice lacking functional iNOS exhibited susceptibility to *Listeria* [97]. Thus, the production of ROI and RNI are critical components in the control of bacterial replication following *Listeria* infection.

**Dendritic Cells:** At early time points post infection with *Listeria*, bacterial growth is controlled by cells of the innate immune response including: neutrophils, macrophages, and NK cells [1]. However, another cell type, DC, are capable of bridging the innate and adaptive immune responses. DC are professional antigen presenting cells (APC) that acquire antigens and present them to antigen-specific T cells to initiate the generation of protective immunity [5, 98, 99]. DC reside in tissues in an immature state where they act as sentinels by actively sampling the environment for pathogenic invaders. In an immature state, DC are highly endocytic and have low surface expression of major histocompatibility complexes (MHC) and costimulatory molecules such as CD40, CD80, and CD86. Immature DC are poor activators of naïve T cells [98-101].
However, upon exposure to inflammatory stimuli, DC undergo a process known as maturation where they shut down their phagocytic properties [102] while increasing their ability to process antigens [98]. Mature DC upregulate MHC and costimulatory molecule expression as well as secrete cytokines such as IL-12, IL-6, and TNF-α. In a mature state, DC are capable of priming naïve T cells [103].

In the context of a *Listeria* infection, DC are vital to the generation of the CD8+ T cell response that is required for clearance of *Listeria* as shown by the lack of a CTL response in the absence of DC [104]. More recently, it has been shown that a subset of DC, specifically the CD8α+ DC, were required for establishing infection in the spleen [105]. This was attributed to the ability of DC to carry *Listeria* from the marginal zone of the spleen into T cell zones of the white pulp [106]. This migration facilitates DC/T cell interactions and results in the development of T cell responses important for pathogen clearance.

Dendritic cells are capable of orchestrating immune responses not only due to their ability to facilitate entry of *Listeria* into the spleen [86, 106] but also due to their ability to recruit NK cells and monocytes to areas of infection. Clustering of NK cells and monocytes around infected DC results in a complex series of events that leads to the differentiation of monocytes and eventual microbial killing [86]. In the absence of DC, clustering of NK cells and monocytes does not occur [86]. This suggests that, in addition to priming T cells, DC play a vital role in the recruitment of innate immune cells to sites of infection and therefore ultimately impact the ability to control bacterial burdens. However, when DC are depleted, *Listeria* are not able to enter the T cell zone of the splenic
white pulp [107]. Thus it remains to be determined if the lack of NK cell and monocyte recruitment is specifically due to the absence of DC or the fact that they bring live bacteria into the T cell zone of the splenic white pulp and the bacterial presence attracts other cell types.

Previously published studies have demonstrated that wild-type *Listeria* was capable of generating protective immune responses. However, a non-hemolytic strain (hly-) of *Listeria* that was retained in vacuoles, did not confer protective immunity [108, 109]. Additionally, it was also shown that protective immunity to *Listeria* was dependent on DC [104]. Therefore, in considering potential reasons for the difference in the ability of these two strains of *Listeria* to generate a protective immune response, we hypothesized that cytoplasmic entry of *Listeria* was necessary to induce DC maturation. Studies from our laboratory have demonstrated that optimal *Listeria*-induced DC maturation requires that the bacteria gain access to the host cytosol as determined by the upregulation of costimulatory molecules on the DC surface as well as the production of cytokines [110]. These studies reveal the importance of DC maturation status on the generation of CD8+ T cell responses, and highlight the contribution of costimulatory molecule expression to the proliferation of antigen specific CD8+ T cells and the contribution of IL-12 and IL-10 to CD8+ T cell IFN-γ production [110]. Therefore, these studies illustrate a potential mechanism why Hly-*Listeria* does not confer protective immunity and therefore has implications on the design of vaccines by demonstrating the importance of DC maturation status on the generation of protective immunity. While optimal DC maturation induced by
*Listeria* requires cytoplasmic entry, the specific mechanism(s) initiating DC maturation have yet to be determined.

While DC infected with Hly- *Listeria* do not undergo the process of DC maturation, there is an additional reason why Hly- *Listeria* infection fails to generate a protective immune response. Wild-type *Listeria* and heat killed *Listeria* (HKLM), which along with Hly- Lm is retained in vacuoles, localize to distinct areas of the spleen. Wild-type *Listeria* appears in the T cell zones of the splenic white pulp following infection while HKLM and Hly- Lm converge in the marginal zone and splenic red pulp [107]. Additionally, studies from our laboratory using a non-hemolytic Hly- *Listeria* mutant have observed similar localization results (unpublished observations) thus highlighting the important contribution of *Listeria* cytoplasmic entry and the downstream cascade of events on the initiation of these responses. Thus, *Listeria* cytosolic entry is important for the localization of bacteria to appropriate areas of the spleen that facilitates cellular interactions that result in CD8+ T cell priming. Therefore, these studies emphasize the requirements for the generation of a protective immune response and provide insight into ways to boost poor T cell responses for the generation and/or enhancement of vaccine efficacy.

**Cytokines of the innate immune response to *Listeria***

**IL-12:** IL-12 is a cytokine that has effects on several cell types of both the innate and adaptive immune response. Firstly, early in the initiation of the immune response, IL-12 is produced by infected macrophages and DC and in combination with IL-18 stimulates the production of IFN-γ by NK cells leading to
the activation of monocytes and bacterial killing [74, 75]. Additionally, IL-12 can be produced by infected DC, macrophages, and neutrophils and is important for the polarization of T cells towards Th1 responses [111]. Studies from our laboratory have also shown that IL-12 is important for impacting the level of IFN-γ produced by CD8+ T cells through the IL-12-dependent production of chemokines CCL1 and CCL17 that enhanced DC/T cell interactions [112]. Therefore, IL-12 is important for initiating cascades that lead to bacterial killing as well as in the generation of T cell responses thus highlighting the role of this cytokine in bridging the innate and adaptive immune responses.

**TNF-α:** Another cytokine that is important in the innate immune response against *Listeria* is TNF-α. Illustrating the significance of this cytokine to the anti-*Listeria* immune response is the increased susceptibility of mice lacking this cytokine or its functional receptor [113-116]. There are several different aspects of the innate immune response to which TNF-α contributes. Firstly, TNF-α has been shown to play a critical role in the activation of macrophages [73]. Additionally, following differentiation of monocytes into TipDC, TNF-α and nitric oxide produced by this cell type are important for microbial killing [4, 81]. Thus, TNF-α contributes to the innate immune response to *Listeria* by activating monocytes and inducing bactericidal functions that control listerial replication.

Another facet of the contribution of TNF-α during a *Listeria* infection is through effects on neutrophils, with a role of TNF-α observed on the recruitment of neutrophils as well as monocytes [117]. Additionally, neutrophil-derived TNF-α has been shown to mature DC, thus contributing to the development of adaptive
immune responses [61, 118, 119]. Therefore, TNF-\(\alpha\) is an important cytokine in the immune response to \textit{Listeria} due to its ability to recruit inflammatory cells to sites of infection but also to influence the maturation state of DC, ultimately leading to the bridging of innate and adaptive immune responses.

**IFN-\(\gamma\):** IFN-\(\gamma\) is a cytokine important in the innate immune response to \textit{Listeria}. IFN-\(\gamma\) is produced predominantly by NK cells in response to the synergistic actions IL-12, TNF-\(\alpha\), and IL-18 produced by infected macrophages [1, 74-76]. Recently, a small subset of neutrophils has also been shown to produce IFN-\(\gamma\) in response to \textit{Listeria} infection [120]. This cytokine activates macrophages and results in the initiation of bactericidal properties [1]. One of the important microbial killing mechanisms utilized by macrophages is the production of nitric oxide that leads to the killing of \textit{Listeria} [97]. Further evidence highlighting the role of IFN-\(\gamma\) in the anti-\textit{Listeria} immune response is the increased susceptibility of mice lacking either this cytokine or its receptor to \textit{Lm} infection [77, 78]. Thus, IFN-\(\gamma\) is essential for the innate response to \textit{Listeria} due to its ability to activate macrophages, the cellular reservoir of \textit{Listeria}.

Additionally, IFN-\(\gamma\) plays an important role in the adaptive immune response generated against \textit{Listeria}. Production of IFN-\(\gamma\) is a vital effector function of CD8+ T cells that leads to the clearance of \textit{Lm} [3]. Thus, IFN-\(\gamma\) is a valuable cytokine of both the innate and adaptive immune responses to \textit{Listeria}.

**Type I IFN response**

Type I IFN is commonly referred to in the context of viral infections for its ability to induce the antiviral state and protect neighboring cells from infection
However, the role of type I IFN in the context of bacterial infection is less well defined. Beneficial and detrimental effects of type I IFN on immune responses to a variety of bacterial pathogens have been observed [23]. Specifically, the production of Type I IFN has negative effects on the immune response to *Listeria* [28-30]. An example connecting type I IFN to bacterial infections is the ability of this cytokine to induce the production of iNOS, a key component of bacterial killing [23]. While the production of type I IFN is associated with a favorable outcome following viral infection, this cytokine, which is produced upon entry of *Listeria* into the cytosol, has both positive and negative effects in the framework of bacterial infections depending on the specific pathogen.

**Adaptive immune responses against *Listeria***

**CD8+ T cell Memory:** When a naïve CD8+ T cell encounters an APC presenting its cognate peptide in the context of MHC class I, expressing costimulatory molecules, and secreting pro-inflammatory cytokines such as IL-12, the T cell will likely become activated. A rapid expansion of the CD8+ T cell population occurs and the T cells acquire effector functions including the production of IFN-γ, TNF-α, and cytolytic activity [121]. Additionally, this Th1 cytokine profile is also key to clearance of *Listeria* infection due to the ability of IFN-γ to activate bactericidal properties of macrophages and prevent bacterial spread [122]. Once the pathogen has been eradicated, the contraction phase is initiated and the effector pool of T cells is reduced via apoptosis by 90-95%. The remaining 5-10% of T cells differentiate into memory T cells. Memory T cells are
characterized by their ability to exert effector functions rapidly and without the requirement for costimulatory molecule expression [123, 124] which allows for the immediate generation of immune responses and more rapid clearance of a secondary exposure to a pathogen. While there is a large body of work that has demonstrated the importance of CD8+ T cells on the clearance and the generation of protective immunity against *Listeria*, it remains to be determined how the maturation status of DC affects the ability to prime naïve T cells.

Preliminary data involving this project has led us to two main hypotheses for this thesis work. First, since DC have the ability to interact with surface PRRs as well as TLRs localized in vacuoles, the adapter molecule MyD88 likely contributes to *Listeria*-induced DC maturation. Additionally, due to the ability of *Listeria* to gain access to the host cytosol and the cytoplasmic surveillance pathways therein, it is also likely that there is a role for type I IFN signaling in the DC maturation process. Therefore, we hypothesize that distinct aspects of the maturation program induced by *Listeria* are regulated by MyD88 and IFNAR. Many of the cytokine responses elicited by *Lm* are genes regulated by MyD88, including IL-12, TNF-α, and IFN-γ [16-19]. Given the sizeable amounts of IFN-β that are produced as a result of *Listeria* cytosolic entry [22], this suggested to us the contribution of type I IFN signaling in the DC maturation process in response to *Listeria*. Examples of IFN-inducible genes include those involved in apoptosis and the activation of innate and adaptive immune cells [18]. An example includes the costimulatory molecule CD40, a molecule that is expressed on DC and is involved in T cell priming [25]. Thus, determining the mechanisms of
Listeria-induced DC maturation will help the field of bacterial immunology understand how innate immune responses to intracellular bacterial pathogens are regulated. Additionally, this work also has implications on vaccine design and development.

In considering the broader implications of the work in this thesis, since Listeria has the ability to gain access to the host cell cytosol and induce the production of large amounts of type I IFN, this bacterial pathogen is capable of initiating responses more characteristic of viral infections. Of growing public health concern are bacterial infections following influenza viral infection. These secondary bacterial infections account for more deaths than the initial influenza infection, especially in populations of aged individuals [125]. Thus, given the seasonal influenza outbreaks as well as the recent emergence of the H1N1 influenza strain, the need for effective treatment options for bacterial infections as a complication of viral infection is an area that requires further attention.

In the absence of type I IFN signaling, mice infected with Listeria demonstrate a resistance phenotype to this intracellular bacterial pathogen and exhibit an altered innate immune cell presence. Therefore secondly, we hypothesize that type I IFN signaling negatively regulates neutrophil recruitment due to altered chemokine regulation. Results of these studies will likely have implications on altering immune responses to treat secondary bacterial infections following viral infection, a public health concern and common cause of influenza related deaths.
References


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CHAPTER II

DIFFERENTIAL ROLES OF MYD88 AND TYPE I IFN RECEPTOR IN MYELOID DENDRITIC CELL MATURATION INDUCED BY *LISTERIA MONOCYTOGENES*

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KL Brzoza-Lewis performed all of the experiments. In vivo infections were performed by CJ Henry and JM Grayson. The manuscript was prepared by KL Brzoza-Lewis and EM Hiltbold.
Abstract:

Listeria monocytogenes (Lm) is an intracellular pathogen with potentially serious consequences for immune-compromised individuals. We have previously shown that Listeria induces maturation of dendritic cells upon infection, and that cytoplasmic entry of the bacteria enhances this response dramatically. The goal of this study was to determine the relative contributions of signaling through cell surface/endocytic pattern recognition receptors (PRR) vs. cytoplasmic PRR to DC maturation induced by Lm. Toward this end, we generated DC from mice lacking either MyD88 (to measure the contribution of several surface and phagocytic TLR) vs. the type I IFN receptor (the receptor for Type I IFN, a cytokine induced upon Lm cytoplasmic entry). Our data reveal that expression of costimulatory molecules CD40 and CD86 induced by Lm is MyD88-independent but IFNAR-dependent. However, following systemic infection in vivo, upregulation of splenic DC costimulatory molecules was both MyD88 and IFNAR-independent. The inflammatory cytokine TNF-α, was completely eliminated in the absence of MyD88 while IL-12, and IL-6 were both reduced but not ablated in MyD88-/- DC. Interestingly, TNF-α was also IFNAR-dependent. The affect of these molecules on T cell priming induced by Lm-infected DC was also investigated. We found that DC lacking IFNAR induced fewer T cells to proliferate while DC lacking MyD88 induced equivalent proliferation but lower levels of IFN-γ production. These findings highlight the importance of both MyD88 and Type I IFN signaling pathways to DC maturation in response to the intracellular pathogen Listeria monocytogenes.
Introduction:

*Listeria monocytogenes* is an intracellular bacterial pathogen causing serious infections in immune compromised individuals. The intracellular life cycle of this bacterium involves cellular invasion, phagosomal escape, cytoplasmic entry, and intercellular spread [1]. Infection with sub-lethal doses of *Listeria* induces a potent innate immune response followed by a robust adaptive response resulting in protective immunity [2]. Dendritic cells bridge the gap between the innate and adaptive response by sensing bacteria, migrating to lymphoid organs, and presenting bacterial antigens to activate naïve T cells [3]. However, the specific pattern recognition receptors that mediate recognition of *Listeria* by dendritic cells and the downstream effects of this recognition on DC maturation are not well understood.

Clearance of the primary bacterial infection requires signals mediated through the toll-like receptor (TLR) signaling adapter, MyD88. Mice lacking MyD88 are susceptible to *Lm* infection and succumb before adaptive immunity can be established [4]. Interestingly, MyD88/-/- mice are able to mount protective T cell responses if they are immunized with either a mutant strain of the bacteria that is unable to spread or are treated with antibiotics [5, 6]. However, while similar numbers of *Listeria*-specific CD8+ T cells were observed in these mice, MyD88/-/- mice had less *Listeria*-specific CD4+ T cells and these cells produced less IFN-γ compared to WT mice [5]. These data indicate that the lack of MyD88 does not prevent DC maturation and CD8+ T cell priming, but the effect of this molecule on the development of long term T cell memory remains to be
determined. These results are particularly intriguing given the studies that implicate CD4+ T cell help in the generation of CD8+ memory responses [7, 8]. Specifically, while it has been reported that MyD88 regulates a number of inflammatory cytokine responses from macrophages and other cell types [4, 9], it is not clear what role this molecule plays in DC maturation.

Upon cytoplasmic entry of *Listeria*, a potent type I interferon response is initiated [10]. It is still unclear which pattern recognition receptor mediates recognition of *Listeria* in the cytoplasm and initiates this response, however, the type I interferon response can serve as a surrogate marker of a specific response initiated in the cytoplasm. Interestingly, mice that lack the type I interferon receptor (IFNAR-/-) are more resistant to listerial infection than wild type controls [11-13], a difference which has been attributed to enhanced TNF-α production [12] and diminished lymphocyte apoptosis [11, 13]. However, protective immunity to *Listeria* is normal in IFNAR-/- mice. They are able to mount protective responses even though the bacteria are cleared more rapidly than in WT mice [12].

Our previous studies have demonstrated that cytoplasmic entry of *Listeria* enhances maturation of myeloid dendritic cells as measured by the expression of inflammatory cytokines and costimulatory molecules [14]. Furthermore, cytoplasmic entry of *Listeria* enhances the ability of these DC to prime naïve CTL responses [14]. With the present study, we set out to determine how two mediators of innate signaling, MyD88 and type I interferon receptor (IFNAR), regulate the maturation of DC in response to listerial infection. Through the use
of dendritic cells generated from the bone marrow of mice lacking either of these molecules, we have identified differential requirements for both of these mediators in DC maturation induced by *Listeria*. We have determined that the adapter molecule, MyD88, is important for the production of cytokines and that type I IFN signaling results in the upregulation of costimulatory molecules. The ability of these signaling mediators to control different aspects of DC maturation has an impact on the ability to prime CD8+ T cells in vitro. Interestingly, IFNAR-/- splenic DC are capable of upregulating CD86 and CD40 and this can be attributed to exogenous factors likely provided by other cell types present in the spleen. Thus, other cells in the spleen may contribute to DC maturation in an indirect manner.
Materials and Methods:

**Mice**  C57/BL6 and OT-1 TCR transgenic mice specific for OVA (257-264) presented by K\(^b\) were purchased from the Jackson Laboratory (Bar Harbor, ME). MyD88/-/- mice were the kind gift of Dr. D. Golenbock, (U. Massachusetts) with the permission of Dr. S. Akira. IFNAR/-/- mice were provided by Dr. S. Mizel (WFUSM) with the permission of Dr. Christian Schindler (Columbia University). All mice were maintained and bred in the animal facility at Wake Forest University School of Medicine.

**Reagents**

**Cytokines:** Recombinant mouse GM-CSF was purchased from Biosource International (Camarillo, CA). **Peptide:** synthetic OVA peptide 257-264 (SIINFEKL) was purchased from the Emory University School of Medicine Peptide Synthesis Facility. **Antibodies:** Fluorescently labeled antibodies against mouse cell surface antigens CD40-PE (clone 3/23), CD80-PE (clone 16-10A1), CD86-PE (clone GL1), I-Ab-PE (clone AF6-120.1), and CD8-PE (clone Ly-2 53-6.7) were purchased from BD Biosciences/Pharmingen (San Diego, CA). Fluorescently tagged anti-IFN-γ used for intracellular cytokine staining was also purchased from BD/Pharmingen. **TLR agonists:** LPS from Salmonella minnesota was purchased from Sigma (St. Louis, MO) and was used at a concentration of 100-300 ng/ml. Poly I:C (used at 25 µg/ml), PAM3CSK4 (used at 200 ng/ml) were purchased from InvivoGen (San Diego, CA). CFSE (carboxyfluorescein succinimidyl ester), was purchased from Invitrogen.
**Bacteria** The *Listeria monocytogenes* strains 10403S (wild-type), strain DP-L2319 (Hly-), which harbours deletions in the genes encoding LLO and two phospholipases (ΔhlyΔplcAΔplcB), and DP-L4056 (*LmOVA*) were obtained from Dr. Daniel Portnoy (University of California, Berkeley, CA). DP-L4056 (*LmOVA*) constitutively secretes ovalbumin protein. Bacteria were grown to stationary phase at 37°C in brain heart infusion broth (BHI), washed 3 times in sterile PBS and diluted to achieve the indicated multiplicity of infection (MOI).

**Dendritic cell propagation** Myeloid DC were generated as previously described [14, 15]. Briefly, bone marrow was removed from the tibias and femurs of 8-10 week old mice. Following red cell lysis and washing, progenitor cells (5x10^5/ml) were resuspended and plated in RPMI containing 10% FCS supplemented with 20 ng/ml GM-CSF (without antibiotics). DC were cultured for 6 days at 37°C in 5% CO₂ and were gently washed and fed with fresh media and cytokine on days 2, 4, and 5. On day 6, the DC were routinely 95% (+/- 5%) CD11c+ and displayed low levels of CD40, CD80, CD86 and MHC class II, characteristic of immature DC (data not shown).

**Infection of DC** *Listeria monocytogenes* (strain 10403s, DP-L2319, or DP-L4056) were grown overnight in brain heart infusion broth to stationary phase. Bacteria were washed 3 times in PBS. Dendritic cells, cultured as above, were harvested and plated in 48 well plates at a density of 5 x 10^5/well and infected at the indicated MOI for 4 hours in the absence of antibiotics. Gentamycin (5 µg/ml) and Chloramphenicol (10 µg/ml) were then added to stop the infections and the cells were cultured for an additional 20 hours to allow time for maturation.
**Measurement of Costimulatory molecule and cytokine expression by DC following bacterial infection**  
DC were plated in 48 well plates at a density of 5 x 10^5 DC/ml in antibiotic-free media. The cells were then infected with the indicated MOI or treated with TLR agonists and incubated for a total of 24 hours. Culture supernatants were then removed and the production of cytokines (IL-6, IL-12, or TNF-α) was measured by ELISA (OptiEIA kits, BD Pharmingen). The cells were then harvested and stained with PE-conjugated antibodies to murine CD40, CD80, and CD86. Non-specific background staining was assessed using isotype control antibodies. DC were co-stained with antibodies to CD11c (myeloid DC) to enable analysis of costimulatory molecules expressed specifically by the DC population.

**T cell priming assay**  
DC (2x10^4/ml, 100µl/well) were plated in 96 well plates and left untreated, or infected with a recombinant strain of *Listeria* that expresses the model antigen ovalbumin DP-L4056 (*LmOVA*) at the indicated MOI. The cells were cultured for 24 total hours before the addition of T cells. Naïve, OVA-specific CD8+ T cells were isolated from the spleens of OT-1 TCR transgenic mice. T cells were stained with CFSE according to manufacturer's instructions and were added to the DC at a ratio of 10 T cells per DC. T cells were harvested 72 hours later and the production of IFN-γ in response to antigen was then measured by incubation with OVA (257-264) peptide (1 µg/ml) in the presence of Golgi Plug reagent for five hours. The cells were then stained, fixed and permeabilized for intracellular cytokine staining using the Cytofix/Cytoperm kit for from BD Biosciences/Pharmingen (San Diego, CA) according to manufacturer's
instructions. Flow cytometric data were acquired using a BD FacsCalibur (BD Biosciences, San Diego, CA). CFSE proliferation data was analyzed as previously described [16] using the FLOJO program for flow cytometric data analysis (Tree Star, Inc. Ashland, OR). The Division Index represents the mean number of divisions that a cell present in the starting population undergoes. Statistical significance was determined using a two tailed, paired student’s T test. p-values of less than 0.05 were considered significant differences.

**In vivo infection** C57BL/6, MyD88-/-, or IFNAR-/- mice were infected i.v. with 5x10^4 *Lm* strain 10403s in 500μl sterile PBS, or mock treated with PBS alone. Twenty four hours post infection, spleens were harvested and digested with collagenase. Cell homogenates were stained with a cocktail of antibodies to differentiate DC from other cell populations. Data were acquired using the BD FACS Canto II cytometer. To focus the analysis on the DC population which is at low frequency, cells expressing CD3 and CD19 were gated out and excluded from analysis. The expression of costimulatory molecules CD80 and CD86 was then assessed on CD11c+ populations. Additionally, a subset of spleen homogenate was treated with golgi plug reagent for 5 hours and intracellular stores of IL-12p40 or IL-6 were determined by intracellular cytokine staining.

**DC-splenocyte co-infection** DC were cultured as previously described [14, 15] and infected with *Listeria* either alone or in the presence of naïve splenocytes (2 splenocytes per DC) collected from the same mouse strain. Twenty four hours post infection, cells were harvested and CD86 surface expression on CD11c+ cells was assessed by flow cytometry.
Results:

Differential requirements for MyD88 in the induction of costimulatory molecule and inflammatory cytokine expression by DC infected with *Lm*

To determine the role of the TLR signaling adapter, MyD88, in the DC maturation response induced by *Listeria monocytogenes*, we utilized bone marrow-derived DC generated from mice lacking this molecule (MyD88-/-) vs. DC from wild type (WT) mice. DC were infected for four hours then the infection was stopped by the addition of antibiotics. The DC maturation response to *Lm* infection (MOI=1) was monitored by measuring costimulatory molecule expression (CD40, CD80, and CD86) by flow cytometry (Figure 3) and cytokine expression by ELISA 20 hours later (Figure 4). Immature DC that were not treated (NT) were used as a negative control and were the basis of comparison for all stimuli. We utilized several TLR agonists known to differ in their dependence on MyD88 as controls, including LPS (300 ng/ml), a TLR4 agonist which triggers both MyD88-dependent and –independent signaling mechanisms, Pam3CSK4 (200 ng/ml), an MyD88-dependent ligand of TLR2, and Poly I:C (25 μg/ml), an MyD88-independent TLR3 agonist. Data for costimulatory molecule intensity are expressed as fold increase in expression (geometric mean fluorescence intensity) over the untreated background for each strain.

*Listeria* infection induced robust expression of CD40 and CD86 expression over untreated background in WT GM-CSF DC, yet we observed only a modest increase in CD80 expression (Figure 3), as we have previously reported [14]. A very similar pattern of response was observed in MyD88 -/- DC
Figure 3: MyD88 is not required for costimulatory molecule upregulation following infection with Listeria. GM-CSF generated DC were infected with Listeria (MOI = 1) or treated with the indicated TLR agonists. Expression of costimulatory molecules on CD11c+ cells was assessed 24 hours later by flow cytometry. A) Representative histograms depict expression of CD40, CD80, and CD86 on wild type (WT) DC or DC lacking MyD88 (MyD88-/-). Gray histograms-untreated DC, black histograms- Listeria-infected DC. B) CD40, C) CD80, and D) CD86 surface expression illustrated as a fold increase over untreated DC. Data are compiled from 5 independent experiments. Open bars indicate WT DC, black bars, MyD88-/- DC. Data are expressed as fold increase in geometric mean fluorescence over untreated DC. Mean and standard deviation of 5 independent experiments are shown. No significant differences (p value < 0.05) were observed between WT and MyD88-/- DC. Red dashed lines indicate background levels of expression.
Figure 4: Inflammatory cytokine production stimulated by *Listeria* infection of DC is reduced in the absence of MyD88−/−. WT and MyD88−/− DC were treated with the indicated TLR-agonists or infected with *Lm* (MOI=1). Twenty four hours post infection, supernatants were collected and (A) IL-12p40, (B) IL-6, (C) TNF-α, and (D) IFN-β concentrations were determined by ELISA. Graphs shown are representative. (E) MyD88−/− DC production of IL-12p40, IL-6, and TNF-α expressed as a percent of the WT response. Data in panel E are compiled from 5 independent experiments.
upon *Listeria* infection in that the expression of all costimulatory molecules was unaffected by the absence of MyD88. LPS treatment of WT DC induced substantial increase in the expression of CD40 and CD80, and CD86 (to a lesser extent). The induction of CD40 by LPS was reduced in the absence of MyD88, yet, the induction of CD80 and CD86 expression was less inhibited in the absence of this adapter molecule. Pam3CSK4 induced the up-regulation of CD40 and CD80 expression yet only background levels of CD86 were observed in WT DC (Figure 3). Up-regulation of each of these molecules was completely ablated in DC lacking MyD88, as expected. Poly I:C treatment induced a slightly different pattern of costimulatory molecule up-regulation, characterized by high levels of CD40 and CD86, but no significant change in CD80 expression. And as predicted, in the absence of MyD88, the induction of all three costimulatory molecules by Poly I:C was equivalent to or better than wild type cells. Taken together, these data illustrate the distinct patterns of costimulatory molecule expression induced by MyD88-dependent vs. MyD88-independent stimuli. Importantly, these findings indicate that costimulatory molecule expression induced by listerial infection did not require MyD88.

**Inflammatory cytokines IL-12, IL-6, and TNF-α but not IFN-β produced by mDC in response to *Listeria* are regulated in part by MyD88**

DC generated from WT or MyD88-/- mice were also used to address the requirement for MyD88 in the induction of pro-inflammatory cytokine expression (IL-12p40, IL-6, TNF-α, and IFN-β) upon *Lm* infection. Twenty four hours post treatment or infection, culture supernatants were collected and the presence of
these cytokines was assessed by ELISA (Figure 4). Infection with *Listeria* induced robust secretion of IL-12, IL-6, TNF-α, and IFN-β in WT DC as we had previously reported [14]. In DC lacking MyD88 however, the production of IL-12, IL-6, and TNF-α was dramatically reduced but not completely ablated. *Lm*-induced IFN-β was not affected in the absence of MyD88 (Figure 4). Stimulation of DC with LPS showed a pattern of MyD88-dependence in that the strong induction of IL-12, IL-6 and TNF-α were ablated in the absence of MyD88 (Figure 4). In fact, the expression of each cytokine induced by LPS was much more strongly and reproducibly diminished in the absence of MyD88 than those cytokines induced by listerial infection. Finally, stimulation of DC with Poly I:C induced little expression of any cytokines tested except IFN-β. In contrast, Pam3CSK4 induced IL-12 and IL-6 in an MyD88-dependent manner, yet this stimulus induced little TNF-α cytokine. Taken together, these data again illustrate the distinct cytokine profiles induced by MyD88-dependent vs. MyD88-independent stimuli. These results also demonstrate that the inflammatory cytokine response to *Lm* infection is mediated in large part by MyD88. However, the reduction in cytokine expression in *Lm*-infected MyD88-/- DC (IL-12 was 25% of WT and IL-6 was 30% of WT, Figure 4E) was much less dramatic than that observed for LPS treatment (IL-12 was 10% of WT and IL-6 was 6% of WT, Figure 4E). This difference suggests that listerial infection triggers cytokine production through pathways in addition to the MyD88-regulated pathway. Interestingly, the production of TNF-α induced by both LPS and *Lm* was equivalently abolished in the absence of MyD88. Given that *Listeria* is such a
complex stimulus, potentially interacting with DC pattern recognition receptors at the cell surface and in the cytoplasm, it is not surprising that other activation pathways are functional here.

**Role of MyD88 in response to phagosomally-retained (Hly-) Lm**

Having observed that a portion of the cytokine response elicited by wild type Lm was still intact in the absence of MyD88, we postulated that this might indicate a role for cytoplasmic receptors in sensing bacteria after release from the phagosome. Thus, we next wanted to determine if the response induced by a phagosomally retained strain of *Listeria* was strictly dependent on MyD88. To address this question we infected DC with higher MOI of Hly- *Lm* (1, 5, or 10) to induce a stronger cytokine response as these bacteria are less potent at inducing inflammatory cytokines [14]. With these higher MOIs of Hly- *Lm*, we did observe a strong, dose-dependent IL-12p40, IL-6, and TNF-α induction (Figure 5) comparable to or better than Hly+ *Lm* at the MOI 1. Again, we observed a diminished, but not ablated IL-12 and IL-6 response in the DC lacking MyD88 infected with WT (Hly+) *Lm*. However, the IL-12 and IL-6 response induced by Hly- *Lm* was much more severely reduced in the absence of MyD88, and the TNF response in these cells was entirely ablated. Thus, we conclude that the cytokine response induced by *Lm* retained within phagosomes is predominantly MyD88-mediated.
Figure 5: DC cytokine production elicited by a high MOI Hly- Lm infection relies on signaling through MyD88. WT and MyD88/-/- DC were treated with LPS or the indicated MOI of either WT Lm or the vacuole-retained Lm mutant strain (Hly-). Twenty four hours post infection, supernatants were collected and levels of (A) IL-12p40, (B) IL-6, and (C) TNF-α were assessed by ELISA. Data are representative of two independent experiments. All responses were significantly different (p < 0.05) between MyD88 and WT DC paired samples.
Role of IFNAR in DC maturation induced by *Listeria*

Type I interferon has been reported to be a potent stimulus for DC maturation and type I interferon is strongly induced by *Listeria* upon entry into the cytosol. Thus, to address the role of a signaling cascade initiated by cytosolic invasion of the bacteria, we next wanted to determine how signaling through the type I interferon receptor impacted the maturation of DC induced by *Lm*. As depicted in the histograms in Figure 6A, we observed little to no change in the level of CD40 and CD80 expression on IFNAR-/− DC upon infection with *Lm* and only a modest increase in CD86 expression over uninfected cells. When data from several experiments were compiled based on fold induction upon infection over background, we observed that the expression of both CD40 and CD86 was significantly impaired in the absence of IFNAR compared to WT DC (Figure 6B and 6D). The differences in CD80 expression were not significant likely due to the low level of induction of this molecule observed overall. Likewise, the induction of CD40 and CD86 by the control stimuli, LPS and Poly I:C was significantly inhibited in the absence of IFNAR. Thus, these studies suggest that full induction of costimulatory molecules CD40 and CD86 upon *Lm* infection requires signaling through the type I interferon receptor.

**IFNAR is not required for the induction of IL-12p40 or IL-6, but is important for TNF-α and IFN-β.**

We also wanted to determine the role of the type I interferon receptor in the cytokine response induced in DC by *Lm*. For these experiments we again
Figure 6: *Listeria*-induced CD40 and CD86 surface expression on GM-CSF DC requires the type I IFN receptor. DC were generated from WT and IFNAR-/- mice and treated with the indicated TLR-agonists as controls or infected with *Listeria*. Twenty four hours post infection, the surface expression of costimulatory molecules was assessed by flow cytometry. A) Representative histograms of costimulatory molecule expression by WT and IFNAR-/- DC following infection with *Listeria*. B) CD40, C) CD80, and D) CD86 expression following treatment shown as a fold increase over untreated background. B-D) Results are compiled from 5 independent experiments. * indicates p value < 0.05, ** indicates p value < 0.01.
measured the cytokines IL-12p40, IL-6, TNF-α and IFN-β 24 h following Lm infection or treatment with control agonists LPS or Poly I:C. We observed that the amount of IL-12 and IL-6 induced by Lm and LPS was not significantly diminished in the absence of IFNAR (Figure 7A and 7B). As observed in Figure 4, there was little to no induction of these cytokines by Poly I:C with the exception of IFN-β. As one might predict, Poly I:C-induced IFN-β was diminished in the absence of IFNAR. Finally, the TNF-α response induced by Lm was severely reduced and the IFN-β response was reduced by half in the IFNAR-/- DC (Figure 7C and 7D). To evaluate the overall effect of IFNAR on the cytokines measured, we calculated the level of each cytokine response observed in the IFNAR-/- DC as a percent of the WT DC response across several experiments. We observed that the IL-12p40 and IL-6 response were not significantly different than WT levels while the TNF-α response was significantly impaired (Figure 7E).

**Naïve T cell proliferation and cytokine production primed by Lm-infected DC are differentially regulated by MyD88 and IFNAR**

The maturation status of DC has implications on the ability of these cells to prime naïve T cells. To determine how MyD88 and IFNAR expressed by DC affected their ability to activate naïve T cells, DC lacking either of these molecules were used as antigen presenting cells in a series of in vitro T cell priming assays. DC were infected with a recombinant strain of *Listeria* expressing the model antigen ovalbumin (*LmOVA*) at an MOI of 1. The cells were allowed 24 hours to mature and to process and present the OVA antigen
Figure 7: The cytokine response induced by *Lm* is selectively regulated by the type I IFN receptor in GM-CSF DC. WT and IFNAR-/- DC were treated with designated TLR agonists or infected with *Lm* for 24 hours and culture supernatants were then collected. Amounts of (A) IL-12p40, (B) IL-6, (C) TNF-α, and (D) IFN-β were determined by ELISA and are representative of five experiments. E) Expression of IL-12p40, IL-6, and TNF-α produced by IFNAR-/- DC represented as a percent of the WT response. Data depicted here were compiled from 5 experiments.
via MHC class I. Naïve OT-1 T cells (specific for the OVA\textsubscript{257-264} epitope presented by H-2 K\textsuperscript{b}) were stained with CFSE and added to the DC. After 3 days of co-culture, the proliferation of the T cells was measured by dilution of CFSE and the production of IFN-\(\gamma\) was measured by intracellular cytokine staining. We observed that T cells primed by either WT or MyD88-/- \textit{LmOVA}-infected DC had similar proliferation profiles in both percent of cells divided as well as number of divisions (Figure 8A). In contrast, a much lower percent of T cells primed by the \textit{LmOVA}-infected IFNAR-/- DC were induced to proliferate (Figure 8A). When we analyzed the percent of T cells divided as a function of the multiplicity of infection, we observed that at the highest MOI tested there was an increase in the percent of T cells that divided when primed by MyD88-/- DC compared to WT. However, we also observed a significantly lower proportion of T cells divided when primed by DC lacking IFNAR (Figure 8B). This suggests that the costimulatory molecules lacking on these DC are critical for the recruitment of naïve T cells into proliferation. This may also be due to a slightly lower level of MHC class I expression on these cells (data not shown).

We then examined the level of IFN-\(\gamma\) produced by the divided T cells upon re-stimulation with OVA peptide. These experiments revealed that the level of cytokine produced on a per-cell basis by T cells primed by DC lacking MyD88 was lower than those T cells primed by WT DC (Figure 8C). This difference was observed at all MOI tested (Figure 8D). Interestingly, while the level of cytokine produced by each T cell was lower in the absence of MyD88 on the DC, the percent of T cells that were above the background level of IFN-\(\gamma\) production was
**Figure 8:** T cell priming capacity of *Lm*-infected DC lacking either MyD88 or IFNAR. WT, MyD88-/-, and IFNAR-/- DC were infected with the indicated MOI of *LmOVA* for 24 hours and then CFSE labeled OT-I TCR transgenic T cells were added to the cultures. 72 hours later, the T cells were restimulated with OVA peptide in the presence of golgi plug for 5 hours and the production of IFN-γ was assessed by intracellular cytokine staining. A) Representative histograms of OT-1 T cell proliferation primed by WT, MyD88-/- or IFNAR-/- DC infected with *Lm-OVA*, MOI 1 as measured by dilution of CFSE. B) Percent of T cells divided after priming by the indicated DC infected with increasing MOI of *LmOVA*. C) Representative contour plots of IFN-γ production by T cells primed by the specified DC infected with an MOI 3. D) Mean fluorescence intensity of IFN-γ produced by divided OT-1 T cells. B, D) Data were compiled from 3 independent experiments. * indicates p value < 0.05, *** indicates p value < 0.001.
not significantly different than WT DC (data not shown). This suggests that the inflammatory cytokines produced in an MyD88-dependent manner upon infection with *Lm* by DC are important in determining the level of IFN-γ produced by T cells upon activation. Interestingly, we observed no significant difference in the level of IFN-γ produced by T cells primed by IFNAR-/- DC infected with an MOI of 0.1 or 1. Thus, the lower level of costimulatory molecules on the IFNAR-/- DC induced a smaller proliferative response, but in the cells that did divide, the level of IFN-γ production was similar to WT.

**Maturation of DC in vivo, role of MyD88 and IFNAR**

To determine how our in vitro results correlated with the maturation of DC following infection in vivo, we monitored the expression of costimulatory molecules and cytokines by CD11c+ splenic DC 24 hours after i.v. infection in WT, MyD88-/- or IFNAR-/- mice. We found that the expression of CD86 and CD80 was increased upon infection of DC from all mouse strains compared to the uninfected controls (Figure 9A and 9C). In contrast to what we observed in vitro with the GM-CSF generated DC, the IFNAR-/- DC showed equivalent or even better induction of CD86 and CD80 over the uninfected background (Figure 9B and 9D). Likewise, the DC from the MyD88-/- mice showed equivalent induction of CD86 and CD80 as WT DC. Thus, these data indicate that perhaps other cell types or their products that are able to induce DC maturation upon infection are present in the IFNAR-/- spleen in vivo.
Figure 9: Costimulatory molecule expression on splenic DC following in vivo *Listeria* infection. WT, MyD88-/-, or IFNAR-/- mice were infected with \(5 \times 10^4\) *Lm* i.v. The animals were sacrificed at 24 hours post infection and the spleens were harvested and the cells stained to assess the surface expression of costimulatory molecules on CD11c+ cells. CD86 and CD80 surface expression is graphed as the mean fluorescence intensity (MFI) (A and C respectively) and as a fold increase over mock (B and D respectively). Data are compiled from 6 mice per group. Students t test compare WT to IFNAR-/- (* indicates p < 0.05, ** indicates p < 0.01, *** indicates p < 0.001). Red dashed line indicated background levels of expression.
A. CD86 Mean Fluorescence Intensity

- WT
- MyD88-/-
- IFNAR-/-

Mock: Low levels
Lm: Increased levels

B. CD86 Fold Increase over Mock

- WT
- MyD88-/-
- IFNAR-/-

Fold increase compared to Mock:

C. CD80 Mean Fluorescence Intensity

- WT
- MyD88-/-
- IFNAR-/-

Mock: Low levels
Lm: Increased levels

D. CD80 Fold Increase over Mock

- WT
- MyD88-/-
- IFNAR-/-

Fold increase compared to Mock:
The production of the inflammatory cytokines, IL-12 and IL-6, upon *Lm* infection was lower in mice lacking MyD88 than WT, but not in those lacking IFNAR (Figure 10). We observed a smaller percent of cells expressing both IL-12p40 and IL-6 in the splenic DC from the MyD88-/- mice while the frequency of cells producing these cytokines was similar to or greater than WT in DC from the IFNAR-/- mice (Figure 10A and 10B). These data are in strong agreement with our in vitro results with the GM-CSF generated DC in that the cytokine response upon *Lm* infection is diminished in the absence of MyD88 and is not affected by the absence of IFNAR. Thus, it appears that MyD88 is critical for the inflammatory cytokine response induced by *Lm* infection both in vivo and in vitro.

**Enhancement of CD86 expression on IFNAR-/- DC through bystander signals from infected splenocytes**

Finally, we wanted to address the conflicting results observed in our in vitro (Figure 6) vs. in vivo data (Figure 9) with regard to the role of IFNAR in the expression of costimulatory molecules. One possibility was that the enhanced maturation of DC in the IFNAR-/- mice in vivo was due to “bystander” signals from other cells of the spleen. Thus, we combined GM-CSF DC and splenocytes in culture, infected with *Listeria*, and measured the maturation of the GM-CSF DC after 24 hours. Two strategies were employed to exclude splenic DC from the analysis of maturation. First, the splenocytes were harvested in the absence of collagenase which results in an almost complete loss of DC from the suspension, and secondly, thanks to a distinct difference in CD11c expression level on the
Figure 10: IL-12p40 and IL-6 cytokine expression by splenic DC ex vivo, following i.v. *Lm* infection. WT, MyD88, and IFNAR-/− mice were infected i.v. with $5 \times 10^4$ *Lm*. Twenty four hours post infection, spleens were harvested and the cells were incubated in the presence of golgi plug for 5 hours. The percent of CD11c+ DC making intracellular IL-12p40 (A) and IL-6 (B) is shown. Data are compiled from 6 mice per group. Students t test of WT compared to IFNAR-/− (***) indicates $p < 0.001$. 

remaining splenic DC vs. the GM-CSF DC, we were able to analyze only the GM-CSF DC that had a high level CD11c expression. Using this system, we observed an increase in the expression of CD86 on GM-CSF DC generated from IFNAR-/- mice when DC were co-cultured with splenocytes from IFNAR-/- mice (Figures 11A and 11B). However, while this enhanced maturation was significant, it did not reach the same level of maturation observed in the splenic DC upon i.v. infection (Figure 9B). As a control, GM-CSF DC from WT mice were co-cultured with splenocytes from WT mice. As expected, WT DC displayed high level maturation alone, and this response was not significantly affected by co-culture with WT splenocytes (Figures 11A and 11B).

The second possibility for the discrepancy in the data in vitro vs. in vivo was that the specific type of DC represented by GM-CSF DC did not accurately correspond to splenic DC in responsiveness to Lm and in requirement for type I interferon signaling. To examine this possibility, we generated DC from the bone marrow of WT and IFNAR-/- mice with the cytokine Flt3L, as these cells have been reported to closely represent the subsets of DC present in the spleen [17, 18]. Interestingly, we observed that Flt3L DC derived from WT and IFNAR-/- mice had similar levels of CD86 expression following Listeria infection (Mitchell, LM, WFU Dissertation 2009). Thus, we conclude that Flt3L and splenic DC have comparable requirements for type I IFN signaling in response to Listeria infection.
Figure 11: CD86 surface expression in response to *Listeria* is enhanced when IFNAR-/- DC are co cultured with splenocytes. A, B) DC were cultured as previously described in GM-CSF and infected with *Listeria* either alone or in the presence of naïve splenocytes collected from the same mouse strain. Twenty four hours p.i., cells were harvested and CD86 surface expression on CD11c+ cells was assessed by flow cytometry. A) Representative histograms of CD86 expression on WT or IFNAR-/- DC infected with *Listeria* in the presence or absence of splenocytes (black lines). Shaded area represents untreated background. Inset numbers are the geometric mean fluorescence intensity of background staining (upper left) and following infection (upper right). B) Compiled CD86 surface expression presented as a fold increase in CD86 compared to untreated DC.
Discussion:

*Listeria monocytogenes* has a unique intracellular life cycle that involves their uptake into phagosomes followed by perforation of the phagosomal membrane and escape into the cell cytoplasm, a step required for survival of the bacteria as well as for replication and spread [1]. Due to this invasive life cycle, *Listeria* has the potential to trigger inflammatory responses, including DC maturation, by triggering pattern recognition receptors (PRR) at several interfaces including the cell surface, within the endocytic pathway, as well as within the cytoplasm. Our previous studies, as well as those of others have demonstrated that *Listeria* infection induces a strong DC maturation response characterized by costimulatory molecule upregulation and inflammatory cytokine production [10, 14, 19]. However, as we previously reported, the ability of *Listeria* to induce maturation of GM-CSF DC (particularly costimulatory molecule upregulation) was dependent on the ability of the bacteria to access the cytosol [14]. If the bacteria were not able to enter the cytosol, the DC response was reduced to a mild inflammatory cytokine response [14].

These data suggested that recognition by cytoplasmic PRR was critical to DC maturation induced by *Listeria*. It has been shown in several studies that the type I interferon is produced only when *Listeria* are able to enter the cytosol [10, 20] and thus this response may serve as a surrogate of cytoplasmic invasion. Additionally, it was not clear what role there might be for signaling through cell surface or phagosomal TLR for *Lm*-induced DC maturation. Thus, to determine the relative contributions to DC maturation of 1) signals initiated upon recognition
at the surface or in the endocytic pathway, we have examined the role of the TLR signaling adapter, MyD88, using MyD88 deficient DC. To determine the role of 2) signals dependent upon cytoplasmic invasion, we have used DC lacking the type I interferon receptor.

Our findings demonstrate the TLR adapter, MyD88 is key to the inflammatory cytokine response induced by *Lm*. While the specific PRR(s) responsible for recognition of *Lm* are still a matter of debate, it has been shown the TLR adapter, MyD88, is required for clearance of *Lm* in vivo in several reports [4, 5, 9]. In our own investigations, we have tested the role of individual TLR including TLR2, TLR4, TLR7, and TLR9 and found that none of these was required for DC maturation induced by *Lm* (data not shown). Likewise, we also tested the possibility that the IL-1R/IL18R signaling pathway might be required for this response, but in DC lacking caspase 1 (and thus unable to generate active IL-1 or IL-18), DC maturation was not different than WT DC upon *Lm* infection (data not shown). Thus, because *Listeria* is a complex organism with multiple potential PAMPs, it is likely that other PRR can compensate in the absence of any one of these receptors.

Interestingly however, the DC inflammatory cytokine response induced by WT *Lm* was not completely dependent on MyD88. Specifically, while the IL-12p40 and IL-6 responses were significantly muted in the absence of MyD88, they were not completely ablated, in contrast to TNF-α (Figure 4). In fact, a substantial level of IL-12 and IL-6 were produced by MyD88/-/- DC, indicating the contribution of other PRR, likely cytoplasmic receptors, to this response. In
support of this notion, when we infected with the mutant \textit{Lm} that was not able to enter the cytosol, (Hly-), we indeed observed that the cytokine response was almost completely ablated in the absence of MyD88. In contrast, there was no contribution of MyD88 to the \textit{Lm}-induced IFN-\(\beta\) response. Furthermore, MyD88 had no effect on the expression of costimulatory molecules induced by \textit{Lm} infection (Figure 3). Thus, the expression of costimulatory molecules and inflammatory cytokines is differentially regulated. These results are in agreement with several related reports in other cell types such as macrophages that also determined that IL-6 production by macrophages utilized both MyD88 and type I IFN signaling [20, 21].

The contribution of IFNAR to DC maturation induced by \textit{Lm} appears to be primarily at the level of costimulatory molecule expression, a phenotype that can be compensated for in vivo. These findings inform our understanding of the resistance of IFNAR-/- mice to \textit{Listeria} and their ability to mount protective T cell responses. The nature of the bystander effect has not been determined, but may stem from the enhanced TNF-\(\alpha\) production observed in IFNAR-/- mice upon \textit{Lm} infection, over that in WT animals [12]. TNF-\(\alpha\) is known to be a potent stimulator of DC maturation and its production by other cell types may enhance DC maturation in vivo [22-24]. Interestingly, unlike the reports of in vivo infection of enhanced TNF-\(\alpha\) production [12], we observed diminished TNF-\(\alpha\) from DC in vitro. While the DC themselves may or may not be able to upregulate their costimulatory molecule expression in response to \textit{Listeria} on their own, the contribution of other bystander cells in the spleen can augment this response.
(Figure 11). However, we were not able to recapitulate the same level of maturation observed in splenic DC in vivo by adding splenocytes to our GM-CSF DC cultures in vitro. This may be due to inefficient interactions occurring in the culture dishes vs. those happening in the context of normal splenic architecture. Alternatively, the GM-CSF DC may represent a distinct DC type with different reactivity than those DC resident in the spleen.

A recent report from Portnoy’s group has carefully analyzed the transcriptional program induced by *Listeria* infection of macrophages. Using macrophages generated from MyD88-/- and IFNAR-/- mice and using both WT and Hly- bacteria, they were able to define three aspects of the response, the vacuolar response of which IL-1 was the representative, the cytosolic response (IFN-β was representative), and a response that was shared between both vacuolar and cytosolic (represented by IL-6) [21]. Our results correlate with these with some notable exceptions. We observed that both IL-12p40 and IL-6 induction by DC were regulated by both vacuolar and cytoplasmic signaling (Figures 4, 5 and 7) however, in macrophages this trend was observed only for IL-6 [21]. Interestingly, macrophage produced IL-12 in response to *Lm* was determined to be part of the vacuolar response only. Thus, the regulation of IL-12 expression by DC vs. macrophages is clearly distinct. These responses are key to the initiation of protective T cell responses as mature DC are thought to be the only cells with the capacity to prime naïve T cells.

Early studies looking at DC maturation were in response to purified TLR-agonists such as LPS [9], however more recent studies have focused on more
physiologically relevant stimuli such as intact bacteria. DCs directly infected with *Salmonella* upregulated costimulatory molecule expression independent of MyD88 or IFNAR signaling, however in the absence of MyD88 and IFNAR, bystander maturation was abolished [25] suggesting that distinct programs of maturation are elicited in response directly to a pathogen or indirectly in response to bystander signals. Interestingly, while directly infected MyD88-/- or MyD88/IFNAR-/- DC are capable of upregulating costimulatory molecules, they have a compromised ability to activate T cells [25]. Since these mice lack MyD88 it is likely that the reduced T cell activation is the result of reduced cytokine production. Therefore, this study suggests that there are two programs of DC maturation, one that occurs in directly infected cells as well as those that mature as a result of bystander signals, commonly type I IFN.

While in vitro studies can establish the involvement of different aspects of DC maturation to T cell priming, it does not address the ability of these T cell responses to clear *Listeria* or the ability to generate protective immunity. A recent study has looked at the contribution of MyD88 and type I IFN signaling to DC maturation and the resulting effects on T cell function [19]. MyD88-/- mice infected with *Listeria* had a reduced total memory CD8 T cell response compared to WT and IFNAR-/- mice. However, similar numbers of IFN-γ producing antigen-specific memory CD8 T cells were recovered from WT, MyD88-/-, or IFNAR-/- mice which correlated with bacterial clearance and ultimately the generation of a protective immune response [19]. While the absence of MyD88 signaling in DC results in the generation of a reduced CD8 T cell memory pool
[19], it is attractive to speculate that this is due to reduced cytokine responses that are important for both the generation and maintenance of T cell populations. However, while it has been demonstrated that DC maturation markers are differentially regulated by the signaling mediators MyD88 and the type I IFN receptor, there appears to be a redundancy in this system as mice lacking either molecule are capable of generating an antigen-specific T cell response that confers protective immunity.

Given the overlapping roles of MyD88 and type I IFN signaling in the ability to generate protective immunity to *Listeria*, determining the roles of these two signaling mediators on DC maturation can be used in the generation of new vaccines or illustrate ways to potentially enhance vaccines that are sub-optimal.
References


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CHAPTER III

TYPE I INTERFERON SIGNALING REGULATES THE COMPOSITION OF INFLAMMATORY INFILTRATES AFTER INFECTION WITH LISTERIA MONOCYTOGENES

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The following manuscript was submitted to The Journal of Leukocyte Biology in May of 2010. KL Brzoza-Lewis performed all of the experiments. Dr. JJ Hoth provided reagents, and KL Brzoza-Lewis and EM Hiltbold prepared the manuscript.
Abstract:

Type I IFN has been shown to be important in the immune response to viral pathogens, however its role in the context of bacterial infections is less well understood. Mice lacking the type I IFN receptor (IFNAR-/-) have been shown to have enhanced resistance to *Listeria* infection. We have now determined that following infection with *Listeria*, the composition of innate cells recruited to the peritoneal cavity and spleen of IFNAR-/- mice reflects an increase in the frequency of neutrophils and a decrease in monocytes frequency compared to WT controls. However, the bone marrow from naïve WT and IFNAR-/- mice had a similar composition of neutrophils and monocytes, indicating that the increased neutrophil frequency observed in IFNAR-/- mice was not due to a difference in the starting numbers of these cells. We also observed no difference in the level of neutrophil function or in level of apoptosis in these cells to explain the composition differences. In order to determine the contribution of the CXC chemokines to the altered neutrophil recruitment in IFNAR-/- mice, we utilized the CXCR2 inhibitor, antileukinate. When IFNAR-/- mice were treated with antileukinate, we observed a reduced recruitment of neutrophils into the spleen following *Listeria* infection, which correlated with increased bacterial burdens at 72 hours post infection. Taken together, these studies are consistent with a model in which type I interferon signaling inhibits the immune response to *Listeria* by negatively regulating the recruitment of neutrophils through a mechanism dependent on CXC chemokines.
Introduction:

*Listeria monocytogenes* (*Lm*) is a gram positive, facultative intracellular bacterial pathogen that causes food borne-illnesses typically acquired from unpasteurized cheeses and prepackaged food products [1]. While of little consequence to healthy individuals, immuno-compromised individuals and pregnant women are at risk for serious infections [1]. *Listeria monocytogenes* has been used extensively as a model organism for studying the development of protective immunity to intracellular bacterial pathogens, reviewed in [2]. *Listeria* has a complex intracellular lifecycle including replication of the bacteria in the cell cytoplasm followed by intercellular spread. A number of virulence factors allow *Lm* to gain access to the host cell cytosol, most notably the pH-dependent pore forming toxin listeriolysin O (LLO) [3]. Once in the cytoplasm, *Listeria* induces the production of IFN-β [4, 5]. IFN-β is only produced upon cytosolic entry, as non-hemolytic mutant strains of *Lm* that are confined to vacuoles fail to induce the expression of this cytokine [6]. IFN-β can act either in an autocrine or paracrine manner by binding to the type I IFN receptor (IFNAR) and stimulating the transcription of IFN-inducible genes.

Type I IFN has been extensively studied in the context of viral infection, however the role of this family of cytokines in bacterial infections is less well defined. Several studies have implicated type I IFN induced by influenza virus infection in enhanced susceptibility to subsequent bacterial infections, such as pneumococcal pneumonia [8-10]. Additionally, recent reports have demonstrated that type I IFN negatively regulates the production of CXC
chemokines, and as a result, neutrophil recruitment [7, 8]. Several studies have shown that mice lacking the type I IFN receptor are resistant to \textit{Lm} infection. Although the mechanisms through which type I interferon enhances \textit{Lm} growth in the murine host are not completely understood, contributing factors may include increased lymphocyte apoptosis [9, 10], inhibition of TNF-\(\alpha\) production, and reduced recruitment of a population of CD11b+ non-dendritic cells [11].

Neutrophils are an innate immune cell type specialized to limit bacterial replication and prevent spread early in the infectious process. A large pool of quiescent neutrophils is present in the bone marrow ready to be called into action following pathogenic insult [12, 13]. These cells are among the first to be recruited to sites of infection and/or inflammation and are attracted by a number of stimuli including CXC chemokines (KC and MIP-2 in mice), complement components, and others [14]. The bactericidal activity of neutrophils is mediated through a number of mechanisms including phagocytosis, production of reactive oxygen and nitrogen intermediates, and production of antimicrobial proteins and peptides [15-17]. Neutrophils also secrete cytokines and chemokines that recruit other cell types such as monocytes and dendritic cells [18]. The importance of neutrophils during \textit{Listeria} infection has been demonstrated in studies using depletion of neutrophils with the RB6-8C5 monoclonal antibody. Exacerbation of bacterial burden was observed when neutrophils were depleted prior to, or at early time points post infection [19] or up to 4 days post infection [20]. Thus, recruitment of neutrophils during \textit{Lm} infection is known to be key to clearance of the infection.
While it is clear that neutrophils are necessary for anti-
Listeria defense [19-24], monocytes and TNF-INOS-producing DC (TipDC) also play important roles in the innate immune response to Lm [25-28]. Several recent studies have explored the recruitment of monocytes out of the bone marrow via the MCP-1/CCR2 interaction [25-27]. CCR2+ inflammatory monocytes are recruited from the bone marrow via MCP-1. They enter the circulation and ultimately, the spleen where they differentiate into TNF/iNOS producing dendritic cells [26, 28], a cell type that is key to limiting bacterial replication [27, 28]. Mice lacking CCR2 are unable to mobilize inflammatory monocytes to sites of infection and are highly susceptible to Lm infection [29]. These studies highlight the importance of inflammatory monocytes and Tip DC in the immune response to Listeria.

A previous study demonstrated that IFNAR-/- mice had increased numbers of TNF-α producing CD11b+ cells (that were not Tip DC) in the spleen following Listeria infection [11]. Given that these mice exhibited an enhanced clearance of Listeria [11], this suggested to us that these cells were likely contributing to the resistance phenotype. Therefore, we hypothesized that upon Lm infection, we would observe a change in the composition of the inflammatory cells recruited to the spleen and/or sites of infection in the absence of type I IFN signaling. Our preliminary experiments revealed that in IFNAR-/- mice there was indeed an increase in a cell population that expressed CD11b and Gr-1 upon Lm infection, as well as the typical polymorphonuclear morphology, indicative of a neutrophil population. We now demonstrate that in the absence of type I IFN signaling, the composition of the inflammatory infiltrate in the spleen and
peritoneal cavity in response to *Listeria* reflected an enhanced frequency of neutrophils and a decreased frequency of monocytes. This differential response was observed up to 3 days post infection. The enhanced neutrophil population observed in the absence of type I IFN signaling was not due to an increased reserve population of neutrophils present in the bone marrow, increased oxidase activity, or decreased apoptosis. However, when we used the CXC chemokine inhibitor, antileukinate, we observed a decreased recruitment of neutrophils into the spleen, suggesting the importance of these chemokines in recruitment of neutrophils. Finally, antileukinate treatment ablated the resistance of IFNAR-/- mice to *Listeria* infection suggesting that the enhanced neutrophil presence contributes to the resistance of IFNAR-/- mice to *Listeria*. 
Materials and Methods:

Mice  C57BL/6 mice were purchased from Charles River Laboratories (Wilmington, MA) and IFNAR-/- mice were a gift of Dr. Christian Schindler (Columbia University). Mice were bred and maintained in the animal facility at WFUSM according to ACUC guidelines.

Antibodies  Fluorescently tagged antibodies against the following mouse molecules were purchased from BD Pharamingen: Gr-1 (clone RB6-8C5), CD11b (M1/70), and Ly6C (AL-21). Cells were stained, fixed, and acquired using the BD FACS Canto II. Analysis was performed using DIVA software (BD Biosciences).

Splenocyte analysis  Spleens were collected from WT or IFNAR-/- mice at the indicated times post infection or from mock treated animals and digested with 2 mg/ml collagenase type D (Worthington). A cell suspension was obtained by homogenization and red blood cells were lysed. Splenocytes were stained with antibodies described above and cells that were Gr-1+ and either Ly6C^{int} or Ly6C^{hi} were quantitated by flow cytometry.

Peritoneal infiltrate analysis:  *Listeria monocytogenes* (*Lm*) strain 10403s was grown to stationary phase by 18h culture at 37°C in brain heart-infusion broth. Bacteria were washed in PBS, diluted to the appropriate concentration (based on the experiment), and were injected intraperitoneally (i.p.) in 1 ml PBS. In mock treated animals and at indicated time post infection, peritoneal exudate cells (PEC) were collected by lavage with 9 ml cold PBS. Exudate cells in the lavage fluid were analyzed for the expression of Gr-1, CD11b, and Ly6C (BD
Biosciences) using the BD FACS Canto II. BD FACS DIVA software was used to quantitate the number of Gr-1+ Ly6C\textsuperscript{int} and Ly6C\textsuperscript{hi} cell populations.

**Isolation of Neutrophils from Bone Marrow** Bone marrow was harvested as previously described [30] and neutrophils were isolated [31] with the following modifications. Bone marrow was flushed from the femurs and tibias of mice with cold PBS. Cell suspensions were loaded onto an isolymph gradient (density 1.077 g/ml) and centrifuged at 1,500 rpm for 40 minutes at room temperature. The cell pellet containing neutrophils and red blood cells (RBC) was collected and RBC were lysed with ACK lysis buffer (Lonza). Isolated populations were greater than 90% neutrophils (data now shown).

**Reactive Oxygen Species (ROS) Detection** Dihydrorhodamine 123 (DHR) (Invitrogen) was used to assess levels of ROS produced by bone marrow neutrophils. Briefly, 5x10\textsuperscript{5} isolated bone marrow neutrophils were stimulated with PMA (Sigma) at the indicated dose for 15 minutes or with serum opsonized \textit{Lm} (MOI 50) for 30 minutes in the presence of 1x10\textsuperscript{5} mM DHR 123. Cells were co-stained with anti-Gr-1 APC (BD Biosciences) and fluorescence of Gr-1+ cells was measured on the BD FACSCalibur.

**Cytokine/Chemokine detection** Cytokine and/or chemokines present in lavage fluid following infection were measured using a cytokine/chemokine protein array (Ray Biotech). Levels of selected cytokines and chemokines were further quantitated by ELISAs according to manufacturer’s instructions. Levels of the CXC chemokines, CXCL1 (KC) and CXCL2 (MIP-2) (R&D Systems) and IL-17A
(Biolegend) were determined by ELISA performed on spleen homogenate according to the manufacturers instructions.

**Antileukinate treatment** Antileukinate, (Ac-RRWWCR-NH₂), a hexapeptide inhibitor of CXCR2 (provided by Dr. Jason Hoth, WFUSM) was injected (1 mg, in 100 ul sterile PBS) i.p. 30 minutes prior to *Lm* infection. This reagent has been shown in several studies to inhibit neutrophil recruitment by binding blocking the interaction of CXC chemokines with CXCR2 [32-35]. For 72 hour experiments, 1 mg antileukinate was re-administered every 24 hours.
Results:

Inflammatory infiltrates in the spleen of IFNAR-/- mice consist of more neutrophils and fewer monocytes following Lm infection

Previous studies have demonstrated an increase in CD11b+ cells in the spleens of IFNAR-/- mice following infection with Listeria, suggesting that type I interferon signaling influences the recruitment of innate cells early during Lm infection [11]. We thus wanted to more closely identify the CD11b+ cells in these mice and their potential role in the observed resistance to Lm infection. CD11b is expressed by a range of cell types, but within the setting of Lm infection, two CD11b+ cell types that are most likely to play a role in early resistance are monocytes and neutrophils. Both cell types can also be tagged by the Gr-1 antibody which binds to both Ly6C, which is highly expressed by monocytes, and to Ly6G, which is expressed by neutrophils [19, 22, 24]. Neutrophils also express Ly6C, but at a lower level than monocytes [36, 37]. Therefore, we examined the expression of CD11b, Gr-1, and Ly6C on the splenocytes of IFNAR-/- mice following infection with Lm to distinguish the monocytes (CD11b+, Gr-1+, Ly6C^hi) and neutrophils (CD11b+, Gr-1+, Ly6C^int) responding to infection.

WT and IFNAR-/- mice were infected with 10^5 Lm i.p. and at 24h post infection, splenocytes were harvested and stained with antibodies to CD11b, Gr-1, and Ly6C. Splenocytes were first gated on cells expressing both CD11b and Gr-1 and 24hpi we observed that WT and IFNAR-/- splenocytes were composed of 6% CD11b+ Gr-1+ cells (Figure 12A). In order to more closely examine the
Figure 12: Inflammatory infiltrates in the spleen of IFNAR-/- mice consist of more neutrophils and fewer monocytes following *Lm* infection. WT and IFNAR-/- mice were infected with $10^5$ *L.m*. i.p. and at the indicated time post infection, splenocytes were harvested and stained. Surface expression of CD11b, Gr-1, and Ly6C was determined by flow cytometry. A) Splenocytes were selected for expression of CD11b and Gr-1 (left panels); Gated cells were then examined for relative expression of Ly6C on Gr-1$^+$ cells at 24h post infection. Numbers in the gates in the left panels indicate the percent of total splenocytes represented by the CD11b$^+$Gr-1$^+$ gated population and in the right panels, the percent of the CD11b$^+$Gr-1$^+$ that are either Ly6C$^{\text{int}}$ (neutrophils), or Ly6C$^{\text{hi}}$ (monocytes). B) Relative expression of Ly6C on Gr-1$^+$ splenocytes at 24 and 72h post infection. Numbers indicate percent of total splenocytes present in each gated population. C) Percent of splenic neutrophils at the indicated times post-infection compiled from several experiments. D) Number of neutrophils in the spleen at the indicated times. E) Percent of splenic monocytes over time. F) Number of splenic monocytes over time. (C-F) Mock treated values are compiled from 5 animals, and values from infected mice are from 5-10 animals. Statistics were calculated using student’s unpaired t test with Welch’s correction.
composition of the CD11b+ GR-1+ population in the spleen following infection, we looked at the levels of Ly6C expression on this gated population with Ly6C^{int} expression indicative of a neutrophil population and Ly6C^{hi} expression indicating a monocyte population. We determined that the CD11b+ GR-1+ population in WT mice was composed of 91% neutrophils (Ly6C^{int}) and 9% monocytes (Ly6C^{hi}). However, the CD11b+ GR-1+ population in IFNAR-/- mice was 98% neutrophils and 2% monocytes, suggesting that IFNAR-/- mice have an altered composition of inflammatory infiltrates in the spleen following infection (Figure 12A). Subsequent analysis of the inflammatory infiltrates will be determined based on their level of LyC6 expression. Differences in neutrophil composition were not observed in the spleen prior to infection between WT and IFNAR-/- mice (Figure 12C and 12D), yet, mock-infected IFNAR-/- animals contained a significantly lower frequency and number of monocytes in the spleen than WT mice (Figure 12E).

To determine how the composition of neutrophils and monocytes in the spleen changed over time in WT vs. IFNAR-/- mice, we quantitated the frequency and total numbers of these cells over a 72h period post infection. Representative dot plots of Gr-1 and Ly6C expression on splenocytes recovered from WT and IFNAR-/- mice 24 and 72 hours post infection are depicted in Figure 12B. Infection elicited significant increases in the number of neutrophils and monocytes by 24hpi in both mouse strains (Figure 12D and 12F). In WT mice, the percent of neutrophils peaked at 48 hours in the spleen and was declining by 72 hours (Figure 12C). However, the percent of neutrophils in the IFNAR-/-
spleens was sustained at 72 hours post infection (Figure 12C). Interestingly, while we observed differences in the percent of splenocytes with a neutrophil phenotype, we did not observe differences in the total numbers of these cells (Figure 12D). This discrepancy can be attributed to a lower number of total cells in the spleen of the IFNAR-/- mice vs. WT at this timepoint (data not shown). In contrast, the monocyte populations in the IFNAR-/- spleens were significantly lower in frequency at 24 and 48h and in number at 24h post infection compared to WT (Figures 12E and 12F). Thus, we have observed that Lm infection of IFNAR-/- mice results in a change in the composition of the CD11b+ cells in the spleen, resulting in increased neutrophil frequency and decreased monocytes.

Recruitment of neutrophils into the peritoneal cavity following Listeria infection is enhanced in the absence of IFNAR

Because the frequency and number of any given cell type in the spleen is the collective result of recruitment and death, we wanted to measure specifically recruitment. Therefore, we employed a peritoneal infection model to investigate cellular recruitment to the site of infection with Lm. WT and IFNAR-/- mice were infected with $10^5$ Lm i.p., and at 24, 48, and 72 hpi, cells recruited into the peritoneal cavity were collected by lavage. To assess the phenotype of peritoneal exudate cells (PEC), we subjected them to cytospin to enable visualization of nuclear morphology at 24 hours post infection (Figure 13A). Cells with segmented or ring shaped nuclei, characteristic of neutrophils, were frequently observed in the PEC recovered from WT and IFNAR-/- mice. Cells
Figure 13: Recruitment of neutrophils into the peritoneal cavity following *Listeria* infection is enhanced in the absence of IFNAR. WT and IFNAR-/− mice were infected i.p. with $10^5$ Lm. At the indicated time post infection, peritoneal lavage was collected and peritoneal exudate cells (PEC) were stained. Cell types recruited into the peritoneal cavity following infection were then assessed by flow cytometry. A) Cytospin preparations of PEC collected at 24 hours post infection. Cells with segmented or ring-shaped nuclei (characteristic of neutrophils) or rounded and uniform (typical of monocytes) were identified in both WT and IFNAR-/− PEC. B) Representative dot plots of Gr-1 and Ly6C expression on PEC collected from WT and IFNAR-/− mice at the indicated time post infection. Numbers in the gates indicate the percent of PEC that are either Ly6C$^{int}$ or Ly6C$^{hi}$ indicative of neutrophils or monocytes, respectively. C) Percent of neutrophils in PEC recovered from WT and IFNAR-/− mice at the indicated time post infection. D) Total number of neutrophils in PEC recovered per ml of lavage fluid. E) Percent of monocytes PEC recovered at the indicated time post infection. F) Total number of monocytes in PEC per ml of lavage fluid. Mock treated values are compiled from 6 animals per group, and time points post infection include 7-12 animals per group. Statistics were calculated using a student’s unpaired t test with Welch’s correction and significant differences are indicated with associated p values.
with rounded nuclei, characteristic of a monocyte morphology were also observed. In order to confirm the identity of these cells, PEC were stained with antibodies to distinguish monocytes and neutrophils as above. Representative dot plots of Gr-1 and Ly6C expression on PEC recovered from WT and IFNAR-/- animals at 24 and 72hpi are depicted in Figure 13B. The gates distinguish cells that are Ly6C^{int} and Ly6C^{hi} (insets indicate percent of total). At 24 and 72 hours post infection, IFNAR-/- mice exhibited an increased percentage of Ly6C^{int} cells recovered from the lavage fluid compared to WT mice. The relative frequencies of these two cell types in the WT and IFNAR-/- mice were similar by our assessment techniques.

To assess the composition of neutrophils and monocytes recruited to the peritoneal cavity, we quantitated the percent of Ly6C^{int} vs. Ly6C^{hi} PEC, respectively (Figure 13C and 13D) by flow cytometry. Prior to infection, neutrophils and monocytes each accounted for less than 1% of the PEC in both WT and IFNAR-/- mice. However, by 24 hours post infection, PEC recovered from IFNAR-/- animals consisted of approximately 80% neutrophils while PEC from WT animals contained only 40% neutrophils (Figure 13C). We also observed an increase in the total number of neutrophils per ml of lavage fluid (Figure 13D) in IFNAR-/- mice compared to WT upon Lm infection at 24, 48, and 72 hours post infection. Conversely, the number of monocytes recovered from the peritoneal cavity following Listeria infection in IFNAR-/- mice, was reduced both in percentage (Figure 13E) and total number of cells (Figure 13F) compared to WT controls at 24 and 48hpi.
The impact of bacterial dose on the relative composition of innate cells recruited upon *Lm* infection

One potential explanation for the altered composition of neutrophils and monocytes observed in the absence of IFNAR was that the dose of bacteria employed in our experiments (10⁵) was higher than bacterial doses used in other studies. The increased bacterial doses used in our studies favored the selective recruitment of neutrophils, perhaps due to increased production of cytokines or chemokines. Thus, to determine how the number of bacteria administered would impact the ratio of neutrophils to monocytes recruited, WT and IFNAR−/− mice were infected with 10⁴, 10⁵ or 10⁶ *Lm* and 24 hpi, PEC and splenocytes were harvested. Again, flow cytometry was used to determine the frequency of neutrophils and monocytes in the peritoneal cavity and spleen following infection.

As in our previous experiments, WT and IFNAR−/− mice contained a similar percent of neutrophils in both the PEC and spleen prior to infection. Following infection, both mouse strains exhibited a dose-dependent increase in the percent of neutrophils found in the PEC and spleen (Figure 14A and 14C). However, regardless of the dose, the percent of neutrophils in the IFNAR−/− PEC was higher than in WT (Figure 14A). Similarly, the percent of neutrophils in the spleen increased in both strains upon infection (Figure 14C). Yet, in this organ the percentages of neutrophils were similar between IFNAR−/− and WT mice except at the lowest bacterial dose, where the IFNAR−/− mice displayed a significantly higher percentage of neutrophils than WT.
Figure 14: The impact of bacterial dose on the relative composition of innate cells recruited upon *Lm* infection. WT and IFNAR-/- mice either mock treated or infected with *Lm* i.p. with the indicated dose. Twenty four hours post infection, PEC were collected by lavage and splenocytes were harvested. Cells were stained and flow cytometry was used to assess the expression of Ly6C on Gr-1^+ recruited cells. A) Percent of neutrophils in PEC at the indicated dose of *Lm*. B) Percent of PEC that are monocytes. C) Percent of neutrophils in the spleen D) Percent of monocytes in the spleen. Data are compiled from 6 animals for mock and 5-8 mice for infections. Statistics were calculated using a student’s unpaired t test.
We observed a different relationship in the percent of monocytes present in the PEC and spleen in WT and IFNAR-/- mice. IFNAR-/- mice exhibited a trend toward lower monocyte frequencies in the PEC than WT at all doses, but this difference was significant only at the highest dose of Lm in the PEC (Figure 14B). Additionally, the percent of monocytes in WT spleens was slightly higher than in the IFNAR-/- spleens at the low and intermediate doses tested (Figure 14D). Therefore, the composition of the peritoneal infiltrate in the IFNAR-/- mice was skewed toward a higher frequency of neutrophils, regardless of the dose of bacteria. However, in the spleen, this trend held true only at the lowest dose of bacteria.

**WT and IFNAR-/- mice have a similar composition of neutrophils and monocytes in the bone marrow**

The bone marrow is a reservoir of mature neutrophils and monocytes that can be rapidly mobilized in the event of an inflammatory stimulus [16, 27, 32]. Based on our observation that inflammatory infiltrates in IFNAR-/- mice showed an altered composition of neutrophils and monocytes upon Lm infection, we wanted to determine if this was due to a similarly altered composition of these cells in the bone marrow. Bone marrow was collected from the tibias and femurs of WT and IFNAR-/- mice that were either mock treated or infected with Lm for 24 hours (Figure 15). The cells were stained and distinguished by flow cytometry as above.
Figure 15: WT and IFNAR-/- mice have a similar composition of neutrophils and monocytes in the bone marrow. Bone marrow was harvested from WT and IFNAR-/- mice that were either mock treated or infected for 24 hours with Lm. The cells were then stained and expression of Ly6C on Gr-1+ cells was determined by flow cytometry. A) Percent of neutrophils in the bone marrow of WT or IFNAR-/- mice following either mock treatment, or 24 hours Lm infection. B) Percent of monocytes in bone marrow. Values are compiled from 4-6 mice per group and were not statistically different based on student’s unpaired t test with Welch’s correction.
Bone marrow from mock treated WT and IFNAR-/− animals was composed of a similar percentage of neutrophils (Figure 15A) as well as monocytes (Figure 15B). While there were slightly more neutrophils in the IFNAR-/− mice, this difference was not significant. The cellular composition 24 hours post *Listeria* infection revealed that the percentage of neutrophils was reduced compared to mock treated animals, but this difference was similar between WT and IFNAR-/− mice (Figure 15A). The bone marrow consisted of approximately 11% monocytes cells in both WT and IFNAR-/− mice and this frequency did not change in the first 24h of infection (Figure 15B).

**Enhanced resistance of IFNAR-/− mice to *Listeria* is not due to enhanced neutrophil ROS production**

Another potential explanation for the enhanced resistance to *Lm* observed in IFNAR-/− mice could be increased function (reactive oxygen species (ROS) production) of the neutrophils in these mice. Dihydrorhodamine 123 (DHR) fluorescence was used to measure neutrophil oxidase function, specifically detecting hydrogen peroxide, hypochlorus acid, and peroxinitrite anions. Neutrophils freshly isolated from the bone marrow of WT or IFNAR-/− mice were stimulated with *Lm*, PMA as a positive control, or no treatment as a negative control. Representative histograms of DHR fluorescence of neutrophils collected from WT or IFNAR-/− mice following treatment with *Lm* are depicted in Figure 16A. We detected a significant shift in the DHR fluorescence of both WT and IFNAR-/− neutrophils upon exposure to *Lm*. Data from several experiments were
Figure 16: ROS production by neutrophils from WT vs. IFNAR-/- mice. Bone marrow was harvested from WT and IFNAR-/- mice and neutrophils were enriched with an isolymph density gradient. Neutrophil oxidase function was tested by adding 5mM DHR to neutrophils concurrently stimulated with 100ng PMA or *Lm*. Twenty minutes post stimulation, DHR fluorescence, as a measure of oxidase activity, was measured by flow cytometry. A) Histograms of DHR fluorescence from WT (left) or IFNAR-/- (right) neutrophils. Gray shaded histograms show level of untreated cells (NT) or and black lines indicate level after stimulation with *Lm*. B) DHR fluorescence depicted as a fold increase over control for WT and IFNAR-/- neutrophils stimulated as indicated. Values are compiled from 3 independent experiments. Responses between WT and IFNAR-/- were determined to be not significantly different using a 2-way ANOVA with Bonferroni post test.
compiled and expressed as a fold increase over non-treated control (Figure 16B). Both WT and IFNAR-/- neutrophils generated a similar ROS response following treatment with 100 ng PMA or with \textit{Lm} (Figure 16B). Thus, neutrophils from WT and IFNAR-/- mice displayed a similar capacity to produce ROS.

**Enhanced numbers of neutrophils observed in IFNAR-/- mice were not due to decreased apoptosis**

One of two potential scenarios may account for the increased percentage of neutrophils in the PEC of IFNAR-/- mice over 72h. The first is increased influx/recruitment of these cells and the second is decreased death of these cells. It has previously been shown that type I IFN signaling enhances lymphocyte apoptosis upon \textit{Lm} infection [5, 13, 14, 33, 34]. Therefore, it was conceivable that the increased frequency of neutrophils observed in the PEC of IFNAR-/- mice was due to reduced neutrophil apoptosis. To determine the extent of apoptosis in our recruited neutrophil populations, WT and IFNAR-/- mice were infected with \(10^5\) \textit{Listeria} i.p. At 24 and 72 hours post infection, PEC were collected and stained with Annexin V and 7-AAD to determine the degree of early and late stage apoptosis by flow cytometry.

Representative dot plots of Annexin V and 7-AAD staining on PEC 72 hours post infection are shown in Figure 17A. Cells present in the upper right quadrant (Annexin V+ and 7-AAD+) display characteristics of cells in late stage apoptosis, while the lower right quadrant includes cells that are Annexin V+ and 7-AAD, or an earlier stage of apoptosis (Figure 17A). We observed that at both
Figure 17: Enhanced presence of neutrophils observed in IFNAR-/- mice was not due to decreased apoptosis. WT or IFNAR-/- mice were either mock treated or infected with $10^5 Lm$ for 24 or 72 hours. At the indicated times post infection, PEC were collected by lavage and stained with Annexin V and 7-AAD to assess levels of apoptosis. A) Representative dot plots of Annexin-V and 7-AAD staining on PEC at 72 hours post infection. Numbers in upper right quadrants represent the percent of PEC that are Annexin-V and 7-AAD double positive indicative of cells in late stage apoptosis. B) Percent of PEC at the indicated timepoint post infection that are Annexin-V and 7-AAD double positive. Data were compiled from 3-6 animals per group.
A. 7-AAD

PEC

72 hr

WT

IFNAR-/-

Annexin V

62.5

11

48.2

14

B.

PEC

Percent of PEC Annexin V & 7-AAD+

Mock 24 hr 72 hr

WT IFNAR-/- WT IFNAR-/- WT IFNAR-/-
24 and 72 hours post infection, PEC from WT and IFNAR-/- animals had no significant difference in the percent of cells in both the early and late stages of apoptosis. Figure 17B depicts the percent of cells in late apoptosis compiled from several experiments. Similar levels of total splenocyte apoptosis were also observed in WT and IFNAR-/- mice (data not shown). Therefore, the augmented frequency of neutrophils observed in IFNAR-/- mice was not due to reduced apoptosis.

**Inhibition of CXCR2 reduced neutrophil infiltrates in the spleen and rendered IFNAR-/- mice more susceptible to *Lm* infection**

Having determined that the altered composition of inflammatory infiltrates in IFNAR-/- mice was not due to differences in level of apoptosis, we next wanted to explore the possibility of differential recruitment. To determine if the chemokines and cytokines involved in neutrophil and monocyte recruitment were expressed at different levels in IFNAR-/- mice vs. WT mice, we measured the expression of several of these in the peritoneal lavage using a cytokine/chemokine array (Figure 18). We observed increased expression of a number of these inflammatory mediators in IFNAR-/- mice upon *Lm* infection, including IL-6 [38, 39], G-CSF [40-43], GM-CSF [41, 42], MIP-2 [44-47], PF-4 [48], and Lix [47, 49] all of which have been shown to impact neutrophil recruitment or function. Therefore, to address the role of CXC chemokines in the altered composition of the inflammatory infiltrates of IFNAR-/- mice, we used antileukinate, a hexapeptide inhibitor of CXCR2. This reagent has been used
Figure 18: Enhanced production of neutrophil-recruiting chemokines and cytokines in IFNAR-/- mice. WT or IFNAR-/- mice were infected with $10^6 Lm$ i.p. and peritoneal lavage was collected 24h later. Lavage fluid was analyzed using a cytokine/chemokine array from RayBiotec (according to manufacturers instructions) as a candidate search for potential differences in cytokine and/or chemokine production between WT and IFNAR-/- mice. Boxes highlight selected chemokines and cytokines that may be differentially expressed.
successfully in models of lung inflammation and injury to inhibit the recruitment of neutrophils following treatment [32, 34, 35, 50, 51].

Groups of IFNAR-/- and WT mice were treated with 1 mg of antileukinate thirty minutes prior to infection with *Lm*. Twenty four hours post infection, we harvested splenocytes and measured the number of neutrophils and monocytes in the spleen (Figure 19). As previously observed, spleens of the IFNAR-/- mice consisted of nearly 6% neutrophils upon infection, yet upon treatment with antileukinate, this was reduced to only 2% (similar to WT level, Figure 19A). A significant reduction was noted not only in the frequency, but also in the total number of neutrophils in the IFNAR-/- mice upon antileukinate treatment (Figure 19B). The effect of antileukinate on neutrophils in WT mice was more modest, however. We did observe a slight reduction in the frequency of neutrophils upon this treatment, but no significant difference in the numbers of these cells (Figure 19A and 19B). The already low frequency and number of monocytes in the infected IFNAR-/- mice was further reduced by anti-leukinate treatment, yet monocytes were not significantly decreased by this treatment in the WT mice (Figure 19C and 19D). Thus, inhibition of CXCR2 by antileukinate dramatically reduced the frequency and number of neutrophils and monocytes in the spleen of IFNAR-/- mice, but had little effect on either population in WT mice. Thus, the composition of inflammatory infiltrates observed in the absence of type I interferon signaling are CXCR2-dependent while other CXCR2-independent mechanisms regulate this response in WT mice.
Figure 19: Inhibition of CXCR2 reduced neutrophil and monocyte infiltrates in the spleen of IFNAR-/- mice. WT and IFNAR-/- mice were infected with 10^4 Lm. Thirty minutes prior to infection, a group of WT and IFNAR-/- mice was injected with 1 mg of antileukinate i.p. to inhibit CXC chemokine activity. Twenty-four hours post infection, splenocytes were harvested and stained. Flow cytometry was used to assess the numbers of neutrophils and monocytes. A) Percent of splenic neutrophils from either WT or IFNAR-/- mice in the presence or absence of antileukinate 24 hours post infection. B) Number of splenic neutrophils C) Percent of monocytes in the spleen. D) Number of splenic monocytes. Each symbol represents one experimental animal and the line indicates the mean value for that group of animals. Significance was determined using a student’s t test with Welch’s correction.
Finally, to determine if CXCR2-mediated cell recruitment might contribute to the resistance of IFNAR-/- mice to *Lm* infection, IFNAR-/- mice were treated with antileukinate and infected with *Lm*. Seventy two hours post infection, spleens and livers were collected to assess bacterial burdens. Antileukinate-treated, *Lm*-infected mice exhibited a two-log increase in the bacterial burden in the spleen (Figure 20A) and an approximately four-log increase in liver CFU (Figure 20B) over *Lm*-infected IFNAR-/- mice. Thus, treatment with the CXCR2 inhibitor, antileukinate, not only reduced the numbers of neutrophils and monocytes observed in the spleen following *Lm* infection of IFNAR-/- mice but also dramatically inhibited clearance of the bacteria.
Figure 20: Inhibition of CXCR2 rendered IFNAR-/− mice more susceptible to

*Lm* infection. IFNAR-/− mice were infected with $10^4$ Lm. Thirty minutes prior to infection, mice were treated with 1 mg antileukinate i.p. or mock treated. The drug was re-administered every 24 hours. Seventy two hours post infection, organs were homogenized and bacterial burdens in A) spleen and B) liver were determined. Each symbol represents one experimental animal. Statistics were calculated using and unpaired student’s t test with Welch’s correction.
Discussion:

The impact of type I IFN signaling on clearance of bacterial infections depends on the pathogen in question. In some cases this response is beneficial [52-56] and in others [9-11, 57, 58], detrimental to the immune response. To our knowledge, this is the first study to show that the type I interferon response skews the composition of inflammatory infiltrates toward higher frequencies of neutrophils and fewer monocytes following systemic infection with *Listeria* (Figure 12 and 13). Thus, type I interferon signaling appears to inhibit the recruitment of neutrophils and/or enhance the infiltration of monocytes. Furthermore, we conclude that this effect is mediated through CXCR2, based on our inhibitor data (Figure 19) and likely stems from negative regulation of CXC chemokine production by type I interferon signaling (Figure 18). It remains to be determined if this difference in cellular composition is due strictly to enhanced production of CXC chemokines in IFNAR-/- mice upon Lm infection or if there is an additional element of negative regulation of monocyte-recruiting chemokines.

Several studies have now examined the role of the type I IFN response in the *Lm* infection model [9-11, 59-61]. These reports demonstrated that type I IFN receptor (IFNAR) deficient mice were more resistant to *Listeria* infection than WT [9-11]. This resistance has been attributed to several important type I interferon-mediated phenomena including apoptosis, cytokine regulation, and recruitment of inflammatory cells. Several reports have demonstrated decreased apoptosis of lymphocytes and macrophages in IFNAR-/- mice, indicating that type I interferon signaling sensitizes these cells to apoptosis [5, 14, 49, 50]. However, our results
did not indicate any significant difference between WT and IFNAR-/− PEC in the number of cells in early or late apoptosis based on Annexin V and 7-AAD staining. Because neutrophils are such a short-lived cell population (12-24h), it may be difficult to capture these cells in different stages of apoptosis ex vivo. Alternatively, neutrophils may not be susceptible to apoptosis sensitization by type I interferon. This remains to be determined in future studies.

The cytokine response to *Lm* is also altered in the absence of IFNAR [13-15, 49]. Mice lacking IFNAR show higher levels of IL-12 and TNF-α (in agreement with our data, Figure 18), yet do not display higher levels of IFN-γ as might be predicted [15]. In fact, it has recently been shown that type I interferon signaling inhibits responsiveness to IFN-γ by down-regulating the receptor for this cytokine [61]. The composition of inflammatory cells in IFNAR-/− mice is likely regulated by this differential cytokine and chemokine response. The recruitment of inflammatory cells upon *Lm* infection in the absence of IFNAR has only been examined in a few studies [15, 29]. One early study in this model reported an increased number of TNF-α-producing CD11b+ cells in the spleen following *Listeria* infection in IFNAR-/− mice [11]. Neutrophils have been shown to be a source of TNF-α [18]. Therefore, we now demonstrate that the composition of the CD11b+ cells recruited upon *Lm* infection is regulated by type I interferon signaling such that neutrophil recruitment is inhibited by this response. Our data, taken together with the studies mentioned above suggest that the mechanism of differential recruitment of neutrophils involves chemokine regulation.
The kinetics and regulation of the chemokine response to *Lm* infection in vivo has not been thoroughly explored. It is clear that this infection results in the emigration of neutrophils and monocytes from the bone marrow, but detecting the chemokine responses that recruit these cells in vivo can be difficult due to the sensitivity of reagents as well as the rapid consumption of these chemokines. However, several studies have provided important insights [29, 51, 52]. A recent study from Pamer's group explored the production of the monocyte-attracting chemokine, MCP-1, by bone marrow macrophages [48]. They demonstrated two waves of MCP-1 expression following infection with *Lm*. The first wave of MCP-1 production was MyD88-dependent and was followed by a stronger, second wave that required signaling through IFNAR. Thus, IFNAR amplified and positively regulated the production of this chemokine which was induced through TLR signaling. However, several reports in other bacterial models have demonstrated that type I interferon signaling inhibits the production of neutrophil-recruiting chemokines such as KC, MIP-2 (in mice) [9] and IL-8 (in human cells) [11, 53]. In fact, direct evidence that type I interferon signaling inhibits the transcription of IL-8 has emerged [11, 53]. Thus, while some chemokines (likely CC chemokines) are positively regulated by IFNAR, our data and those of others, indicate that CXC chemokines are negatively regulated by this receptor which results in the recruitment of the appropriate effector cells to the site of infection.

*Lm* infection has been shown to induce the mobilization of monocytes and neutrophils from the bone marrow and monopoiesis [27, 54]. Another recent study from Pamer's group examined the recruitment of monocytes to the spleen
in the absence of IFNAR, among other innate signaling mediators [26]. Their conclusion with regard to IFNAR was that it (alone) did not affect the recruitment of monocytes upon Lm infection. In contrast, we observed fewer monocytes in the infiltrates of IFNAR-/- mice. However, some key differences between their study and ours may explain our conflicting conclusions. Serbina, et al did not examine recruitment using the peritoneal infiltrate model (where we saw the greatest differences), they did not quantitate neutrophils (again, our most striking differences were in this cell type), and they used a lower dose of bacteria (which we have shown can impact the types of cells recruited). It is likely that the higher doses of Listeria we used in our experiments induced a cytokine/chemokine profile that skewed toward the recruitment of neutrophils compared to the lower doses of Listeria that were observed to promote monopoiesis [25]. Thus, the role of the IFNAR in regulating the composition of inflammatory infiltrates may have been previously under-appreciated.

The significance of the interplay between the type I interferon response and neutrophil-attracting CXC chemokines is most visible in the realm of polymicrobial infections [55]. It is well established that many respiratory bacterial infections such as pneumococcal pneumonia are exacerbated by previous or concurrent infection with viruses such as influenza or RSV [9, 56, 57]. While clearance of extracellular bacterial infections depends on strong neutrophil influx, coupled with antibody and complement, this response is inhibited following a strong type I interferon response [56, 57]. Therefore, these studies suggest that type I IFN is negatively regulating chemokine expression and that CXC
chemokine expression is imperative for the recruitment of neutrophils. Our study highlights the contribution of neutrophil recruitment mediated by CXC chemokines to the control of Lm infection, and more specifically, suggests that enhanced neutrophil populations are a potential mechanism for the resistance of IFNAR-/- mice to Listeria.
References


CHAPTER IV

DISCUSSION

The interaction of the bacteria, *Listeria monocytogenes*, with cells of the innate immune system such as dendritic cells initiates a series of events leading to clearance of the bacteria and development of the adaptive immune response. Due to its unique intracellular lifecycle, *Listeria* enters sites within the cell that bacteria are routinely taken into (phagosomes) as well as the cytosol, a site from which most bacteria are restricted. Likewise, *Lm* has the potential to trigger specific immune responses through TLR/MyD88 in the endocytic pathway and specific immune responses (such as the type I interferon response) generated by PRR localized in the cytosol. Here, I will discuss how my findings illuminate this process by defining the regulatory roles of the signaling molecules, MyD88 and the type I interferon receptor (IFNAR). I will discuss the infectious process in sequence to delineate the steps at which my findings specifically enhance our understanding of this process. Finally, I will finish by discussing broader implications of this work and how it relates to the field of bacterial immune responses. Our initial goal was to determine how these regulatory mechanisms impacted the development of protective immunity to intracellular bacterial pathogens, thus I will also discuss how my studies have addressed this goal.

Step 1. *Listeria* binds to the cell surface and is engulfed into a phagosome.
One of the first sites at which host DC encounter *Lm* following systemic infection, is the spleen [1-3]. Here, DC and other phagocytic cells within the marginal zone are known to sample the circulation and may become infected [3]. In these cells, *Lm* will bind to the surface and be taken into phagosomes through phagocytosis. Due to the presence of several PAMPS on the bacteria, the DC will likely attempt to target *Lm* for destruction through rapid phagolysosomal fusion, an MyD88-dependent enhanced transport mechanism [4, 5]. However, *Lm* has a potent escape mechanism to evade this killing pathway [6, 7] (see step 2, below). During this early phase of the infection, the bacteria are likely to trigger the activation of several TLR that are localized either on the plasma membrane (TLR2, TLR4, or TLR5) or within the endocytic pathway (TLR9) [8-10]. The activation of these various TLR depends on the availability of their respective ligands (LTA, LLO, flagellin, hypomethylated DNA) [11]. Some of these may be masked on intact bacteria, yet may be revealed upon degradation of the cell wall within the phagosome [12]. Importantly, each of these TLR is known to utilize the signaling adapter molecule, MyD88 either exclusively (TLR2, 5, and 9) or in concert with the adapter, TRIF (TLR4) [13].

**The impact of phagosomal detection and signaling through MyD88 on the innate immune response to *Listeria***

While the role of individual TLR, such as TLR2, in the response to *Listeria* remains a matter of debate [14-18], it is clear that MyD88 is essential for clearance of the bacteria during the innate phase of the immune response [14, 17, 18]. Our findings indicate that this mediator is involved in the production of
many inflammatory cytokines that are known to be critical to the Lm immune response such as TNF-\(\alpha\), IL-12, IL-6 (Chapter 2, Figure 4), and IL-1 (data not shown). Thus, it is not surprising that mice lacking MyD88 succumb rapidly to infection because many of the effector cytokines that activate macrophages and other phagocytes are lacking [14]. In contrast, the costimulatory molecules induced as a consequence of Lm infection were not diminished in cells lacking MyD88. These findings highlight an important difference in the regulation of these two classes of molecules.

Our results demonstrating differential regulation of pro-inflammatory cytokines and costimulatory molecules are consistent with previous studies of MyD88’s role in these responses in macrophages [19-21]. However, an important difference in our studies was that we utilized whole, live bacteria while previous reports used LPS and other purified TLR agonists [19-21]. Thus, it is likely that our observations of the varying degrees of MyD88 “dependence” of certain cytokines may be attributed to the additional complexity of our system (multiple TLR agonists as well as multiple potential PRR triggering in the cytosol). For example, based on our data it is likely that TNF-\(\alpha\) is produced in an MyD88-dependent manner upon recognition of the bacteria in the phagosome. In contrast, IL-6 and IL-12 must be produced due not only to phagosomal recognition, but also based on recognition in the cytosol. These predictions are generally consistent with a recent paper exploring the transcriptional programs generated in macrophages upon Lm infection initiated by phagosomal vs. cytosolic signaling [22]. The transcription factors mediating MyD88-initiated
transcription of these inflammatory cytokines differ based on the cell type and on
the specific promoters of each cytokine [23, 24]. However, they share a common
dependence on the transcription factors NF-κB and MAPK [19]. Thus, defining
the specific mechanisms mediating the transcriptional regulation of pro-
inflammatory cytokines induced by bacterial infection will require further,
extensive investigation.

Another consequence of bacterial sensing through TLR and MyD88 in the
phagosome is the induction of chemokines. These chemoattractants may recruit
other cells of the innate immune system, to enhance bacterial clearance [25], or
T cells, to enhance their interaction time and potential for activation [26]. The
role of MyD88 in the production monocyte-attracting chemokines has recently
been reported [27-30]. MCP-1 is a key chemokine in the mobilization of
inflammatory monocytes from the bone marrow during *Lm* infection and mice that
lack its receptor, CCR2, are highly susceptible to *Lm* infection [31]. Interestingly,
Pamer’s group found that MCP-1 was induced by *Lm* in an MyD88-dependent
manner, but this response was modest and transient [28]. A second, stronger
wave of MCP-1 production followed the first and required bacterial cytoplasmic
entry as well as the type I interferon receptor [28]. Thus, the chemokine
response to *Lm* is tightly regulated and a strong response requires activation at
multiple levels. Previous studies from our lab have also demonstrated that the T
cell-attracting chemokines, CCL1 and CCL17, are produced by *Lm*-infected DC
in an IL-12 dependent manner [26]. While the role of MyD88 in the induction of
these chemokines was not specifically addressed, it is likely that the low level of
IL-12 produced by *Lm*-infected, MyD88-deficient cells would diminish their production. Thus, chemokines produced in an MyD88-dependent manner are key to the recruitment of not only innate cells for bacterial clearance, but also for T cells for the development of protective CTL responses.

One of the later steps in DC maturation is their migration to the T cell zones of lymphoid organs. In the case of systemic *Lm* infection, DC infected in the marginal zone of the spleen migrate to the peri-arteriolar lymphoid sheath (PALS, also known as the T cell zone) of the spleen [1, 2, 32]. Here, they nucleate the formation of organized clusters of innate cells, flanked by T cells [32]. These clusters consist of DC and *Lm* surrounded by neutrophils and monocytes, flanked by a ring of NK cells. These clusters serve as localized sites for production of inflammatory cytokines, IFN-γ and bactericidal compounds such as ROI and NO [32]. These clusters are also essential for antigen presentation and T cell priming following *Lm* infection [2]. Surprisingly, it remains to be determined if DC migration is an MyD88-dependent or –independent aspect of maturation. Likewise, the role for MyD88 in migration of DC to the PALS and formation of innate cell clusters has only received a cursory examination to date [32]. Mice lacking MyD88 show small innate cell clusters in the PALS and display low levels of IFN-γ production within these clusters [32]. This report, taken together with our findings, leads us to postulate that DC migration is mediated by MyD88 at least in part, likely through the expression of an MyD88-dependent chemokine receptor. Furthermore, the reduced level of IFN-γ seen in the clusters of MyD88−/− spleens is likely due to reduced IL-12 production.
Testing these potential hypotheses will provide critical insights into the mechanisms driving DC migration and initiation of antigen presentation upon bacterial infection.

**The impact of phagosomal detection and signaling through MyD88 on the development of protective T cell responses to Listeria**

As a key regulator of inflammatory cytokine production, one might expect MyD88 to have a powerful impact on the nature of the T cell response generated to *Lm*. In my in vitro studies, MyD88 expressed by DC was shown to regulate the level of IFN-γ production by naïve CD8+ T cells yet, T cell proliferation was not affected by MyD88. These results suggest that the costimulatory molecules, thought to regulate proliferation were sufficiently expressed in the absence of MyD88, while the inflammatory which cytokines regulate the strength of the IFN-γ response were lacking. Similar results were also obtained in recently published work from the Wick laboratory in vivo [33]. This study did not measure levels of IL-12. However, it is likely that reduced IL-12 production in MyD88-/- mice is primarily responsible for the reduced IFN-γ production observed in both studies, given our data and the well established role of IL-12 in IFN-γ production.

Interestingly, they also showed that there were less CD8+ *Lm*-specific memory T cells in the absence of MyD88, yet no difference was observed in clearance of lethal *Lm* challenge [33]. Thus, our results and those of other groups indicate that MyD88 regulates not the number of effector T cells in the primary response [34], but enhances CTL effector function and likely their long term survival.
One of the primary questions we had when we began these studies was: why does Listeria that fails to access the cytosol also fail to induce protective CTL responses to subsequent challenge? In other words, why does bacterial recognition and signaling exclusively from the phagosome fail to prime CTL? My findings in combination with other recent reports have provided a reasonable explanation to this problem. My first observation in the laboratory was that Hly-Lm (phagosomally confined) do not induce a full program of DC maturation [35]. We observed a modest expression of cytokines, but no costimulatory molecule up-regulation in DC infected with phagosomal Lm, which was later confirmed by Leiberman’s group [36]. I subsequently determined that virtually all of the responses induced by phagosomal Lm were mediated by MyD88. These findings led us to postulate that in vivo, DC infected by Hly-Lm would fail to up-regulate costimulatory molecules and that the cytokines and chemokine responses mediated by MyD88 were not sufficient to stimulate DC maturation and/or T cell priming.

Several related reports around this time also examined the localization of Lm in the spleen following infection with WT or Hly-Lm. They showed that the majority of WT Lm was found in the T cell zones of the spleen after 24h of infection while Hly-Lm was restricted to the marginal zones at this time [1, 2, 32]. Thus, not only were the Hly-Lm-infected DC not mature, they also failed to enter the T cell zones where they would have potential to prime T cells. While these factors might seem sufficient to explain the poor immune response to Hly-Lm, a recent study from the Portnoy group has provided further evidence of immuno-
suppression induced by this bacterial strain [37]. This report demonstrates that Hly- *Lm* not only fail to induce T cell responses, but actually inhibit T cell responses to WT *Lm* in a dose-dependent manner. This inhibition was attributed primarily to IL-10 production by Hly- *Lm* infected cells [37]. Thus, cytokines induced in an MyD88-dependent manner by *Lm* confined to the phagosome include the immunosuppressive cytokine, IL-10 and have the potential to negatively regulate immune responses. These findings offer compelling insights and invite further study to determine if these MyD88-mediated responses are involved in the development of regulatory T cell responses.

**Step 2. Escape from the phagosome and bacterial cytoplasmic invasion**

While the progressive acidification of the phagosome is key to bacterial killing and degradation it also creates the ideal environment for the activity of *Listeria*’s pore-forming toxin, Listeriolysin O (LLO). LLO is a pH-dependent toxin that functions optimally at pH 5-5.5 [38]. Thus, following uptake of extracellular bacteria (~pH 7.0) the phagosome transitions through the range of 5-5.5 on the way to a pH of at or below 4.0 in a typical phagolysosome. Through the activity of LLO and two phospholipases, the bacteria disrupt the phagosomal membrane and enter the cytosol [39]. Here, the bacteria find a niche rich in nutrients in which to multiply and spread. Using a virulence factor, ActA, *Lm* polymerize the host cell actin into comet-like tails that it uses to propel itself around the cell and ultimately into neighboring cells [40-43]. Escape from the phagosome not only enables the bacteria to evade the toxic environment of the phagolysosome, but also keeps them protected from several extracellular immune defense
mechanisms such as antibody and complement. However, entry into the cytosol makes the bacteria visible to cytoplasmic PRR, initiating downstream effects of this recognition [12, 22, 44, 45]. Finally, entry into the cytosol is a key step in the maturation and migration of DC from the marginal zones of the spleens into the T cell zones where protective CTL responses are primed [1, 35, 36].

**Cytosolic signaling induced by *Listeria* triggers the type I IFN response**

Due to the ability of *Listeria* to enter the host cell cytoplasm, this opens up the potential for the host cell to detect *Listeria* through a variety of cytoplasmic PRR. These PRR include but are not limited to the cytoplasmic DNA sensor DNA-dependent activator of IFN-regulated genes (DAI) [46] or the cytoplasmic RNA sensors retinoic acid inducible gene-I (RIG-I) and melanoma differentiation association antigen-5 (MDA-5) [47, 48]. Additionally, nucleotide-binding oligomerization domain (NOD) proteins reside in the host cytosol and are capable of detecting bacterial peptidoglycan [49]. Interestingly, mice that lack NOD2 are susceptible to *Listeria* only when infection occurs via intragastric inoculation and not through intravenous or intraperitoneal infection routes [50]. These results suggest that detection of *Listeria* by the cytosolic sensor NOD2 is not essential in the context of experimental infection. However, in a physiological context, through the ingestion of contaminated food products, recognition of *Listeria* by NOD2 appears to be valuable, thus, exhibiting site specific importance. While the specific cytoplasmic receptor that recognizes *Listeria* remains unknown, regardless of the mechanism of induction, a major consequence of cytoplasmic entry of *Lm* is the induction of a strong type I IFN
response [44]. This response is strictly dependent on cytoplasmic invasion of the bacteria and does not require LLO expression [44]. Thus, we have examined the type I interferon response using IFNAR as a surrogate marker for responses initiated upon *Lm* entry into the cytosol and cytosolic signaling.

Our studies in vitro indicated that IFNAR regulated the DC maturation response induced by *Lm* at the level of costimulatory molecule expression. However, these results were not observed in vivo. The level of costimulatory molecule expression on splenic DC was similar between WT and IFNAR-/- DC following *Lm* infection in vivo. These somewhat perplexing results indicated that our in vitro system did not fully represent the activation of IFNAR-/- DC in vivo. To address these contrasting findings, we tested the hypothesis that splenic DC express costimulatory molecules upon *Lm* infection in an IFNAR-independent manner thanks to the contributions of neighboring cells. We co-cultured infected IFNAR-/- DC with infected splenocytes and measured maturation of the DC. We did observe an increase in the maturation of these DC, but not to the same level as that observed by splenic DC in vivo. Thus, we conclude that bystander cells can enhance IFNAR-independent DC maturation. Yet, it remains to be determined if there are other factors that contribute to this effect in vivo, or if our experimental conditions simply did not provide the optimal environment to observe this phenomenon.

When we consider what IFNAR-independent factors may be produced by infected splenocytes that enhance DC maturation, TNF-α tops the list. This cytokine has been shown to cause DC maturation in vitro on its own [51, 52] and
TNF-α production is known to be increased in IFNAR-/- mice upon *Lm* infection [53]. Other DC maturation-inducing candidates include reactive oxygen intermediates produced in response to bacterial infection. Several studies have shown that reactive oxygen intermediates are a key element of DC maturation [54-56]. Both TNF and ROI are produced abundantly by neutrophils, suggesting that this cell type may contribute to the maturation of DC and to the resistance to *Lm* infection in IFNAR mice.

Three concurrent papers published several years ago demonstrated that IFNAR-/- mice were resistant to infection with *Listeria* and this was attributed primarily to decreased lymphocyte apoptosis [53, 57, 58]. While these data and other studies since then have shown differences in the level of lymphocyte apoptosis in the absence of type I IFN signaling as well as the contribution of LLO to lymphocyte apoptosis [57-60], it seemed probable that other mechanisms could also be contributing to the resistance of IFNAR-/- mice to *Listeria*. One study determined that upon *Lm* infection of IFNAR-/- mice there was an increased population TNF-α producing CD11b+ cells in the spleen that were not DC [53]. While CD11b is expressed by several cell types, we considered the possibility that this enhanced cell population was largely comprised of neutrophils. Preliminary experiments determined that there was an enhanced frequency of neutrophils in the spleen of IFNAR-/- mice at 24 hours post infection with *Listeria* compared to WT. Thus, we hypothesized that enhanced numbers of neutrophils observed in the absence of type I IFN signaling contributed to the resistance phenotype observed in IFNAR-/- mice following infection with *Listeria*.
The role of the type I interferon response in the composition of \textit{Lm}-induced inflammatory infiltrates

Once the bacteria have invaded the cytosol, they begin to multiply and spread. One major mechanism for control of bacterial spread is recruitment of activated phagocytes. For example, prior to activation, quiescent macrophages are permissive to \textit{Lm} cytoplasmic penetration \cite{61}. However, after activation, through exposure to bacterial PAMPs and cytokines such as IFN-\(\gamma\) and TNF-\(\alpha\), macrophages restrict the bacteria to the phagosome and kill internalized bacteria through production of RNI \cite{61}. Likewise, recruitment of neutrophils to sites of infection is critical for the clearance of \textit{Lm} due to their potent capacity to produce ROI and other bactericidal compounds \cite{62, 63}. The recruitment of monocytes and neutrophils is mediated by distinct classes of chemokines. In general, monocytes are recruited by CC chemokines such as MIP-1 (CCL2) and neutrophils are recruited by CXC chemokines such as KC and MIP-2 (CXCL1, and CXCL2 respectively) \cite{64-66}. While the mechanisms regulating induction of these chemokines remain to be fully elucidated, MyD88 and IFNAR are known to play specific roles \cite{28}. I previously referred to the regulation of the monocyte-attracting chemokine MCP-1 by MyD88 and IFNAR (Discussion page 128, paragraph 2). I will now discuss how my results contribute to our understanding of the regulation of CXC chemokines by IFNAR.

CXC chemokines are a family of chemoattractive cytokines that are important for the recruitment of neutrophils \cite{67}. Using a peritoneal infection model to look specifically at cell recruitment in response to \textit{Lm} infection, we
determined that there was an enhanced and prolonged recruitment of neutrophils in the absence of type I IFN signaling. The enhanced neutrophil recruitment observed in IFNAR-/- mice was not due to an increased starting population of neutrophils in the bone marrow (Chapter 3, Figure 15) or decreased levels of apoptosis (Chapter 3, Figure 17). We did however, observe an increase in the expression of several neutrophil-attractive chemokines in the peritoneal fluid of Lm-infected IFNAR-/- mice (Chapter 3, Figure 18). Similar results have also been reported in a mouse model of polymicrobial sepsis, in which increased neutrophil recruitment into the peritoneal cavity at early timepoints post infection was attributed to increased levels of MCP-1 and LTB₄ in IFNAR-/- mice [68]. Importantly, IFN-β has been shown to also inhibit gene expression of IL-8 in humans [69] and MMP-9 at the transcriptional level [70]. Interpreting this data collectively, we conclude that type I IFN signaling induced by bacterial infection negatively regulates the production of CXC chemokines at the transcriptional level, resulting in reduced neutrophil recruitment. Our next goal was to determine if CXC chemokine-mediated recruitment of neutrophils contributed to the enhanced resistance to Lm infection observed in IFNAR-/- mice.

To define the role of CXC chemokines in the enhanced neutrophil recruitment observed in response to Listeria in IFNAR-/- mice, we treated WT and IFNAR-/- mice with the CXCR2 inhibitor, antileukinate [71-74]. In the presence of antileukinate, we observed reduced neutrophil trafficking into the spleens of IFNAR-/- mice compared to WT controls in which the effect of antileukinate was modest (Chapter 3, Figure 19). These results suggest that the
increased neutrophil population observed in IFNAR-/- mice (Chapter 3, Figure 13) was due to the enhanced CXCL1 and CXCL2 expression and that other redundant mechanisms regulate this response in WT mice. Therefore, these studies imply that enhanced CXC chemokine production contributes to the enhanced neutrophil influx detected in mice lacking type I IFN signaling. To determine if the increase in CXC chemokine-mediated neutrophil influx observed in the absence of type I IFN signaling contributes to the resistance of IFNAR-/- mice to *Listeria*, we treated mice with antileukinate and assessed bacterial burdens at 72 hours post infection. Antileukinate treatment of IFNAR-/- mice rendered them very susceptible to *Lm* infection. These findings highlight a novel mechanism contributing to the resistance of this mouse strain to *Listeria*. CXC chemokines have also been shown to be vital in the neutrophil-mediated immune response against *Pseudomonas aeruginosa* and *Klebsiella* infections [67, 75]. These studies as well as our own, emphasize the essential contribution that CXC chemokines play in orchestrating immune cell recruitment that is imperative for the control of many bacterial pathogens (Figure 21).

There are many positive effects of type I IFN on cell types in response to *Listeria*, including the generation of early sources of IFN-γ that lead to the activation of macrophages, the cellular reservoir of *Listeria* [76]. However, protective immunity to *Listeria* is mediated by CD8+ T cells. Type I IFN has been demonstrated to augment the generation T cell responses both indirectly, by activating DC [77] as well as directly, by providing “signal 3” required for priming naïve T cells [36, 78]. However, even with enhancement of immune responses
Figure 21: Molecular Regulation of Innate Immunity to Listeria. The goal of this thesis work was to investigate the regulation of innate immune responses to Listeria. In order to assess the contribution of phagosomal sensing to the innate immune response induced by Listeria, we utilized mice lacking the adapter molecule MyD88. We determined, both in vitro and in vivo, that phagosomal sensing of Listeria induced the production of pro-inflammatory cytokines by DC. Sensing via the endocytic pathway was also important for the initial chemokine burst that resulted in the recruitment of both monocytes and neutrophils. In order to determine the contribution of cytosolic sensing in the innate immune response to Listeria, we utilized mice lacking the type I IFN receptor as a surrogate for the cytosolic response. In an in vitro model, type I IFN signaling and thus cytosolic sensing of Listeria, was important for the upregulation of the costimulatory molecules CD40 and CD86 on the surface of DC. However, in an in vivo system, we did not observe this effect suggesting that in vivo, other cell types or their products (cytokines) are compensating for this response. We also determined that type I IFN signaling negatively regulates CXC chemokine production, thus preventing the recruitment of neutrophils. Studies from other laboratories have observed a positive regulation of CC chemokines by type I IFN signaling, resulting in the recruitment of monocytes. Thus, distinct aspects of the Listeria-induced innate immune response are regulated by phagosomal and cytosolic sensing mechanisms.
Molecular regulation of innate immunity to *Listeria*

**Phagosomal sensing:**
- MyD88
- Regulates:
  - Pro-inflammatory cytokines
  - Initial chemokine burst

**Cytosolic sensing:**
- IFNAR
- Costimulatory molecules:
  - *in vitro*: Type I IFN signaling regulates
  - *in vivo*: Bystander effect
  - Positive regulation of CC chemokines
  - Negative regulation of CXC chemokines

**Monocyte recruitment**

**Neutrophil recruitment**
attributed to type I IFN signaling, it sensitizes macrophages and lymphocytes to
death [57, 58, 79]. Interestingly, type I IFN has been shown to have effects on
multiple cell types involved in the coordinated response to Lm [76] thus
demonstrating immunomodulatory effects of type I IFN and demonstrating a link
between the innate and adaptive immune systems.

The contribution of neutrophils to the adaptive immune response

While numerous studies have demonstrated the beneficial role of CXC
chemokine mediated neutrophil recruitment on bacterial pathogen clearance [67,
68, 75], a growing body of work also suggests that neutrophils are capable of
influencing the generation of adaptive immune responses through interactions
with DC [80, 81]. The interaction of neutrophils and DC is complex, however it
has been shown that neutrophils and DC physically interact via binding of the C-
type lectin DC-SIGN on DC and Mac-1 or CEACAM1 on the surface of
neutrophils [81-84]. Interaction of DC and neutrophils results in two important
outcomes. First, binding of these two cell types prolongs the life of the
neutrophils [82, 85, 86]. Secondly, interaction of neutrophils with DC has been
shown to mature DC in both mice [51] and humans [83] with neutrophil-derived
TNF-α credited as the maturation stimulus [51, 87]. These studies describing the
ability of neutrophils to mature DC suggest a potential mechanism that
contributes to the in vivo maturation phenotype of splenic IFNAR-/− DC. The
requirement for cell contact and/or cytokines secreted by neutrophils suggests a
likely reason why our in vitro generated IFNAR-/− DC did not upregulate
costimulatory molecules. Additionally, interaction of neutrophils with DC through
any of the mechanisms described above also explains the enhancement of IFNAR-/- DC expression of costimulatory molecules observed following co-culture with splenocytes (Chapter 2, Figure 11).

Neutrophils also have the capacity to regulate adaptive immune responses by skewing the polarization of naïve T cells to a Th1 phenotype by influencing the cytokines produced by DC [51, 83]. Further illustration of neutrophils skewing adaptive immune responses towards the Th1 phenotype can be observed as activated neutrophils produce chemokines such as MIP-1α, MIP-1β, MIG, and IP-10 that act on receptors expressed on Th1 cells but not Th2 cells [88]. The significant contribution of neutrophils in the process of instructing DC can be observed in studies where in the absence of neutrophils, the dominant Th1 polarized phenotype was shifted to a Th2 phenotype. This shift towards a Th2 phenotype resulted in the increased susceptibility of mice to various pathogens including: *Helicobacter pylori* [89], *Candida albicans* [90], and *Legionella pneumophila* [91]. Given the capacity of neutrophils to shape the immune response, it is likely that an experimental model that results in enhanced neutrophil numbers in response to infection, such as the one described in this thesis, would be ideal to study the specific contributions of neutrophil: DC interactions on the development of protective immunity. Therefore, these studies highlight the important contribution that neutrophils have on the orchestration of immune responses, not only with regard to innate responses, specifically in bacterial killing, but also indirectly by influencing DC and shaping adaptive immune responses.
Neutrophils are also capable of contributing to adaptive immunity by enhancing antigen presentation by DC [92]. Neutrophils responding to infectious stimuli undergo the process of apoptosis and can be phagocytosed by DC capable of cross-presentation. This ultimately results in the neutrophil-derived antigens presented by DC to CD8+ T cells [92-94]. The ability of DC to present the neutrophil-derived antigens to CD8+ T cells is especially important in the context of Listeria infection as the primary clearance mechanism of protective immunity is mediated by CD8+ T cells [95-97]. Thus, the enhanced neutrophil populations present in the absence of type I IFN signaling observed in this thesis work may contribute to the development of Listeria specific CD8+ T cell responses through increased levels of cross presentation and is the subject of future studies.

The yin and yang of type I IFN- a balance between help and harm

In the context of a Listeria infection, type I IFN signaling negatively regulates the innate immune response [53, 57, 58]. However, infection with other pathogens, in particular, viruses requires type I IFN signaling for clearance [76, 98, 99]. This dichotomy of favorable vs. harmful can also be observed when looking at other immune phenomena such as autoimmune responses. Type I IFN has been shown to be critically involved in the pathogenesis of systemic lupus erythematosus (SLE), an autoimmune disorder characterized by autoantibody production [100-103]. SLE studies using mouse models are currently targeting type I IFN and this therapeutic approach has shown promising results while also causing less side effects than traditional immunosuppressive
therapies [101]. The rationale behind targeting type I IFN in this disease is due to the ability of this cytokine to act as an immune activator that promotes DC maturation, activates T cells, and stimulates the production of antibodies [101, 104, 105]. Thus, type I IFNs have a yin and yang effect on various aspects of the immune response with a delicate balance between helpful and harmful depending on the type of pathogenic insult or response to self.

**Susceptibility to bacterial infections secondary to viral infection**

It has been appreciated for some time that viral infection predisposes patients to secondary bacterial infection, which often times has fatal outcomes [106-111]. For example, patients infected with HIV are susceptible to bacterial pneumonia secondary to the viral infection. These secondary bacterial infections have been shown to increase the mortality of HIV patients, with pneumonia currently the leading secondary cause of death among persons dying of HIV infection [107, 109, 110, 112]. As a result, the lung is the most frequent organ involved in AIDS-associated diseases [113], however HIV-infected individuals also have an increased incidence of gastroenteritis, urinary tract infections, and sepsis [108, 114-116]. While HIV patients have altered cell mediated immunity, they have an increased risk of bacterial infections due to altered neutrophil function [108], with defects observed on chemotaxis, phagocytic capacity, and bacterial killing [116-118]. However, treatment of HIV patients with the cytokine G-CSF was able to reverse the viral induced neutrophil dysfunction, and has the potential to become helpful in the prevention and treatment of secondary infections [118]. In our studies, we have determined that type I IFN signaling
negatively regulates the expression of CXC chemokines involved in neutrophil recruitment. Therefore, altering chemokine expression through the addition of exogenous CXC chemokines would lead to enhanced neutrophil recruitment and may help overcome the neutrophil dysfunction and contribute to bacterial pathogen clearance.

While influenza infection can be lethal, a large number of deaths result from bacterial pneumonia secondary to the influenza infection especially in populations of very young or elderly individuals [119]. Bacterial superinfections following viral infection occur in response to many different viruses however the role of viruses in initiating secondary infections has been most comprehensively studied using influenza [108]. It has been appreciated for some time that viral infections predispose hosts to bacterial infection. For example, a study published in 1982 wanted to determine if IFN production played a role in otitis media infections. Middle ear fluid was collected from pediatric patients and cultured. Bacteria were isolated from 70% of the specimens and IFN was detected in most of those specimens with virus detected in only 5% of samples [120]. This study established a link between IFN, a byproduct of viral infection, and bacterial burdens. Numerous studies have used models investigating the role of primary viral infections on the exacerbation of secondary bacterial infections either in animal models directly infecting with bacteria or studying human patients that developed secondary pneumonia following viral infection. Suffice it to say that it is very well established that secondary bacterial pneumonia occurs and a major contributing factor is neutrophil dysfunction with abnormalities observed in
chemotaxis, phagocytosis, oxidative activity, and bactericidal activity depending on the study [106, 108, 121-123].

Recently, a study assessed the contribution of viral infection on the development of secondary pneumococcal pneumonia. Influenza infected IFNAR-/- mice exhibited improved survival and clearance of secondary *Streptococcus pneumoniae* infection in the lungs and blood compared to WT controls [124]. The authors attributed the weaker response observed in WT animals to impaired production of the neutrophil chemoattractants CXCL1 and CXCL2 after secondary bacterial challenge which resulted in insufficient neutrophil recruitment [124]. Exogenous CXCL1 and CXCL2 given to WT mice enhanced the neutrophil response and resulted in bacterial burdens that were similar to those recovered from IFNAR-/- mice. Conversely, when CXCR2, the receptor for CXCL1 and CXCL2, was blocked, the protective phenotype observed in IFNAR-/- mice to secondary pneumococcal infection was reversed [124]. These data suggest that type I IFN signaling negatively regulates CXC chemokine expression, which is important for neutrophil recruitment and clearance of secondary bacterial pneumonia. Studies from our lab have also demonstrated enhanced production of CXCL1 and CXCL2 leading to enhanced neutrophil recruitment in response to *Listeria* in the absence of type I IFN signaling that has a dramatic effect on bacterial burdens (Chapter 3, Figure 20).

Secondary bacterial infections occur following a number of primary viral infections including but not limited to: influenza virus, respiratory syncytial virus (RSV), hepatitis virus, human immunodeficiency virus (HIV), and
cytomegalovirus (CMV) [108]. Since susceptibility to bacterial infection is
induced by many distinct viruses, it suggests that a common host response to
virus (likely the type I IFN response) confers this susceptibility. Our data has
shown that type I IFN negatively regulates CXC chemokine production and
neutrophil recruitment upon Lm infection. Since Listeria is capable of entering
the host cell cytoplasm, this bacterial pathogen arguably behaves like a virus by
interacting with cytosolic receptors and inducing the production of large amounts
of type I IFN, leading to the transcription of IFN-inducible genes [36, 45]. Several
studies have demonstrated that treatment with G-CSF and/or GM-CSF can
reverse the virus-induced neutrophil dysfunction and thus makes these cytokines
potential therapeutic agents [118, 125, 126]. While we did not directly quantitate
levels of G-CSF or GM-CSF, a global survey of cytokine/chemokine expression
in our model system indicated that expression of these cytokines was enhanced
in the absence of type I IFN signaling. Thus, I propose that inhibition of the type I
interferon response offers promise as treatment for secondary bacterial
pneumonia following viral infection. This could be achieved through treatment
with blocking antibodies to type I IFN.

Further demonstration for a role of cytokine modulation can be found in a
study where mice pretreated with virus exhibited increased bacterial burdens and
augmented IL-10 compared to controls with bacterial pneumonia attributed to
increased IL-10 production and reduced neutrophil presence in the lung [111].
Therefore, future studies assessing the expression of cytokines and chemokines
in the absence of type I IFN signaling are warranted as altered
cytokine/chemokine profiles may contribute to or prevent not only the neutrophil dysfunction observed following viral infections, but may also impact the other cell types recruited to the lung following infection that may play a role in fighting the bacterial insult.

In summary, the work presented in this thesis has implications on the generation of vaccines based on the contribution of various components involved in DC maturation that ultimately leads to naïve T cell priming. Mechanisms resulting in enhanced neutrophil recruitment may also contribute to the field of vaccine development due to the ability of neutrophils to augment immune responses through interactions with DC. Finally, the negative regulation of the immune response observed by type I IFN may lead to the discovery of cytokines, chemokines or other cell-derived factors that may be targeted in a manner that helps resolve the public heath concern of secondary bacterial pneumonia.
References


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