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The IL-23/IL-17 axis is detrimental during autoimmune disorders, but is protective against certain pathogens, particularly extracellular bacteria. We have previously shown that IL-23 is required for host resistance against *Listeria monocytogenes* (LM) and for neutrophil recruitment to the liver, but not the spleen, during infection. Despite efficient neutrophil recruitment to the spleen, IL-23p19 knockout (KO) mice have an increased bacterial burden in this organ compared to C57BL/6 (B6) mice. Interestingly, specific depletion of neutrophils abrogated the differences in bacterial burden in the liver, but not the spleen of B6 and IL-23p19 KO mice, suggesting that IL-23 may regulate the recruitment/function of another cell type in the spleen. Accordingly, LM-infected IL-23p19 KO mice had fewer inflammatory monocytes in the spleen as well as a reduction in monocyte-recruiting chemokines compared to B6 mice. Therefore, IL-23 is necessary for the optimal recruitment of inflammatory monocytes to the spleen during LM infection. Phagocytic cells express receptors for IL-23 and IL-17A suggesting that the activity of these cells could be regulated by IL-23 or IL-17A. However, it is not known whether IL-23 could impact the function of phagocytic cells during LM infection. Inflammatory monocytes, neutrophils, and macrophages from B6 and IL-23p19 KO mice displayed equivalent phagocytic potential. Although exogenous stimulation with IL-23

and IL-6 increased the production of ROS from B6 neutrophils, the absence of IL-23 did not impact the ability of inflammatory monocytes and neutrophils to make ROS during LM infection. Additionally, the expression of myeloperoxidase (MPO), inducible nitric oxide (iNOS), and TNF-α by inflammatory monocytes, neutrophils, and peritoneal macrophages was not altered by the lack of IL-23 during LM infection. Therefore, IL-23 is not required for the optimal function of phagocytic cells during LM infection. The pro-inflammatory mediators, TNF-α and $NO^{\cdot}$, are essential for protection against LM. Surprisingly, there was a reduction in the overall levels of TNF-α and $NO^{\cdot}$ in the spleen of IL-23p19 KO mice compared to B6 mice during LM infection. However, exogenous stimulation with IL-23 or IL-17A did not induce or enhance the production of these pro-inflammatory mediators from splenocytes isolated from LM-infected B6 mice. Interestingly, the reduction in overall production of TNF-α and $NO^{\cdot}$ in the spleens of LM-infected IL-23p19 KO mice, corresponds with reduced numbers and percentages of TNF-α and iNOS-expressing monocytes. This deficient recruitment of inflammatory monocytes resulted in decreased production of monocyte-derived TNF-α and $NO^{\cdot}$, leading to increased bacterial burdens in the spleens of LM-infected IL-23p19 KO mice. Collectively, our data reveal that the enhanced susceptibility of IL-23p19 KO mice is not due to functional impairment of phagocytic cells, instead it is caused by the inefficient recruitment of neutrophils to the liver, and inflammatory monocytes to the spleen, during LM infection.

THE ROLE OF IL-23 IN REGULATING THE FUNCTION AND RECRUITMENT OF PHAGOCYTIC CELLS DURING *LISTERIA MONOCYTOGENES* INFECTION

DISSERTATION

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TABLE OF CONTENTS

Page

CHAPTER

CHAPTER

LIST OF FIGURES

LIST OF ABBREVIATIONS

Name	Abbreviation
Analysis of variance	ANOVA
Antigen-presenting cell	APC
Brain-heart fusion	BHI
C57Bl/6	B6
Colony-forming unit	CFU
C-type lectin receptor	CLR
Dendritic cell	DC
Dulbecco's modified eagle medium	DMEM
Enzyme-linked immunosorbent assay	ELISA
Extracellular superoxide dismutase	ecSOD
Fetal bovine serum	FBS
Granulocyte-colony stimulating factor	G-CSF
Green fluorescent protein	GFP
Granulocyte/macrophage-colony stimulating factor	GM-CSF
Hank's balanced salt solution	HBSS
Heat-killed *Listeria monocytogenes*	HKLM
Hydroethidine	HE
Inducible nitric oxide synthase	INOS
Interferon-gamma	IFN-γ
Interleukin	IL
intraperitoneal	i.p.
Intravenous	i.v.
Listeria monocytogenes	LM
Mean fluorescence intensity	MFI
Myeloid-derived suppressor cell	MDSC

Myeloperoxidase	MPO
Natural Killer cell	NK
Neutrophil extracellular trap	NET
Nicotinamide adenine dinucleotide phosphate	NADPH
Nucleotide oligermerization domain	NOD
Pattern recognition receptor	PRR
Phosphate-buffered saline	PBS
Polymerase chain reaction	PCR
Post-infection	p.i.
Reactive nitrogen species	RNS
Reactive oxygen species	ROS
Real-time polymerase chain reaction	RT-PCR
Red blood cell	RBC
Retinoic acid-inducible gene-like I receptor	RLR
Regulatory T cell	Treg
Severe combined immunodeficiency	SCID
Toll-like receptor	TLR
Tumor necrosis factor	TNF

CHAPTER I

INTRODUCTION

Specific Aims

Interleukin-23 (IL-23) is a pro-inflammatory cytokine that is secreted by activated dendritic cells and macrophages. IL-23 is required for the generation and maintenance of IL-17-producing T cells. The recruitment of immune cells via the IL-23/IL-17 axis has been shown to be important for defense against several pathogens, but detrimental during autoimmune disorders. We have previously demonstrated that IL-23 is required for resistance against *Listeria monocytogenes* infection, and for bacterial clearance from both the spleen and liver. Importantly, IL-23 is required for the optimal recruitment of neutrophils to the liver during *Listeria monocytogenes* infection. Interestingly, the recruitment of neutrophils to the spleen of mice lacking IL-23 was unaltered compared to C57Bl/6 (B6) mice. However, there was an increased bacterial burden in this organ compared to B6 mice. This suggested that IL-23 regulated the recruitment and function of another immune cell in the spleen during *Listeria monocytogenes* infection. Therefore, we hypothesize that IL-23 enhances the recruitment and function of phagocytic cells during *Listeria monocytogenes* infection.

Specific Aim 1: Does IL-23 mediate the recruitment of inflammatory monocytes during *Listeria monocytogenes* (LM) infection?

We have previously demonstrated, by utilizing mice lacking IL-23 (IL-23p19 KO), that IL-23 is required for resistance against LM, and for promoting bacterial clearance from the spleen and liver. Additionally, IL-23p19 KO mice had reduced recruitment of neutrophils to the liver compared to B6 mice. First, we will determine if this reduced neutrophil recruitment to liver of IL-23p19 KO mice is causal for increased bacterial burden in this organ. This will be determined by monitoring survival and bacterial burdens from neutrophil-depleted B6 and IL-23p19 KO mice. Studies have indicated that inflammatory monocytes are the predominant mediators of innate anti-listerial defense in the spleen. We will determine whether the absence of IL-23 impacts the recruitment of inflammatory monocytes to the spleen during LM infection. Flow cytometric analysis will be performed to determine the percentages of inflammatory monocytes in the spleen, liver, and blood of IL-23p19 KO mice in comparison to control B6 mice, under homeostatic and infected conditions. Next, we will measure the levels of inflammatory monocyte recruiting chemokines, CCL2 and CCL7, in the serum, organ homogenates, and overnight cell culture supernatants. Finally, the requirement of IL-23 for the generation and maintenance of inflammatory monocytes in the bone marrow will be determined.

Specific Aim 2: Does IL-23 directly or indirectly enhance the function of phagocytic cells during LM infection?

The receptors for IL-23 (IL-23R) and IL-17A (IL-17RA) are expressed by inflammatory monocytes, neutrophils, and macrophages suggesting that the function of these cells can be modulated by IL-23. Therefore, we will determine whether the absence of IL-23 impacts the function of phagocytic cells including inflammatory monocytes, neutrophils, and peritoneal macrophages during LM infection. First, an *in vitro* phagocytosis assay will be performed on inflammatory monocytes, neutrophils, and peritoneal macrophages from B6 and IL-23p19 KO mice. Next, the production of pro-inflammatory mediators from inflammatory monocytes, neutrophils, and peritoneal macrophages isolated from B6 and IL-23p19 KO mice will be determined. Flow cytometry will be utilized to measure the following pro-inflammatory mediators: TNF-α, reactive oxygen species (ROS), inducible nitric oxide synthase (iNOS), and myeloperoxidase (MPO). Additionally, the effect of exogenous stimulation of cells with IL-23 and IL-17A on the production of TNF-α and nitric oxide ($NO^{\cdot}$) will be assayed. Finally, an RT-PCR array will be performed on spleen to determine how the absence of IL-23 impacts the expression of genes associated with the innate immune response during LM infection.

Listeria monocytogenes

Listeria monocytogenes (LM) is a Gram-positive ubiquitous soil bacterium that is a common contaminant of meat and dairy products. The risk of severe infection with LM is high among immunocompromised individuals including the elderly, pregnant women, fetuses, and newborns. Clinical manifestations of LM infection in susceptible individuals are sepsis, meningitis, and abortion of fetus in pregnant women (1). LM has the highest mortality rate at approximately 30% among food-borne infections (2). LM, classified as a BSL-2 pathogen, is widely used as a model organism to study host-pathogen interactions. Therefore, significant research on LM has aided in a better understanding of the pathogenicity of LM, and the nature of the host biological response against LM.

The natural route of infection by LM is via the gastrointestinal tract. Upon ingestion of contaminated food, LM utilizes its protein, internalin A, to bind to E-cadherin expressed on the host epithelial cell, and this interaction facilitates the internalization of LM by receptor-mediated endocytosis. LM disrupts the vacuolar membrane by secreting two proteins: phospholipase C (PLC) and the hemolysin, listeriolysin O (LLO), thus allowing the pathogen to escape into the cytosol. LM adapts to the host cytosolic environment by producing the proteins including hexose phosphate transporter and actin polymerizing protein (ActA). ActA is structurally similar to host wiskott-aldrich syndrome protein, and therefore, it interacts with host cell actin-related protein 2/3 (Arp2/3) to propel LM to the neighboring cells (2). In this fashion, LM spreads from cell-to-cell, while avoiding exposure to extracellular milieu. The transcytosis of LM from the epithelial cell to the lamina propia is thought to cause the systemic dissemination of LM (3). The lamina propia is drained by the mesenteric lymph nodes that empty into the thoracic duct. From the thoracic duct, LM disseminates into the bloodstream, and colonizes its primary target organs, the

spleen and liver. It is important to note that in mice, due to a single amino acid variation in E-cadherin, LM cannot bind effectively to this host protein. Therefore, the oral route of LM infection in mice does not result in a reproducible, consistent infection, nor does it induce a robust immune response (4). To circumvent this issue, in mouse models, LM infection is established by intravenous (i.v.) or intraperitoneal (i.p.) injection of LM. The i.v. and i.p. models of LM infection in mice are widely used to study the nature of the immune response induced by LM *in vivo*.

The pathogenicity of LM is dependent on the expression of various virulence factors including LLO, ActA, and PLC. It has been demonstrated that mutants of LM lacking these virulence factors cannot effectively establish infection in mice (5). The intensity of infection established by LM is dependent on two factors: the virulence of LM and the effectiveness of the host immune response. Studies in mice with a sub-lethal dose of LM have demonstrated that bacterial load in the spleen and liver peaks 2-3 days post-infection. However, within 10-14 days LM is cleared due to the generation of both innate and adaptive immune responses (2). The fact that LM infection can be fatal in individuals with a compromised immune system demonstrates the requirement of a functional immune system, in order to provide protection against pathogenic microorganisms.

Recognition of LM by the immune system

The first step in initiating an immune response is the recognition of the potential pathogen by both immune and non-immune cells. This pathogen recognition is accomplished by evolutionary conserved pattern recognition receptors (PRR) that bind to different pathogen associated molecular patterns (PAMPs). PRRs have been classified based on their location and

ligand specificity into several types including Toll-like receptors (TLR), Nucleotide oligomerization domain (NOD), NOD-like receptors (NLR), Retinoic acid-inducible gene-(RIG)-like I receptors (RLR), and C-type lectin receptors (CLR) (6). Generally, the ligation of a PRR triggers a cascade of signaling pathways that result in the production of pro-inflammatory mediators. LM-derived products including flagellin, peptidoglycan, lipotechoic acids, and DNA initiate signaling via different PRRs. For instance, flagellin is recognized by TLR5, CpG DNA motifs are recognized by TLR9, and peptidoglycan and lipotechoic acids are recognized by TLR2. Signaling via TLRs can be mediated by either MyD88-dependent or independent mechanisms. Absence of MyD88 renders mice highly susceptible to LM infection indicating signaling via TLRs is critical for resistance against LM (7). However, mice deficient in TLR2 are resistant or moderately susceptible to LM infection suggesting other non-TLR2 dependent mechanism of immune recognition of LM must exist (7). NOD1 and NOD2 are cytosolic sensors that recognize components of peptidoglycan present in LM. Another set of cytosolic PRRs trigger the assembly of a multicomponent complex, the inflammasome, which is required for the maturation and secretion of pro-inflammatory cytokines, IL-1 and IL-18 (8). LM infection triggers the activation of inflammasomes, NLRC4 and AIM2, that sense peptidoglycan and DNA, respectively (9). Thus, synergistic and redundant mechanisms of pathogen recognition aid in the generation of an effective immune response.

Innate immune response against LM

Macrophages

Macrophages are derived from common monocyte/dendritic cell precursors, and have been identified based on the expression of cell surface markers, F4/80 and CD11b. Macrophages

are very effective phagocytic and antigen presenting cells. Based on the phenotype, two distinct subsets of macrophages have been identified in the mouse peritoneal compartment: large peritoneal macrophages ($F4/80^{hi}CD11b^{hi}$), and small peritoneal macrophages ($F4/80^{lo}CD11b^{lo}$). These macrophage subsets differ in their expression of surface markers including, Ly6C and MHC Class II as well as in their ability to respond to LPS stimulation, suggesting that these macrophages are functionally distinct (10). However, little is known about how infection or other types of stimuli modulate the differentiation of these subsets of macrophages in the peritoneal cavity. Based on their function, macrophages have been classified into two types: classically activated (M1) and alternatively activated (M2) macrophages. M1 macrophages produce pro-inflammatory mediators including IFN-γ, TNF-α, reactive oxygen and nitrogen species (ROS/RNS), and therefore, are effective at providing protection against microbes. M2 macrophages display an anti-inflammatory phenotype by producing IL-10 and arginase, and are required for resolving inflammation and promoting tissue repair (11, 12). Because of their ability to undergo extensive oxidative burst upon activation, M1 macrophages are critical for defense against intracellular pathogens such as LM.

The early containment of LM in the bloodstream is imperative to prevent bacterial dissemination. Depletion of both marginal zone macrophages in the spleen and kupffer cells in the liver increases the susceptibility of mice to LM infection (13). Studies have reported that selective depletion of splenic marginal zone macrophages (MZM) resulted in the spreading of LM to the peripheral organs, including lungs and kidneys (14). The absence of kupffer cells renders hepatocytes more permissive for LM replication, and ultimately, these cells become apoptotic. Macrophages activated with IFN-γ undergo extensive oxidative burst, and are able

prevent the escape of LM into the cytosol. Therefore, macrophages are essential for preventing systemic spread of LM.

Monocytes

Monocytes are cells of myeloid lineage, and are precursors for macrophages and dendritic cells (DC). These cells have been classified as inflammatory and resident monocytes based on the level of expression of Ly6C and chemokine receptors, CCR2 and CX_3CR1. The murine inflammatory monocytes identified as $Ly6C^{hi}CCR2^{+}CX_3CR1^{lo}$ resemble the classical human monocytes, $CD14^{hi}CD16^{+}$, and the resident murine monocytes identified as $Ly6C^{lo}CX_3CR1^{hi}CCR2^{-}$ correlate with the non-classical human monocytes, $CD14^{lo}CD16^{-}$. As the name indicates, inflammatory monocytes produce pro-inflammatory mediators and are recruited to sites of infection or injury. It has been suggested that under steady state conditions, $Ly6C^{hi}CX_3CR1^{lo}$, inflammatory monocytes can differentiate into $Ly6C^{lo}CX_3CR1^{hi}$, resident monocytes, but during infection, $Ly6C^{hi}CX_3CR1^{lo}$ are rapidly recruited to the site of infection (15, 16). Inflammatory monocytes have been demonstrated to be essential for protection against infection with multiple pathogens including *Toxoplasma gondii*, *Citrobacter rodentium, Aspergillus fumigatus,* and herpes simplex virus (17). However, inflammatory monocytes have also been implicated as detrimental during inflammatory conditions such as atherosclerosis (18), rheumatoid arthritis (19), and breast cancer (20).

The historic observation that LM infection increases the number of monocytes in circulation led to its name “monocytogenes” (21). The importance of monocytes during LM infection was established by demonstrating that impairing monocyte recruitment to infected sites by genetic deletion of CCR2 (22), the receptor for monocyte-recruiting chemokines, CCL2 and

CCL7 (23), enhanced the severity of LM infection. Cytosolic invasion of LM induces a MyD88-independent recruitment of inflammatory monocytes to the spleen during a systemic infection (24). The continuous recruitment of inflammatory monocytes to the spleen can be separated into two phases. In the initial phase, monocytes pre-existing in the bone marrow are mobilized immediately to the infected tissue and in the second phase, there is an infection-induced robust expansion of monocytes in the bone marrow to sustain the recruitment to the periphery. Additionally, there is a selective expansion of monocytes during LM infection (25).

Chemokines in the circulation are thought to initiate the mobilization of immune cells from the bone marrow to the periphery. Recent studies have demonstrated that localized chemokine production by bone marrow stromal cells can facilitate the mobilization of immune cells, particularly inflammatory monocytes, during infection. CXCL12-activated reticular (CAR) cells present in the bone marrow stroma recognize bacterial ligands and produce the monocyte-recruiting chemokines, CCL2 and CCL7. The concerted action of these two chemokines promotes the emigration of inflammatory monocytes during LM infection (26). Mice lacking CCL2, CCL7, or CCR2 had reduced percentages of monocytes in the periphery due to retention of these cells in the bone marrow, and this subsequently led to increased bacterial burden in the spleen and liver. Interestingly, adoptive transfer experiments with monocytes from CCR2 KO mice demonstrated that trafficking of monocytes is chemokine-independent in the spleen, but ICAM-dependent in the liver (27). This differential requirement for monocyte trafficking to the infected LM foci in the spleen and liver can be attributed to the structural differences between these two organs. A recent study reported that mice deficient in STING/MPYS, an adaptor for type I IFN signaling, displayed normal percentages of inflammatory monocytes in the spleen, but not the liver. In fact, this deficiency in the recruitment of inflammatory monocytes to livers of

STING/MPYS KO mice was cell intrinsic (28). Further research is required to understand this organ-specific regulation of cell recruitment under disease conditions.

The activation of inflammatory monocytes at the site of infection is dependent on MyD88 signaling triggered by cytosolic invasion of LM (24). Upon activation, inflammatory monocytes produce TNF-α and iNOS in the spleen, and have been referred to as "TNF-α/iNOS-producing dendritic cells" (TipDCs) (29). Furthermore, inflammatory monocytes have been shown to activate memory CD8 T cells and NK cells by producing IL-18 and IL-15 (30). It has been reported that early IFN-γ from NK cells promotes the differentiation of inflammatory monocytes during LM infection. It was thought that innate immune cells such as monocytes might not play much of a role during the adaptive phase of the immune response. Notably, inflammatory monocytes also contribute during secondary LM infection. It has been shown that CCL3-derived from memory CD8 T cells activates inflammatory monocytes to undergo extensive oxidative burst to kill LM (31). Therefore, inflammatory monocytes mediate anti-listerial defense during both innate and adaptive phases of the immune response.

Neutrophils

Neutrophils are granulocytic cells derived from the myeloid lineage that are identified based on the expression of Ly6G. Neutrophils are rapidly recruited from the circulation in response to infection or injury. These cells contain granules that produce anti-microbial molecules including cationic antimicrobial peptides and myeloperoxidase. Additionally, they produce pro-inflammatory mediators, undergo oxidative burst and produce neutrophil extracellular traps to clear pathogens. Neutrophils can also display an anti-inflammatory phenotype and promote tissue repair (32).

The correlation between the number of neutrophils in infected foci and resistance against LM infection hinted that neutrophils were involved in providing innate protection. Studies have proposed that neutrophils promote clearance of LM by lysing LM-infected hepatocytes (33), or by engulfing apoptotic hepatocytes (34). It has been suggested that neutrophils could act as antigen presenting cells and prime CD8 T cells during LM infection (35). However, these studies depleted neutrophils by utilizing a non–specific anti-Gr-1 antibody, which depleted multiple cell types including neutrophils, monocytes, memory CD8 T cells, and plasmacytoid DCs (36). Our laboratory re-examined the role of neutrophils by performing depletions with the highly specific anti-Ly6G antibody. Contrary to the other study, the depletion of neutrophils did not impact the priming of CD8 T cells during LM infection. Comparison of survival outcomes of LM-infected mice that were depleted with anti-Ly6G antibody indicated that neutrophil-depleted mice were highly susceptible to a high dose LM infection compared to isotype-treated B6 mice. Importantly, clearance of bacteria from the liver requires neutrophils at all doses of LM infection tested (36). However, only a high infectious dose of LM necessitated neutrophil-mediated bacterial clearance from the spleen. This suggests that other immune cells might contribute to the immune response in the spleen during LM infection.

Natural Killer cells

Natural killer (NK) cells are large granular lymphocytes derived from the common lymphoid progenitor. NK cells express various inhibitory and activating receptors to recognize healthy versus, infected or tumorigenic cells, respectively. They produce cytotoxic granules containing granzymes and perforin to lyse target cells. In some diseases, NK cells are a

predominant source of TNF-α and IFN-γ. These cells are required for anti-tumor and anti-viral activity (37).

The activation of NK cells during LM infection requires contact with infected DCs and IL-18 (38). Since NK cells are the major producers of IFN-γ, it is believed that these cells could be important during LM infection. However, depletion of NK cells improved the clearance of LM (39). Additionally, NK cell deficient mice had normal IFN-γ levels, and survived sub-lethal LM infection (40). Recent reports have indicated that high levels of IFN-γ from $CD27^+$ NK cells reduce the mobilization of $CXCR2^+$granulocytes to LM-infected organs, thereby exacerbating the outcome of LM infection (41). Thus, the contribution of NK cells towards resistance against LM is debatable.

γδ T cells

Unconventional γδ T cells express TCRs containing gamma and delta chains, and do not express CD4 or CD8 on their surface. γδ T cells recognize a wide range of microbial products including lipids, and produce cytolytic molecules, such as granzyme and Fas ligand. During LM infection, γδ T cells in the spleen and liver produce IL-17A in an IL-23-dependent fashion, and promote neutrophil mobilization to these organs (42, 43).

Dendritic cells

Dendritic cells (DC) are derived from myeloid or lymphoid precursors. Activated DCs that have captured antigen in the tissue migrate to the nearest lymph node to present antigen to naïve T cells. In addition, activated DCs produce inflammatory mediators including IL-12, IL-

23, IL-10, IL-6, or TGF-β to promote the differentiation of naïve T cells into different subsets (44). CD8α^{+} DCs, a subset of DCs present in spleen, facilitate the uptake of LM, and transport live LM from the marginal zone to the T cell areas (45). Depletion of DCs resulted in a reduction in the CD8 T cell response, establishing that these cells are required for priming LM-specific CD8 T cells (46). Additionally, infected DCs can also activate NK cells in a contact-dependent fashion (38). This demonstrates that DCs can prime cells belonging to both the innate and adaptive immune system during LM infection.

Adaptive immune response against LM

CD4 T cells

CD4 T cells are derived from the common lymphoid progenitor. These cells express the αβ TCR, and are restricted to MHC Class II molecules. Naïve CD4 T cells are activated by antigen presenting cells. Based on their cytokine profile, these cells have been classified into different subsets such as Th1, Th2, Th17, and Tregs (47). During a primary LM infection, macrophage derived IL-12 induces naïve CD4 T cells to differentiate into IFN-γ producing Th1 cells (48). One of the major functions of CD4 T cells is to provide help for activating CD8 T cells. Although, the depletion of CD4 T cells did not impact the generation of effector CD8 T cell response, there was a reduction in the development of memory CD8 T cell response (49).

CD8 T cells

CD8 T cells are generated from the common lymphoid progenitor, and are MHC Class I restricted lymphocytes. These cells contain cytotoxic granules containing granzyme and perforin.

CD8 T cells produce TNF-α and IFN-γ in response to antigenic stimulation, and are required for defense against intracellular pathogens and tumors (50).

Comparison of LM infection outcomes in mice lacking either CD4 or CD8 T cells revealed that mice lacking CD8 T cells displayed increased LM burden during primary and secondary infections compared to B6 mice (51). During a primary LM infection, MHC class Ib restricted CD8 T cells responding to N-formylated peptides expand rapidly. However, upon reinfection, MHC class Ia restricted CD8 T cells specific for other LM derived proteins such as LLO are the major responders. Memory CD8 T cells can also respond in an antigen-independent manner by producing IFN-γ upon stimulation with IL-12 and IL-18, and they can protect against LM infection (52, 53). Thus, distinct populations of CD8 T cells could be differentially activated during LM infection. Antigen–specific effector CD8 T cells deficient in TNF-α and IFN-γ production were able to provide protection against LM infection, suggesting that CD8 T cells are not a critical source for these cytokines (54, 55). Interestingly, transfer of CCL3-deficient effector CD8 T cells to naïve mice resulted in reduced bacterial clearance during LM infection, indicating CD8 T cell derived-CCL3 is essential for protective immunity (56). Follow-up studies demonstrated that CCL3 mediated this protection by enhancing the recruitment and activation of mononuclear phagocytes and neutrophils (57). Thus, even during the adaptive phase of immune response, the innate immune cells, including neutrophils and monocytes, are required for conferring protection.

Pro-inflammatory mediators

Reactive oxygen and nitrogen species

Reactive oxygen and nitrogen species (ROS/RNS) are potent anti-microbial agents. The phagocyte oxidase complex in an inactive state consists of the membrane components, ($gp91^{phox}$ and $p22^{phox}$), and a set of cytosolic components, ($p47^{phox}$, $p67^{phox}$, $p40^{phox}$, and Rac2). Phagocytosis triggers the association of cytosolic components with membrane components to form the active subunit of NADPH oxidase (58). Phagocytosis is the process by which cells, including macrophages and neutrophils, engulf and eliminate invading pathogens. In the first step of phagocytosis, the antigen is engulfed in a membrane bound vesicle called a phagosome. Following which the lysosome containing hydrolytic enzymes fuses with the phagosome to form a phagolysosome, where the pH drops to acidic, and the lytic contents of the lysosome are emptied into the phagosome. This combination of acidic pH and lytic enzymes is toxic to the microbe contained within the phagosome. The maturation of the phagosome occurs in a sequential process, and this event is followed by the production of reactive oxygen species (59). The NADPH complex subunits assemble at the surface of the phagolysosomal membrane and generate superoxide in the lumen. Superoxide is converted into different reactive molecules by various enzymes. The enzyme extracellular superoxide dismutase (ecSOD) converts superoxide into hydrogen peroxide that is further converted into hypochlorous acid by MPO. Another molecule, $NO^{\cdot}$ that is generated by inducible nitric oxide synthase iNOS, combines with superoxide to form peroxynitrite, which inactivates proteins by nitroslyating tyrosine residues (60). Thus, oxidative burst in phagocytes results in the production of multiple oxidative molecules including superoxide, hydrogen peroxide, $NO^{\cdot}$, and hypocholorous acid that are collectively referred to as ROS/RNS.

Oxidative burst is one of the main mechanisms for killing intracellular bacteria. Macrophages, inflammatory monocytes, and neutrophils are the major source of ROS/RNS during LM infection. IFN-γ enhances anti-listerial activity of macrophages by augmenting their oxidative burst. Mice lacking the $p47^{phox}$ subunit of NADPH oxidase or iNOS are susceptible to LM infection (61). In contrast, a recent study reported that in TLR2-stimulated macrophages, $NO^{\cdot}$ facilitated the cell-to-cell spread of LM (62). This suggests that $NO^{\cdot}$ could also be harmful to the host. Our lab has demonstrated that ecSOD activity is detrimental for host survival during LM infection. EcSOD activity increased the recruitment of neutrophils to the liver; however, these cells produced less TNF-*α* and superoxide (63). These studies indicate that ROS/RNS can be both beneficial and detrimental to the host during bacterial infections.

Interferon α and β

Interferon (IFN)-α and β belong to the type I family of IFNs that can be secreted by both non-immune cells and immune cells during bacterial and viral infections. Historically, these cytokines were associated with viral infections; however, studies have demonstrated that these cytokines are involved in non-viral infections as well. Signaling via the type I IFNAR inhibits proliferation of cells, and induces expression of multiple interferon inducible genes associated with apoptosis (64). Type I IFN response seems to be absolutely required for anti-viral immunity, in contrast to bacterial infections where it can be detrimental to the host (65).

The production of type I IFN requires cytosolic recognition of LM, and occurs via a NOD and TLR-independent mechanism mediated by cytosolic DNA sensors. Recent reports have identified that Lys-M expressing cells belonging to the monocyte /macrophage lineage, but not plasmacytoid DCs, are the major producers of type I IFNs (66). Type I IFNs induce the

apoptosis of macrophages and T cells during LM infection. Additionally, these cytokines desensitize macrophages to IFN-γ by down-regulating its receptor. In addition, mice lacking the receptor for type I interferons display reduced susceptibility, and are able to effectively clear LM infection (67). Therefore, type I IFN response is detrimental to host survival during LM infection.

IFN- γ

IFN-γ is a type II interferon that is secreted by Th1 cells, macrophages, DCs, NK cells, and CD8 T cells. Activation with IFN-γ increases MHC Class I presentation and enhances oxidative burst. IFN-γ is necessary for defense against infections with intracellular pathogens (68).

The early innate IFN-γ response is absolutely essential for the control of LM infection. Neutralization of IFN-γ (69) or knock out of IFN-γ receptor (70), renders mice highly susceptible to LM infection likely due to the fact that IFN-γ activates macrophages to enhance LM killing. Studies utilizing SCID mice that lack T cells and B cells have demonstrated that NK cells are the predominant producers of IFN-γ at early time points (71). However, CD8 T cell derived IFN-γ production plays a predominant role in the clearance of LM (53). In addition, IL-12 and IL-18 can induce the secretion of IFN-γ from memory CD8 T cells in an antigen non-specific fashion (52).

TNF-a

TNF-α is a pro-inflammatory cytokine discovered for its role in inducing death of tumor cells. It is secreted in response to infectious and inflammatory stimuli by macrophages, CD4 T

cells, CD8 T cells, and NK cells. TNF-α has a broad spectrum of functions including activating of endothelial cells, promoting vasodilation, and activating other immune cells. Although TNF-α is an important cytokine for anti-microbial defense, excess levels of TNF-α in the circulation can lead to severe inflammation resulting in septic shock (72).

It has been demonstrated that mice deficient in p55 subunit of TNFR succumb to a very low dose of LM infection (73). Additionally, neutralization of TNF-α decreased the resistance during a secondary LM infection. Therefore, TNF-α provides protection during the innate and adaptive phases of the immune response. The cellular sources of TNF-α during LM infection include macrophages, inflammatory monocytes, neutrophils, and CD8 T cells. By utilizing mice that selectively lack TNF-α gene in neutrophils and macrophages, it has been demonstrated that TNF-α derived from neutrophils and macrophages is critical for resistance against LM (74). Moreover, neutrophil depleted mice displayed a reduction in the overall levels of TNF-α in serum and organ culture supernatants (36). Studies suggest TNF-α can mediate anti-listerial defense by promoting the lysis of infected LM-infected hepatocytes (33), or by enhancing the bactericidal activity of macrophages.

IL-6

IL-6 is a pro-inflammatory cytokine that is produced during the acute phase response. Functions of IL-6 include production of acute phase proteins in the liver, differentiation of B cells, and hypothermia. It plays a role in enhancing the mobilization of neutrophils and macrophages and promotes the differentiation of Th17 cells (75). Mice deficient in IL-6 are highly susceptible to LM infection (76), and exogenous administration of IL-6 increases the resistance to LM infection (77). Studies identified monocytes, macrophages, and T cells as the

source of IL-6. Although, the absence of IL-6 did not impair the functional ability of NK cells, macrophages and T cells, it resulted in neutrophilia (76).

IL-10

IL-10 is a prototypic anti-inflammatory cytokine that dampens the host immune response and promotes the resolution of inflammation. IL-10 is secreted by Tregs, M2 macrophages, and myeloid-derived suppressor cells. IL-10 negatively regulates Th1 responses and suppresses macrophage activation (78). Pathogens induce IL-10 production to evade host immune responses. Macrophages infected with LM produced IL-10, and neutralization of this cytokine led to increased IL-12 production. Furthermore, neutralization of endogenous IL-10 controlled LM burden in the spleen and liver indicating that IL-10 is detrimental during LM (79).

IL-18

IL-18 is a pro-inflammatory cytokine that is produced upon activation of the inflammasome. Active IL-18 is produced upon caspase-1 mediated cleavage of pro-IL-18. Sources of IL-18 include non-immune cells and immune cells including macrophages, DCs, and T cells (80, 81). IL-18 has been shown to be critical for resistance against both primary and secondary LM infections (82). Seminal studies have demonstrated that it acts along with IL-12 to induce antigen non-specific IFN-γ production from NK cells and memory CD8 T cells (52).

IL-12

IL-12 is a pro-inflammatory cytokine produced by activated macrophages and dendritic cells. It is made of two subunits, the common p40 subunit and the IL-12p35 subunit. IL-12 promotes the differentiation of the Th1 subset of T cells (83). LM is a potent stimulator of IL-12 production, and administration of IL-12 increased the resistance of mice to LM infection (84). Additionally, the lack of IL-12 reduced the production of IFN-γ and skewed T helper responses towards the Th2 phenotype (48). IL-12 promotes anti-listerial immunity by inducing the production of IFN-γ, which in turn activates macrophages to kill LM.

IL-17A and IL-17F

IL-17A, commonly referred to as IL-17, and IL-17F are pro-inflammatory cytokines that belong to the IL-17 family of cytokines. Activated cells including Th17 cells, γδ T cells, neutrophils, and macrophages produce IL-17 under inflammatory conditions (85-87). The maintenance of IL-17-producing T cells is dependent upon the pro-inflammatory cytokine, IL-23. The well-characterized function of IL-17 is the induction of secretion of chemokines from epithelial cells, thereby facilitating the recruitment of immune cells to the sites of inflammation (85).

Systemic infection with LM induces the secretion of IL-17A and IL-17F, from the spleen and liver, in an IL-23 dependent fashion. γδ T cells in LM-infected spleen and liver have been identified as the primary source of IL-17A (43). The absence of IL-17A or IL-17RA renders mice susceptible to LM infection suggesting that signaling via IL-17RA is required for protection against LM (42, 43). It has been demonstrated that IL-17 promotes the secretion of

neutrophil-attracting chemokines including GM-CSF and G-CSF during LM infection (88). Furthermore, mice deficient in IL-17RA displayed reduced percentages of neutrophils in the liver during LM infection suggesting that IL-17-mediated neutrophil recruitment is essential for protection against LM in this organ (43).

IL-23

IL-23 is a pro-inflammatory cytokine that belongs to the IL-12 family of cytokines and is made up of two subunits, a common IL-12p40 and a unique p19 subunit. Activated antigen presenting cells, including macrophages and dendritic cells, produce IL-23 in response to immune stimulation. IL-23 is required for the production of IL-22, and the maintenance of IL-17A- and IL-17F-producing T cells. Although, IL-23 and IL-12 belong to the same cytokine family, their functions do not seem to overlap. For example, mice lacking IL-23 were resistant to the development of the autoimmune disorder, experimentally-induced autoimmune encephalitis (EAE), compared to mice lacking IL-12. The immunopathology in the EAE model caused by IL-23 was due to the influx of T cells and macrophages (89). Our lab has demonstrated that IL-23 negatively regulated IL-12–induced IFN-γ production from CD8 T cells and NK cells, further establishing that IL-23 is functionally distinct from IL-12 (90).

The first study that indicated a role for IL-23 in the mobilization of immune cells, demonstrated that the transgenic over-expression of the IL-23p19 subunit resulted in an increase in the number of neutrophils in the circulation (91). In contrast, the complete absence of IL-23 reduced the delayed type hypersensitive response and mice deficient in IL-23 resembled mice lacking IL-17 (92). Ever since, several studies have reported that IL-23, IL-17A, and IL-17F promote the mobilization of neutrophils through the induction of cytokines and chemokines,

including IL-6, G-CSF, GM-CSF, CXCL1, CXCL2, and CXCL8 (85). The prompt recruitment of immune cells to the site of injury or infection is pivotal for eliminating pathogens and protecting the host. The importance of IL-23 in promoting protection has been demonstrated during infection at mucosal surfaces with pathogens including *Klebsiella pneumoniae* (93), *Citrobacter rodentium* (94), and *Mycobacterium tuberculosis* (95). However, very little is known about the role of IL-23 during systemic infection with a Gram-positive, intracellular bacterium such as LM.

The role of IL-23 in regulating the recruitment of immune cells

The mechanism of protection mediated by IL-23 seems to vary with the type of pathogen. The regulation of immune responses by IL-23 depends upon its downstream cytokines, IL-17 and IL-22, and their respective effector functions. IL-23 is required for promoting immunity against chronic infection with *Mycobacterium tuberculosis*, which is an acid-fast, intracellular bacterium that colonizes the lung. In this model, IL-23 via IL-17A and IL-22, induced the production of CXCL13 from lung stromal cells. CXCL13 promoted the formation of B cell follicles in the infected lesions enabling better localization of T cells to the granuloma (96). During infection with *Klebsiella pnuemoniae*, a Gram-negative, extracellular bacterium, IL-17A-mediated neutrophil recruitment to the lung was shown to be important for protection (93, 97). In a similar fashion, IL-23 enhanced neutrophil recruitment during infection with a Gram positive, extracellular bacterium, S*treptococcus pneumoniae* (98). In an interesting model of cross-protection, mice infected with *Mycoplasma pneumoniae* during a primary infection were protected against secondary LM infection. This protection was due to the increased recruitment

of neutrophils to the spleen and liver mediated by IL-17 (99). Thus, the IL-23/IL-17 axis is important for mobilizing neutrophils during infections.

IL-23 can induce the production of IL-22 from different cells, including T cells, natural killer cells, and dendritic cells. IL-22 is known to stimulate the production of anti-microbial peptides and to protect tissues from damage. The IL-23/IL-22/anti-microbial peptide axis has been shown to be important during infections with extracellular bacteria such as *Citrobacter rodentium* (94) and *Klebsiella pneumoniae* (100). In these models, IL-23 induced the production of antimicrobial peptides, and was required for protection of the host. Interestingly, during LM infection, IL-23 was required for the production of IL-22; however, mice lacking IL-22 displayed bacterial burden similar to B6 mice. Additionally, serum levels of alanine aminotransferase (ALT), a marker for tissue damage were not increased in mice lacking IL-22 (101). Therefore, the IL-23/IL-22 axis is dispensable for bacterial clearance from the spleen and liver, host survival, and for protecting tissues from damage during LM infection.

By utilizing mice lacking IL-23, our lab has established that IL-23 is required for resistance against LM (43). During LM infection, IL-23p19 KO mice displayed increased bacterial burden in both the spleen and liver compared to B6 mice. Additionally, the absence of IL-23 resulted in reduced production of IL-17A and IL-17F. Importantly, the recruitment of neutrophils was impaired in the liver, but not in the spleen of IL-23p19 KO mice. This indicates that IL-23-dependent production of IL-17A and IL-17F provided protection by mobilizing neutrophils to the liver. Furthermore, injection of IL-17A restored the bacterial clearance from both the spleen and liver of IL-23p19 KO mice, possibly by enhancing the recruitment of neutrophils to the liver, and by an unknown mechanism in the spleen (43). Therefore, it remains

to be investigated how IL-23 mediates the recruitment or function of another immune cell type in the spleen during LM infection.

The role of IL-23 in regulating the function of phagocytic cells

Phagocytic cells including macrophages, dendritic cells, neutrophils and monocytes are necessary for defense against pathogens. Functions of phagocytic cells include pathogen engulfment, production of pro-inflammatory mediators, and pathogen killing. The receptors for IL-23 and IL-17 are expressed by phagocytic cells suggesting that the function of phagocytic cells could be modulated by IL-23 or IL-17 (87, 102). Fig.1 illustrates the multiple ways by which IL-23 could potentially regulate the function of phagocytic cells. In a simple, direct fashion, IL-23 could bind to the IL-23R on a phagocytic cell. In the indirect pathway, IL-23-induced downstream effector molecules, IL-17A and IL-17F or chemokines could activate phagocytic cells via their cognate receptors.

Recent studies in a colitis model have shown that a population of neutrophils produce IL-22 and anti-microbial peptides in an IL-23 dependent manner, and are required maintaining the integrity of colon epithelium (103). In another report, IL-23 in combination with IL-6 enhanced the production of ROS from bone marrow neutrophils stimulated with heat-killed *Aspergillus fumigatus*. Additionally, these neutrophils also produced IL-17A, expressed the receptor for IL-17A, and were activated in an autocrine fashion (86). In mice infected with the fungus, *Cryptococcus neoformans* treatment with IL-23 increased their survival, and lead to enhanced infiltration of DCs and T cells to the sites of infection (104). Additionally, IL-23 treatment increased the expression of CD80 and CD86 in peritoneal macrophages following *Cryptococcus neoformans* infection (105). These studies provide evidence that IL-23 can alter

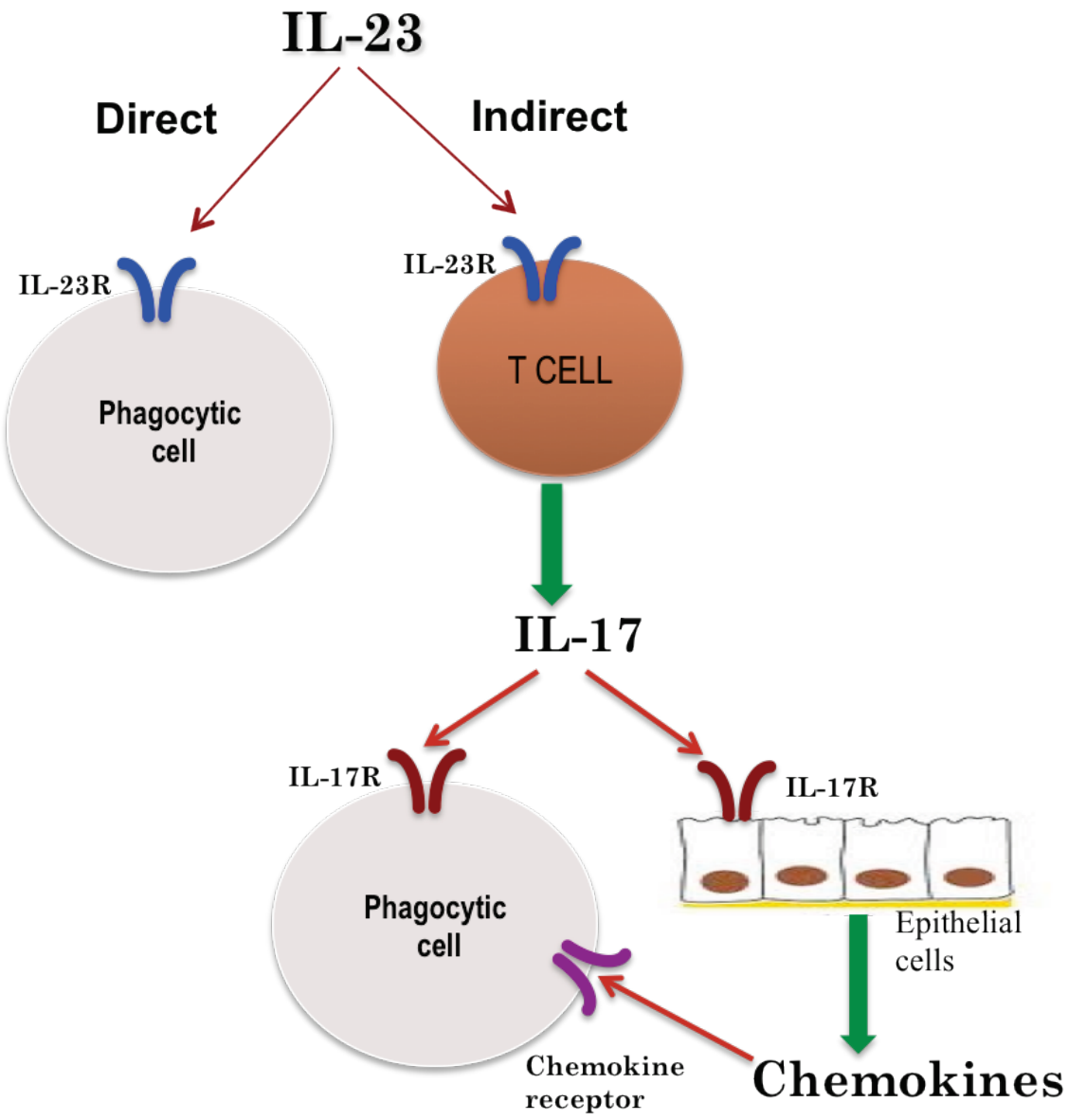


Figure 1: The regulation of functions of phagocytic cells by IL-23

the function of phagocytic cells during infection. The stimulation of macrophages with IL-17A enhanced the capacity of these cells to phagocytose apoptotic neutrophils (106). This increase in phagocytosis was attributed to the increase in the receptor, lectin-like oxidized low-density lipoprotein receptor 1 (LOX-1) on macrophages. Furthermore, IL-17A enhanced the production of IL-3, IL-6, IL-9, and IL-12 from peritoneal macrophages (87, 107) as well as enhanced the killing capacity of macrophages in a *Bordetella pertussis* model (108). In addition to increasing the activation state of macrophages, exogenous priming of neutrophils with IL-17A increased their ability to migrate in response to fMLP stimulation (109). Based on these studies that demonstrate that activation of phagocytes by IL-23 or IL-17A enhances their function, one might speculate phagocytes from IL-23p19 KO mice could be functionally impaired compared to those from B6 mice. Therefore, it needs to be investigated whether IL-23 can modulate the function of phagocytes including inflammatory monocytes, neutrophils, and peritoneal macrophages during LM infection.

Significance

The IL-23/IL-17 axis is beneficial for providing protection against multiple pathogens including LM, but can be detrimental during autoimmune disorders. Multiple therapies target the IL-23/IL-17 axis as a treatment option for autoimmune disorders. However, therapeutically blocking the IL-23/IL-17 axis could render individuals immunocompromised, and make them susceptible to pathogens such as LM. Therefore, it is necessary to study the immune responses regulated by this pathway to design better therapeutics. Inflammatory monocytes protect the host against infections; however, these cells can also promote diseases such as breast cancer and rheumatoid arthritis. It is important to gain an understanding of the mechanisms governing their recruitment and function, to either enhance or inhibit their response based on the disease condition.

CHAPTER II

MATERIALS AND METHODS

Mice

C57Bl/6 (B6) mice were purchased from the National Cancer Institute (NCI). IL-23p19 KO were originally obtained from Dr. Nico Ghilardi and backcrossed on a B6 background for at least 8 generations. IL-23p19 KO mice were generated by replacing the entire coding region for the IL-23p19 gene with a construct expressing enhanced green fluorescent protein (GFP). IL-23p19 deficient mice were fully fertile and displayed normal phenotype similar to their wild-type control B6 mice (92). Gender and age matched (6-12 weeks) mice were used for all the experiments. All the mice were provided food and water *ad libitum*, and were housed in sterile microisolator cages with sterile bedding at the University of North Texas Health Science Center AAALAC accredited animal facility. All animal studies were performed in compliance with the U.S. Department of Health and Human Services Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee at the University of North Texas Health Science Center.

LM infections

LM 10403 serotype 1 was grown on brain-heart infusion (BHI) agar plates (BD Bacto, Sparks, MD), and virulent stocks were maintained by passage through B6 mice. For infections,

log phase cultures of LM were grown in BHI broth, washed twice, and diluted to the required concentration in sterile PBS. The route of infection was i.p. for experiments with peritoneal macrophages and i.v. for all the other experiments. A dose of ~10^4 LM was used for survival studies, day 1 and day 3 p.i experiments. For bacterial burden determination at day 5 p.i., a dose of ~5 X 10^3 LM was used. To enumerate bacterial burden, organ homogenates were resuspended in the desired volume of sterile double-distilled water, serially diluted, and 50μL of each dilution was plated on BHI agar plates. After 24 hours of incubation at 37°C without CO_2, colony-forming units (CFUs) of LM were counted.

Organ processing and tissue culture

For collecting serum, blood collected from the retro-orbital plexus of mice was kept on ice for 8 hours, centrifuged at 18,000 X g for 30 minutes, and the clear, blood-free supernatant was harvested. To isolate peripheral blood leukocytes, blood from the lateral tail vein was collected in Hanks' Balanced Salt Solution (HBSS) containing 2% FCS and heparin, centrifuged at 4000 RPM for 6 minutes, followed by RBC lysis with Tris-Ammonium Chloride (pH 7.2). Splenocytes were isolated by grinding the spleen between two sterile glass slides. A discontinuous density gradient centrifugation was performed to isolate liver leukocytes. Liver homongenate resuspended in 35% percoll was layered on 67.5% percoll, and centrifuged at 600 X g for 20 minutes, and cells at the interface were harvested. Bone marrow cells were isolated by flushing femurs and tibiae with HBSS + 2% FBS, and performing collagenase digestion as previously described (23). RBC lysis of splenocytes, liver leukocytes, and bone marrow cells was performed with Tris-Ammonium Chloride (pH 7.2). Peritoneal macrophages were harvested by washing the peritoneal cavity with 1x PBS, and centrifuging the peritoneal exudate.

For tissue culture, cells were resuspended in phenol-red free DMEM media supplemented with 10% FCS (Atlanta Biologicals, Norcoss, GA), L-glutamine, vitamins, and penicillin/streptomycin (Invitrogen-Gibco, Carlsbad, CA). Splenocytes or liver leukocytes were cultured overnight at 37°C in humidified air containing 5% CO_2 in the presence or absence of heat-killed LM (HKLM) at a multiplicity of infection (MOI) of 50:1. For cytokine titration experiments, cells were cultured with varying concentrations of IL-23 or IL-17A (Biolegend, San Diego, CA) in the presence or absence of HKLM stimulation. For organ homogenates, spleens were homogenized in ice-cold PBS containing 0.01% Triton X-100 (Sigma Aldrich, St.louis, MO), centrifuged at 10,000 X g for 30 minutes, and supernatants were collected for analysis (23). Serum, cell-free overnight culture supernatants, and organ homogenates were stored at -80°C for further analysis.

Neutrophil depletion

Depletions were performed using anti-Ly6G antibody (clone 1A8) (Bio-X-Cell, West Lebanon, NH), which has been previously shown to exclusively deplete neutrophils (36, 110). To deplete neutrophils, mice were injected i.p. with 500μg of anti-Ly6G antibody or 500μg of isotype control antibody one day prior to, and three days post-infection (p.i.). Depletions were confirmed by flow cytometry.

Quantification of TNF-α, CCL2, CCL7 and $NO^{\cdot}$

Serum, cell-free overnight culture supernatants, and organ homogenates were used for these assays. TNF-α ELISAs were performed with antibody pairs and recombinant TNF-α from Ebioscience. To measure chemokine levels, the following ELISA kits were used: CCL2 kit from

BD Biosciences and CCL7 kit from Bender Med-Systems. $NO^{\cdot}$ concentrations in supernatants were determined with a Nitric Oxide Quantitation Kit (Active motif, Carlsbad, CA). Cytokine/chemokine levels were determined by comparison with standard curves. Data were analyzed using a Biotek EL808 spectrophotometer.

Flow cytometry based assays

For cell staining, the following antibodies were used from BD Biosciences (San Diego, CA): anti-CD16/CD32 (2.4G2), anti-Ly6G PE (1A8), anti-CD11b-biotin or PE-Cy7 (M1/70), anti-TNF-α PE-Cy7 (MP6-XT22), streptavidin-PE; Biolegend (San Diego, CA): anti-Ly6C PE or Alexa Fluor 647 (HK1.4), anti-TNF-α Alexa Fluor 647 (MP6-XT22); Caltag Laboratories/Invitrogen (Carlsbad, CA): streptavidin PE-TR; Ebioscience (San Diego, CA): anti-F4/80 PE-Cy7 (BM8), streptavidin–PE; Hycult biotech (Plymouth Meeting, PA) Anti-MPO-biotin (8F4); Santa Cruz Biotechnology: goat-anti-iNOS (M-19); Jackson ImmunoResearch Laboratories (West Grove, PA): goat IgG isotype control, anti-goat IgG-PE; Beckman Coulter (Brea, CA): anti-CD45 ECD (I3/2.3). All data were acquired and analyzed on a Beckman Coulter FC500 flow cytometer.

Cell surface staining

Splenocytes, liver leukocytes, or peritoneal macrophages were incubated with saturating amounts of antibodies, and anti-CD16/32 to block Fc receptors in staining buffer (PBS + 2% FBS + 0.1% sodium azide) for 15 minutes at 4°C. Cells were fixed with either 1% Paraformaldehyde or BD Stabilizing fixative. Cell populations of interest were identified based on their expression of cell surface markers as follows: inflammatory monocytes ($Ly6C^{hi}CDllb^{int}$), neutrophils ($Ly6G^{+}CD11b^{+}$ or $Ly6C^{int}CD11b^{hi}$), and macrophages ($F4/80^{+}CD11b^{+}$).

Intracellular staining for TNF- α, iNOS and MPO

To detect intracellular TNF-α, cells were cultured with HKLM in the presence of GolgiPlug containing brefeldin A (BD Pharmingen) for 3 hours. Splenocytes, liver leukocytes, or peritoneal macrophages were stained for surface markers, and then fixed and permeabilized for 20 minutes at 4°C with Cytofix/Cytoperm kit (BD Biosciences). After washing with permeabilization buffer, splenocytes, liver leukocytes or peritoneal macrophages, were stained with saturating amounts of anti-TNF-α for 15 minutes at 4°C. For iNOS staining, cells were stained for surface markers, fixed and permeabilized, and incubated with saturating amounts of goat anti-iNOS or goat isotype control antibody, followed by anti-goat IgG-PE for 20 minutes at 4°C. For MPO staining, cells were stained for surface markers, fixed and permeabilized, and incubated with saturating amounts of biotinylated-MPO for 30 minutes, followed by streptavidin-PE for 20 minutes at 4°C.

Phagocytosis assay

To detect phagocytosis, pHrodo conjugated *E.coli* bioparticle assay kit (Life technologies, Carlsbad, CA) was used according manufacturer's instructions. Splenocytes, liver leukocytes and peritoneal macrophages resuspended in DMEM were incubated for 30 minutes at 37ºC without CO_2. After incubation, the cells were spun down and resuspended in HBSS containing opsonised pHrodo conjugated E.coli biopartcles for one hour at 37ºC without CO_2, and then stained for cell surface markers.

ROS production

To determine ROS production using hydroethidine (HE) (Polysciences), splenocytes and liver leukocytes were cultured with HKLM stimulation in the presence of 16μM HE at 37ºC with

5% CO_2. After 3 hours, cells were stained for cell surface markers as previously described. To measure ROS production using H_2DCFDA (Life technologies), bone marrow cells were first cultured with varying concentrations of IL-6 and IL-23 in the presence of HKLM for 2 hours, and then incubated with 10μM H_2DCFDA at 37ºC with 5% CO_2 for 1 hour. Following which cells were stained for surface markers as previously described in the cell surface staining section.

Real-time PCR array

RNeasy Plus Mini kit (Qiagen, Valencia, CA) was used to isolate mRNA from spleens of B6 and IL-23p19 KO mice infected with LM for one day. The quantity and quality of mRNA was determined using NanodropND-100 spectrophotometer. The mRNA was converted into cDNA using RT^2 first strand cDNA synthesis kit (Qiagen, Valencia, CA). The cDNA master mix prepared according to the manufacturer's instructions and added to the PCR array with primers for 84 genes associated with inflammation and autoimmunity. The array was run on an Applied Biosystems StepOne Plus 96-well RT-PCR machine. Data was analyzed with Qiagen's web-based PCR analysis software.

Statistical analyses

Each experiment was repeated one time with 4-5 mice per group unless stated otherwise. ANOVAs or Student's *t*-tests were conducted on the data where appropriate. For *post hoc* analyses, Bonferroni *t*-tests were used. LM CFU data were log transformed prior to analysis, and are represented as such in the figures. The survival curves between groups were compared using Kaplan–Meier plots and log rank tests. A p value of 0.05 or less was considered significant in all cases.

CHAPTER III

THE ROLE OF IL-23 IN REGULATING THE RECRUITMENT OF INFLAMMATORY MONOCYTES DURING LM INFECTION

Depletion of neutrophils further increases the susceptibility of IL-23p19 KO mice to LM infection

We have recently established that IL-23 is required for protection against systemic LM infection, and that IL-23p19 KO mice have reduced recruitment of neutrophils to the liver compared to B6 mice (43). In order to determine if the reduced neutrophil recruitment to the liver results in increased susceptibility of IL-23p19 KO mice, neutrophils were depleted from B6 and IL-23p19 KO mice, and a survival study was performed. Neutrophil depletions were performed using the anti-Ly6G antibody that is highly specific for neutrophils (36, 110), and depletions were confirmed in the spleen, liver, and blood by performing flow cytometric analysis (Fig. 2). In agreement with our previous publications, there was a reduced recruitment of neutrophils to the livers, but not the spleens, of IL-23p19 KO mice compared to B6 mice (43), and treatment with the anti-Ly6G antibody efficiently depleted neutrophils from the spleen, liver, and blood in both B6 and IL-23p19 KO mice (36). As shown in Fig. 3, there were no differences in survival between neutrophil-depleted B6 mice, isotype-treated B6 mice, or isotype-treated IL-23p19 KO. However, a much higher percentage (70%) of neutrophil-depleted IL-23p19 KO mice

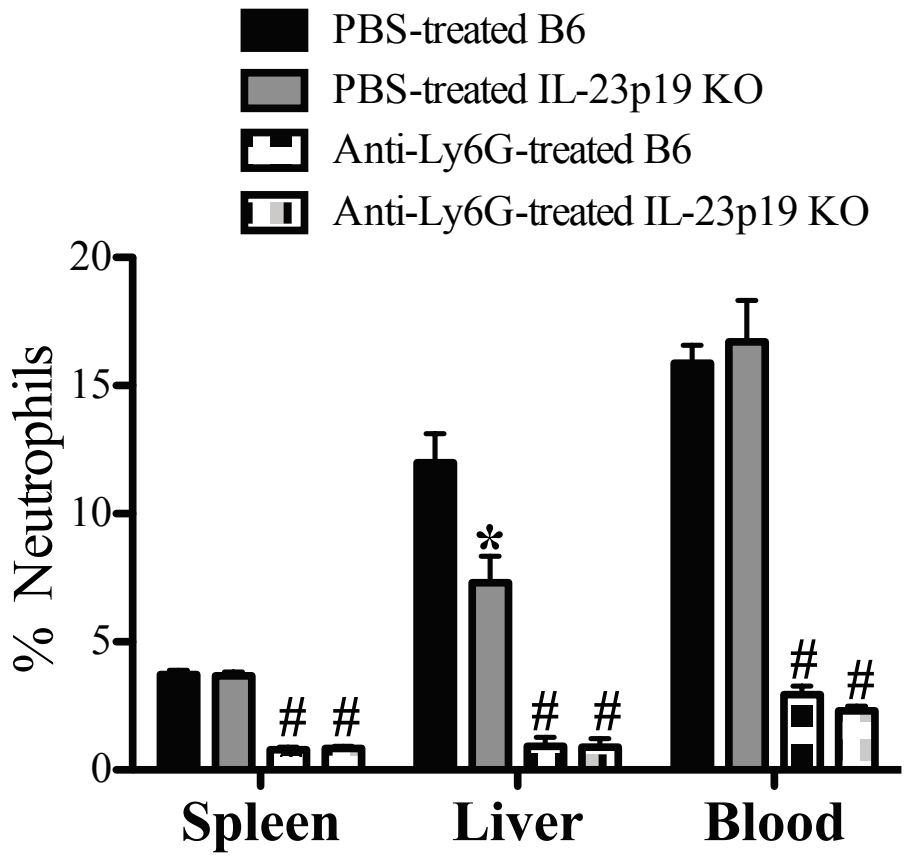


Figure 2: Treatment with the anti-Ly6G antibody depletes neutrophils from the spleen, liver, and blood.

B6 and IL-23p19 KO mice were infected with ~10^4 LM for one day. These mice were treated with PBS or anti-Ly6G antibody one day prior to infection. The neutrophil ($Ly6C^{int}CD11b^{hi}$) percentages were determined in the spleen, liver, and peripheral blood by flow cytometric analysis. A two-way ANOVA was performed to determine statistical differences between groups. An asterisk (*) indicates a significant difference from PBS-treated B6 mice, and a pound symbol (#) indicates a significant difference from the respective PBS-treated mice. All data are expressed as mean ± SEM (n=5/group). These data are representative of two independent experiments.

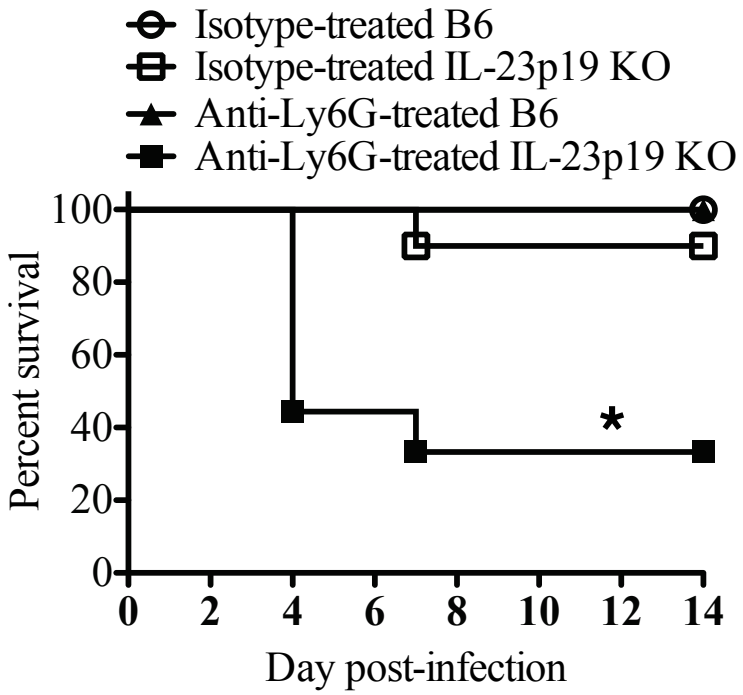


Figure 3: Depletion of neutrophils enhances the susceptibility of IL-23p19 KO mice to LM infection

B6 and IL-23p19 KO mice were infected with ~10^4 LM. These mice were treated with anti-Ly6G or isotype antibody one day prior to and 3 days p.i. The susceptibility of the mice was monitored for 14 days p.i. These data are combined from two independent experiments (n=9-10/group). A logrank analysis was performed to determine statistical differences between groups. An asterisk (*) indicates a significant difference from all the other groups.

succumbed to infection compared to the other groups. These data indicate that depletion of neutrophils in IL-23p19 KO mice increases their susceptibility to infection. Importantly, the fact that neutrophil-depleted B6 and IL-23p19 KO mice do not show equivalent susceptibility to LM suggests that differences other than deficient neutrophil recruitment must exist between B6 and IL-23p19 KO mice.

Depletion of neutrophils abrogates the differences in bacterial burden in the livers, but not the spleens, of B6 and IL-23p19 KO mice during LM infection

We have previously demonstrated that IL-23p19 KO mice have higher bacterial burdens in the spleen and liver than B6 mice at day 5 post-LM infection (43). To investigate if the increased bacterial burden in the IL-23p19 KO mice is due to deficient neutrophil recruitment, spleen and liver CFUs from LM-infected neutrophil-depleted B6 and IL-23p19 KO mice were determined. Because most of the neutrophil-depleted IL-23p19 KO mice succumb to infection by day 4 p.i. at ~10^4 LM, a sub-lethal dose of ~3000 LM was used for this study. As shown in Fig. 4, in accordance with our previous study (43), the isotype-treated IL-23p19 KO mice had higher CFUs in the spleen and liver compared to the isotype-treated B6 mice. Additionally, depletion of neutrophils in both the B6 and IL-23p19 KO mice compromised their ability to efficiently clear bacteria, compared to the respective isotype-treated control mice (36). However, it is important to note that the depletion of neutrophils resulted in significantly higher bacterial burden in the spleens of IL-23p19 KO mice compared to B6 mice, in contrast to the livers, wherein depletion equalized the bacterial burden between the B6 and IL-23p19 KO mice (Fig. 4). This enhanced bacterial burden in the spleens of neutrophil-depleted IL-23p19 KO mice could lead to increased

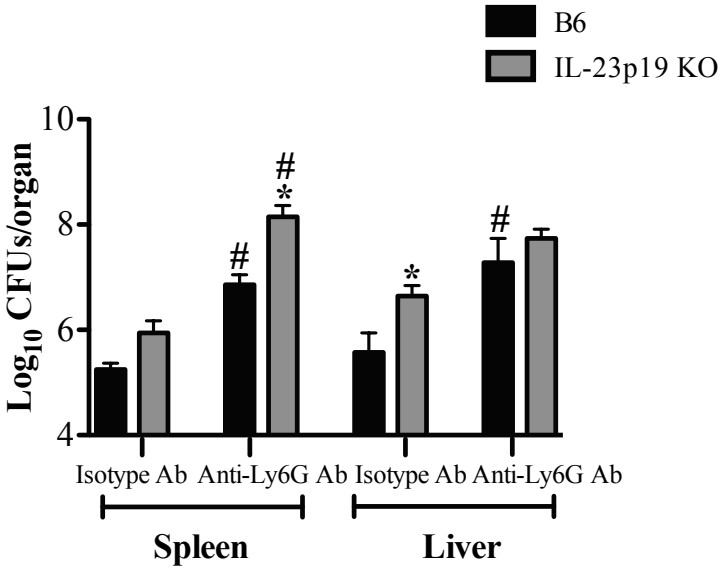


Figure 4: Depletion of neutrophils eliminates the differences in bacterial burden in the livers, but not the spleens, of B6 and IL-23p19 KO mice during LM infection.

B6 and IL-23p19 KO mice were infected with ~3000 LM for 5 days. These mice were treated with anti-Ly6G or isotype antibody at one day prior to and 3 days p.i. At day 5 p.i., spleens and livers were harvested and the bacterial CFUs were enumerated in the spleen and the liver. A two-way ANOVA was performed to determine statistical differences between groups. An asterisk (*) indicates a significant difference from B6 mice and a pound symbol (#) indicates a significant difference from isotype-treated controls. All data are expressed as mean ± SEM (n=5/group). These data are representative of two independent experiments.

susceptibility of these mice to LM infection. Collectively, these data strengthen our previous findings that the reduced neutrophil recruitment to the livers of IL-23p19 KO mice is causal for the increased LM burden in this organ (43). However, in the spleen, IL-23 may regulate the recruitment or function of an additional cell type, which may account for the differences in spleen CFUs between LM-infected IL-23p19 KO and B6 mice.

IL-23 is required for the optimal recruitment of inflammatory monocytes to the spleen during LM infection

Inflammatory monocytes are recruited to the spleen during LM infection and are required for resistance (111). To investigate whether IL-23 is required for the recruitment of inflammatory monocytes, mice were left uninfected or infected for 1 or 3 days with LM, and percentages of inflammatory monocytes in the blood, spleen, and liver were determined by flow cytometric analysis. The representative dot plots (Fig. 5) from day 1 LM–infected splenocytes depict inflammatory monocytes that were identified based on the expression of the surface markers Ly6C and CD11b as $Ly6C^{hi}CD11b^{int}$ cells, which were clearly reduced in the spleens of LM-infected IL-23p19 KO mice. Prior to infection, monocyte percentages were decreased in the blood of IL-23p19 KO mice compared to B6 mice (Fig. 6A). However, there were no differences in monocyte percentages in the spleen or liver (Fig. 6A), or total numbers of monocytes in the spleens (Fig. 6B) between uninfected B6 and IL-23p19 KO mice. Upon LM infection, there was a reduction in the monocyte percentages in the blood, spleens, and livers of IL-23p19 KO mice compared to B6 mice at day 1 p.i. (Fig. 6C). Likewise, the total numbers of monocytes in the spleens of IL-23p19 KO mice was reduced compared to B6 mice (Fig. 6D). However, at day 3 p.i., the monocyte percentages did not differ in the blood and livers of B6 and IL-23p19 KO

mice, but both the percentage and total numbers of monocytes in the spleens were reduced in the IL-23p19KO mice compared to B6 mice (Figs. 6E and 6F). These results indicate that IL-23 is required for the optimal recruitment of monocytes to the spleen during infection with LM.

IL-23 is required for the optimal production of inflammatory monocyte-recruiting chemokines during LM infection

Reports have identified CCL2 and CCL7 as the key chemokines responsible for the emigration of inflammatory monocytes from the bone marrow to the blood during LM infection (22, 23). To investigate if IL-23 mediated the production of inflammatory monocyte-recruiting chemokines, CCL2 and CCL7, we determined the levels of these chemokines in B6 and IL-23p19 KO mice that were either uninfected or infected for 1 day with LM. CCL2 and CCL7 concentrations were measured in serum, spleen homogenates, and supernatants from splenocytes and bone marrow cells stimulated overnight with HKLM or left unstimulated. There were no significant differences in CCL2 production between uninfected B6 and IL-23p19 KO mice in the serum, spleen homogenates or overnight culture supernatants from spleen and bone marrow (Fig. 7). However, upon LM infection, there was a significant reduction in the amount of CCL2 and CCL7 in the serum of infected IL-23p19 KO mice compared to B6 mice (Fig. 8A). Additionally, CCL2 concentrations were reduced in the spleen homogenates from IL-23p19 KO mice compared to B6 mice (Fig. 8B). There were no significant differences between B6 and IL-23p19 KO mice in the levels of CCL2 in the serum and spleen homogenates, or CCL7 in the serum at day 3 p.i. (data not shown). Although the production of CCL2 did not differ between B6 and IL-23p19 KO mice under unstimulated culture conditions, it was significantly reduced in

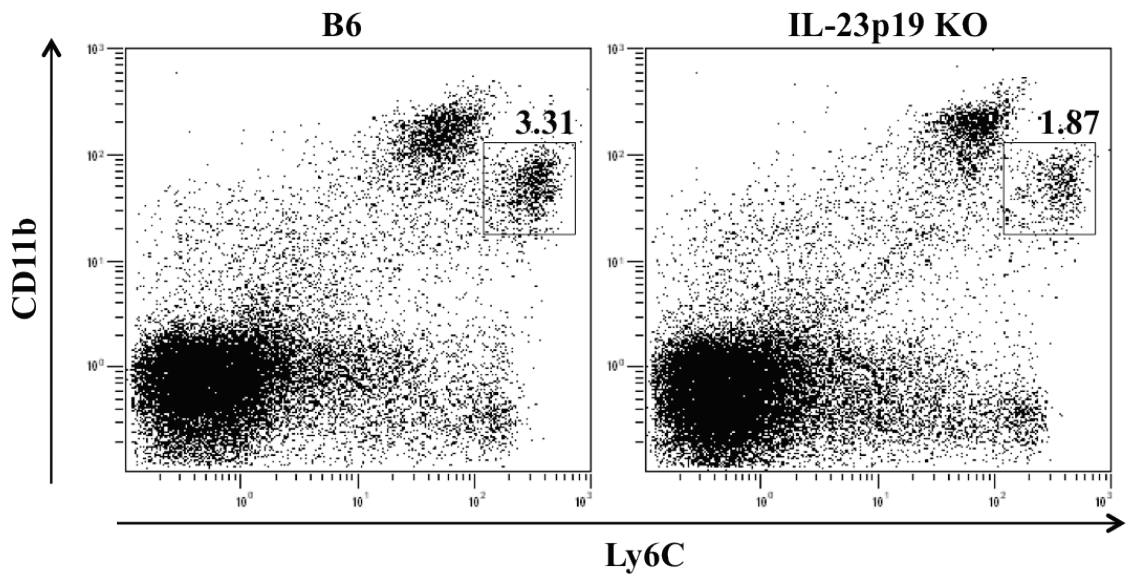


Figure 5: IL-23 is required for the optimal recruitment of inflammatory monocytes during LM infection.

B6 and IL-23p19 KO mice were infected with ~10^4 LM for one day. Flow cytometry was performed to determine the percentages of inflammatory monocytes that were identified as cells expressing high levels of Ly6C and intermediate levels of CD11b ($Ly6C^{hi}CD11b^{int}$) in splenocytes from day 1 LM-infected mice.

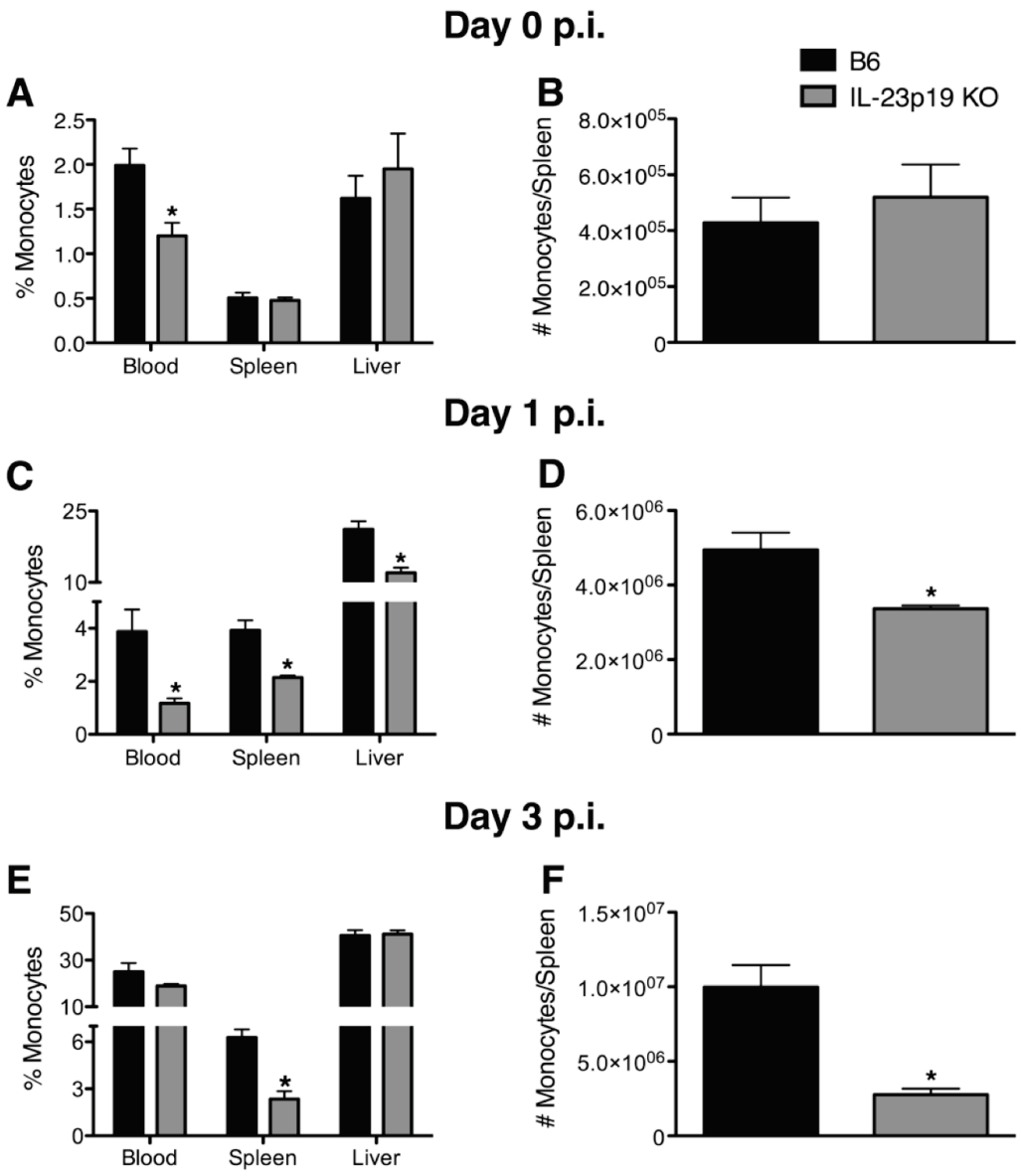


Figure 6: IL-23 is required for the optimal recruitment of inflammatory monocytes during LM infection.

B6 and IL-23p19 KO mice were infected with ~10^4 LM. The percentages of inflammatory monocytes in the blood, spleen, and liver were determined at day 0 (A), day 1 (C) and day 3 p.i. (E). The numbers of inflammatory monocytes in the spleen were examined at day 0 (B), day 1 (D) and day 3 p.i. (F). A t-test was performed to determine statistical differences between groups. An asterisk (*) indicates a significant difference from B6 mice. All data are expressed as mean ± SEM (n=5/group). These data are representative of two independent experiments.

the supernatants from HKLM-stimulated splenocytes and bone marrow cells of IL-23p19 KO mice compared to B6 mice (Figs. 8C and 8D). Collectively, these data demonstrate that early production of the inflammatory monocyte-recruiting chemokines, CCL2 and CCL7, is regulated by IL-23 during LM infection.

IL-23 is not required for generation or maintenance of inflammatory monocytes in the bone marrow

Inflammatory monocytes are derived from myeloid progenitors in the bone marrow, and the generation of these cells in the bone marrow requires growth factors such as M-CSF. It is well known that the IL-23/IL-17 axis mediates the production of growth factors, including G-CSF and GM-CSF (85). Therefore, we examined if the reduction in inflammatory monocyte percentages in IL-23p19 KO mice was due to a defect in the generation or maintenance of these cells in the bone marrow. The percentage of inflammatory monocytes was determined in bone marrow cells isolated from B6 and IL-23p19 KO mice at day 0, day 1, and day 3 p.i. As expected, there was a marked increase in the inflammatory monocyte percentage at day 3 p.i. compared to uninfected and day 1 p.i., due to LM infection induced monopoiesis. However, the percentages of inflammatory monocytes did not differ between B6 and IL-23p19 KO mice at any of the time points (Fig. 9). Therefore, IL-23 is not required for the generation or maintenance of inflammatory monocytes in the bone marrow during homeostasis or LM infection.

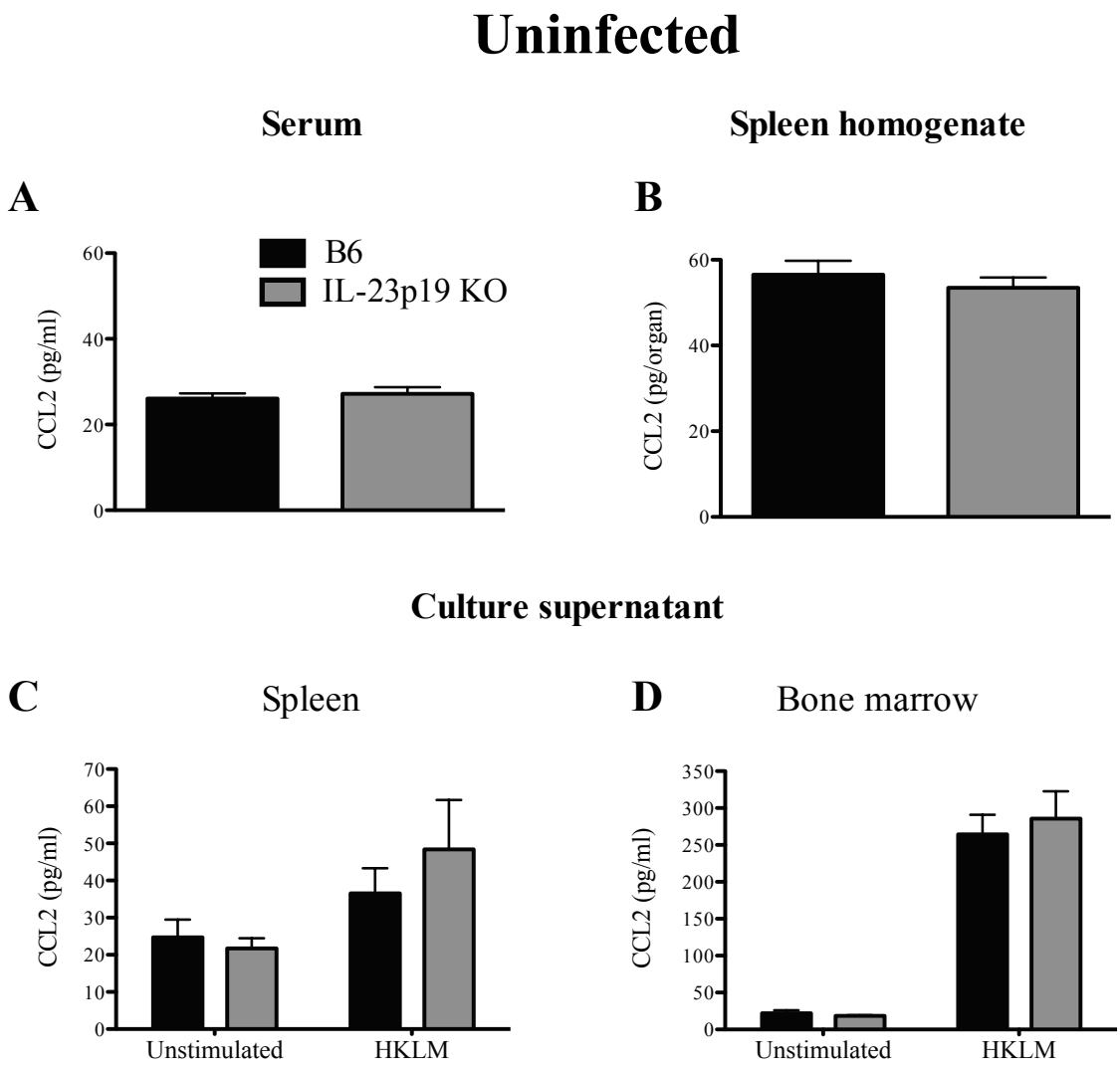


Figure 7: IL-23 does not regulate the production of CCL2 under homeostatic conditions.

Serum, spleens, and bone marrow cells were harvested from naïve B6 and IL-23p19 KO mice. ELISAs were performed to determine the concentrations of CCL2 in the serum (A), spleen homogenates (B), and supernatants from overnight cultures of splenocytes (C) and bone marrow cells (D) stimulated with HKLM or left unstimulated. A t-test (A & B) and two-way ANOVA (C & D) were used to determine statistical differences between groups. There were no differences between B6 and IL-23p19 KO mice. All data are expressed as mean ± SEM (n=4-5/group). These data are representative of two independent experiments.

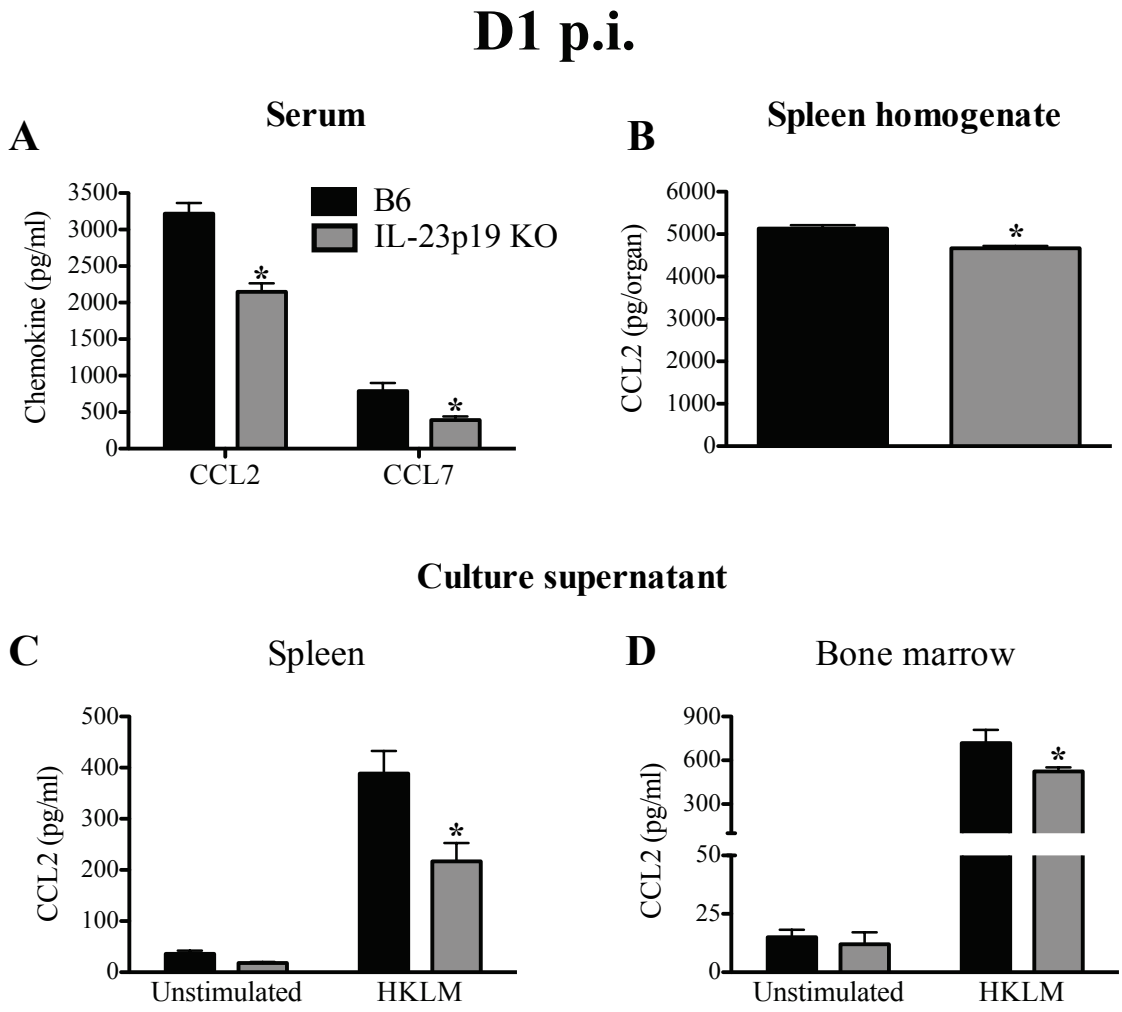


Figure 8: IL-23 is required for the optimal production of CCL2 and CCL7 during LM infection.

B6 and IL-23p19 KO mice were infected with ~10^4 LM for 1 day. ELISAs were performed to determine the concentrations of CCL2 and CCL7 in the serum (A), CCL2 in spleen homogenates (B), and CCL2 in supernatants from overnight cultures of splenocytes (C) and bone marrow cells (D) that were stimulated with HKLM or left unstimulated. A two-way ANOVA (A) and t-tests (B, C, and D) were used to determine statistical differences between groups. An asterisk (*) indicates a significant difference from B6 mice. All data are expressed as mean ± SEM (n=5/group). These data are representative of two independent experiments.

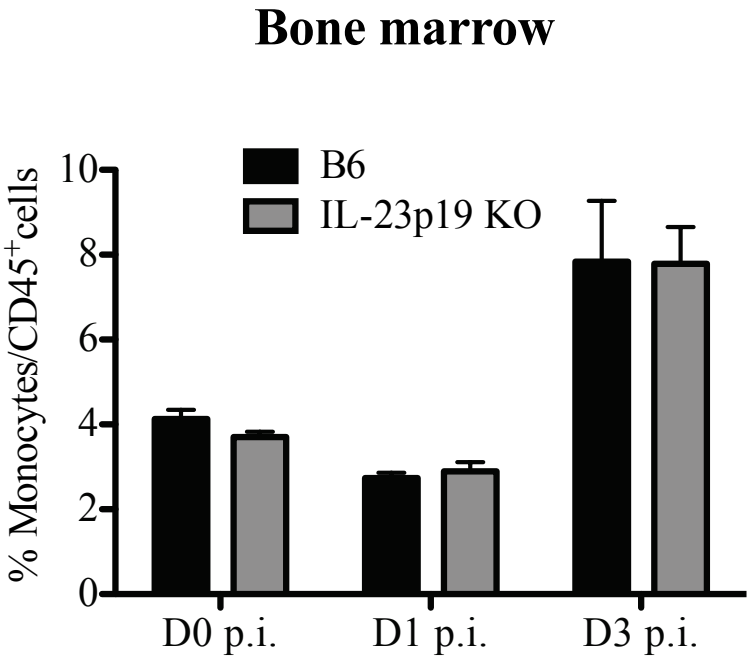


Figure 9: IL-23 is not required for the generation or maintenance of inflammatory monocytes in the bone marrow.

B6 and IL-23p19 KO mice were left uninfected or were infected with ~10^4 LM for 1 or 3 days. Flow cytometry was performed to determine the percentages of inflammatory monocytes in the bone marrow. All data are expressed as mean ± SEM (n=5/group). These data are representative of two independent experiments

Summary of Chapter III

The data presented in this chapter demonstrate that IL-23 is essential for the optimal recruitment of inflammatory monocytes to the spleen during LM infection. Our neutrophil depletion studies establish that IL-23 is required for promoting neutrophil recruitment to the liver; however, it regulates the recruitment and function of another immune cell in the spleen during LM infection (Figs. 3 and 4). Further analysis identified that IL-23 mediates the recruitment of inflammatory monocytes to the blood, spleen, and liver during LM infection. Importantly, IL-23 is necessary for continued recruitment of monocytes to the spleen during LM infection (Figs. 5 and 6). Furthermore, IL-23 is required for the optimal production of inflammatory monocyte-recruiting chemokines, CCL2 and CCL7, during LM infection (Figs. 7 and 8). Interestingly, IL-23 does not regulate the generation or maintenance of inflammatory monocytes in the bone marrow (Fig. 9). These data recognize a novel role for IL-23 in mediating the recruitment of immune cells in an organ-specific fashion during a bacterial infection.

CHAPTER IV

THE ROLE OF IL-23 IN REGULATING THE FUNCTION OF PHAGOCYTIC CELLS DURING LM INFECTION

IL-17RA is expressed by inflammatory monocytes and neutrophils during LM infection

Prior studies have demonstrated that the receptors for IL-23 and IL-17A are expressed on phagocytic cells during different disease states (86, 87, 102). However, it has never been investigated whether IL-23R or IL-17RA are expressed on phagocytic cells during LM infection. Since IL-23 and IL-17A are produced during LM infection (43, 112), it is possible that LM infection induces phagocytic cells to express these cytokine receptors. To detect the expression of IL-23R and IL-17RA on phagocytic cells during LM infection, flow cytometry was performed on splenocytes and liver leukocytes from B6 and IL-23p19 KO mice infected with LM for one day. Due to technical issues associated with the detection of IL-23R expression, we examined the expression of IL-17RA only. As shown in the histogram in Fig. 10A, at day 1 post-LM infection, there was a higher percentage of inflammatory monocytes and neutrophils expressing IL-17RA in the spleen and liver of infected B6 mice compared to naïve B6 mice. This suggests that infection may increase the expression of IL-17RA on inflammatory monocytes and neutrophils. Next, we determined if the production of IL-23 was required for the enhanced expression of IL-17RA on inflammatory monocytes and neutrophils during LM

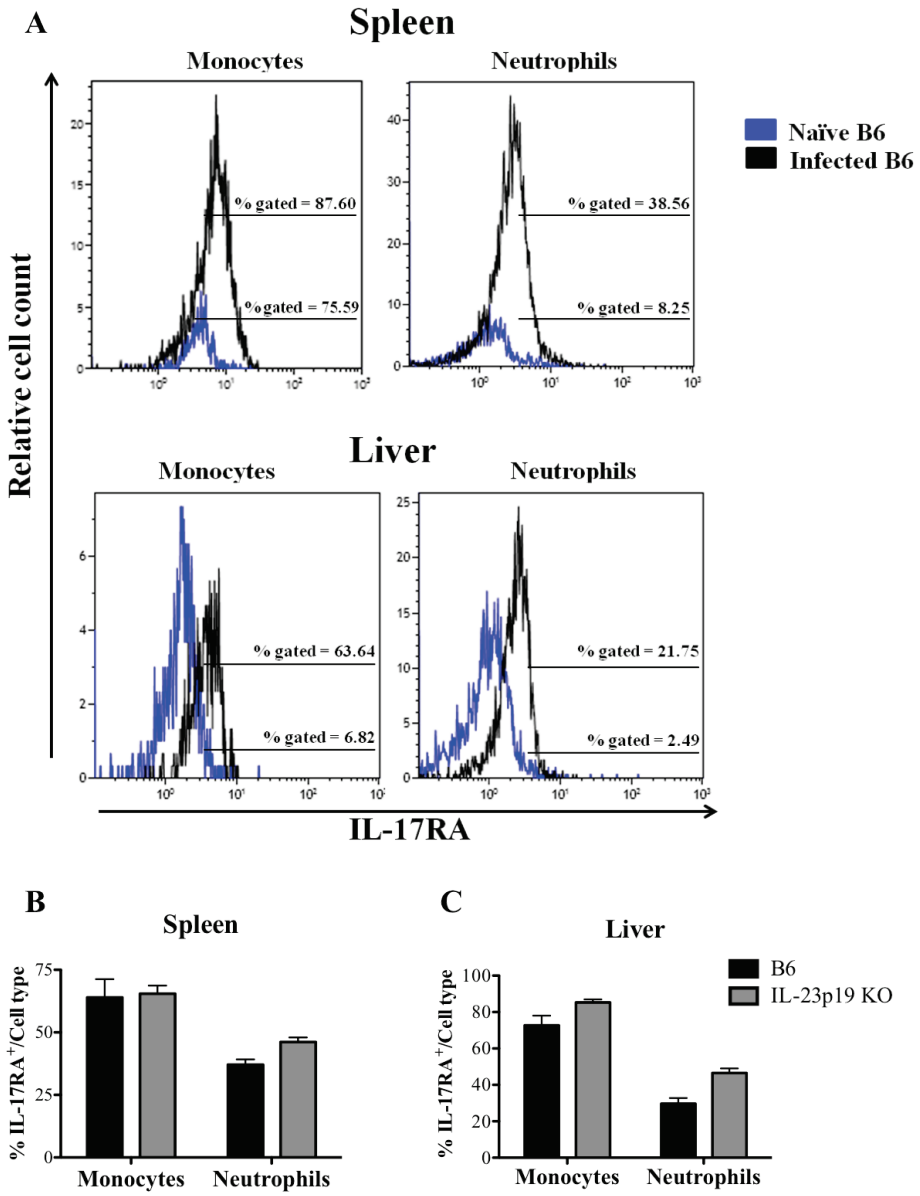


Figure 10: IL-23 does not impact the expression of IL-17RA during LM infection.

B6 and IL-23p19 KO mice were infected with ~10^4 LM for one day or left uninfected. Overlay histograms of IL-17RA expression on inflammatory monocytes and neutrophils in the spleen and liver from naïve and infected B6 mice (A). The percentages of inflammatory monocytes and neutrophils expressing IL-17RA were determined in the spleen (B) and liver (C). A two-way ANOVA was performed to determine statistical differences between groups. All data are expressed as mean ± SEM (n=5/group). These data are representative of one independent experiment.

infection. There was a greater percentage of inflammatory monocytes expressing IL-17RA compared to neutrophils in the spleen and liver. Importantly, the absence of IL-23 did not result in a reduction in the percentages of IL-17RA-expressing inflammatory monocytes or neutrophils in the spleen and liver of B6 and IL-23p19 KO mice during LM infection (Figs. 10B and 10C). These data indicate that inflammatory monocytes and neutrophils from both B6 and IL-23p19 KO have the potential to respond to IL-17A stimulation during LM infection.

IL-23 is not required for optimal phagocytosis by inflammatory monocytes, neutrophils, and macrophages

One of the early events that initiate pathogen clearance is the engulfment or phagocytosis of the pathogen by phagocytic cells including inflammatory monocytes, neutrophils, and macrophages. However, very little is known about the factors that regulate this process during infection. It has been demonstrated that exogenous stimulation of macrophages with IL-17A increases their ability to engulf apoptotic neutrophils suggesting that this cytokine could modulate phagocytosis (106). Since the production of IL-17A is dependent on IL-23, it is possible that IL-23 could indirectly regulate phagocytosis. To determine if IL-23 is required for optimal phagocytosis, flow cytometry based phagocytosis assay was performed on inflammatory monocytes and neutrophils in the spleen and liver, and peritoneal macrophages, from naïve B6 and IL-23p19 KO mice. This assay utilizes a pH sensitive dye conjugated to *E.coli* bioparticles. The dye fluoresces at low pH conditions such as in a lysosome, enabling the detection of

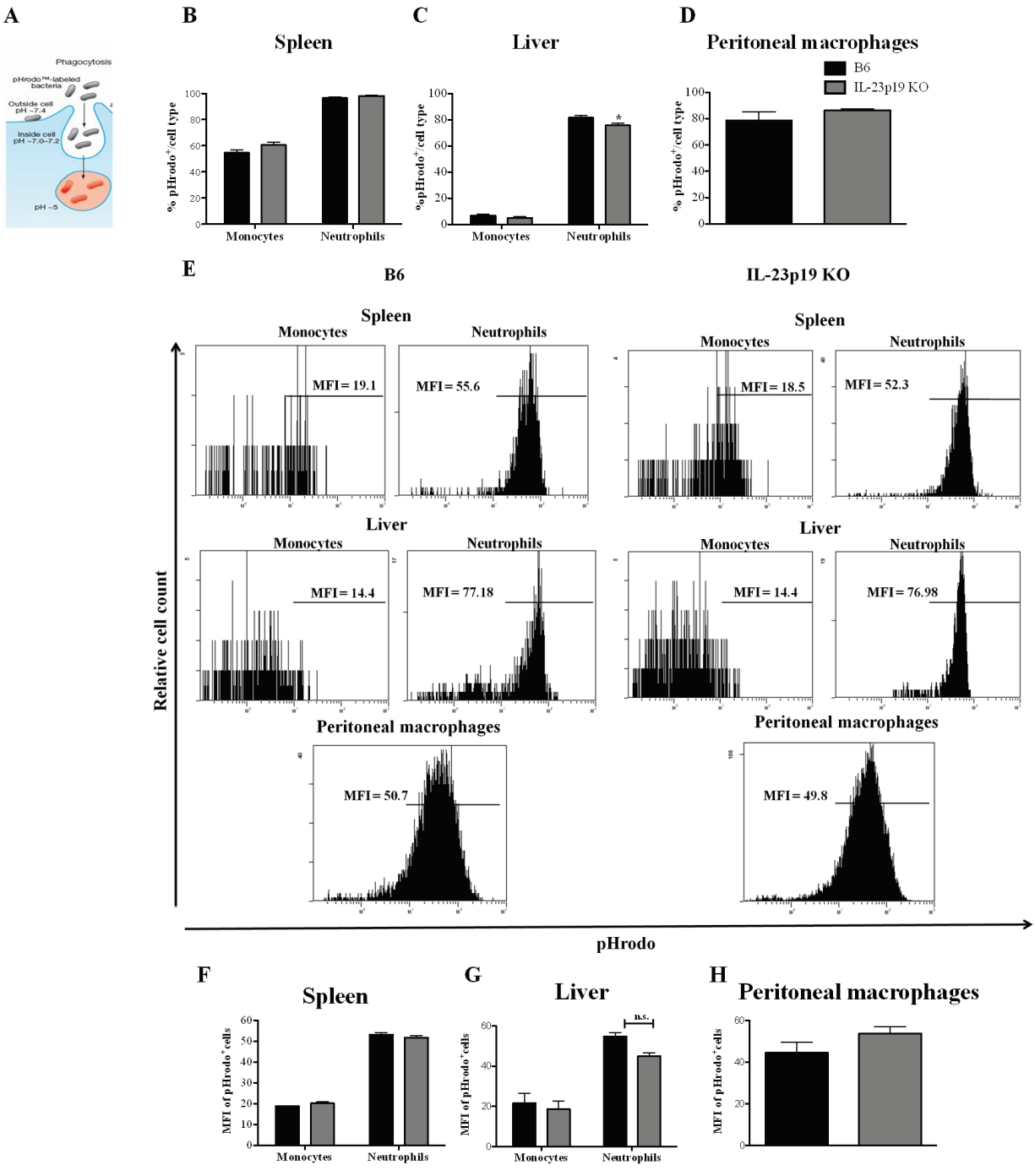

A
Phagocytosis
pHrodo™-labeled bacteria
Outside cell pH ~7.4
Inside cell pH ~7.0–7.2
pH ~5
B
Spleen
% pHrodo+/cell type
Monocytes
Neutrophils
C
Liver
%pHrodo+/cell type
*
Monocytes
Neutrophils
D
Peritoneal macrophages
B6
IL-23p19 KO
% pHrodo+/cell type
E
B6
IL-23p19 KO
Spleen
Monocytes
Neutrophils
MFI= 19.1
MFI= 55.6
MFI= 18.5
MFI= 52.3
Liver
MFI= 14.4
MFI= 77.18
MFI= 14.4
MFI= 76.98
Peritoneal macrophages
MFI= 50.7
MFI= 49.8
Relative cell count
pHrodo
F
Spleen
MFI of pHrodo+cells
Monocytes
Neutrophils
G
Liver
n.s.
MFI of pHrodo+cells
Monocytes
Neutrophils
H
Peritoneal macrophages
MFI of pHrodo+cells

Figure 11: IL-23 is not required for optimal phagocytosis.

B6 and IL-23p19 KO mice were left uninfected. Splenocytes, liver leukocytes, and peritoneal macrophages were harvested, and incubated with pHrodo conjugated *E.coli* bioparticles (A). Flow cytometry was performed to determine the percentages of pHrodo^{+} inflammatory monocytes (Ly6C^{hi}CD11b^{int}) and pHrodo^{+}neutrophils (Ly6C^{int}CD11b^{hi}) in both the spleen (B) and liver (C), and pHrodo^{+} peritoneal macrophages (F4/80^{+}CD11b^{+}) (D). Representative histograms of pHrodo^{+}cells is shown (E). The MFI of pHrodo^{+} inflammatory monocytes and pHrodo^{+}neutrophils in both the spleen (F) and liver (G), and pHrodo^{+}peritoneal macrophages (H) were also determined. A two-way ANOVA was performed to determine statistical differences between groups. An asterisk (*) indicates significant difference from B6 mice. All data are expressed as mean ± SEM (n=5/group). These data are representative of two independent experiments.

phagocytosis by flow cytometry (Fig. 11A). In the spleen, there were no differences in the percentages of inflammatory monocytes or neutrophils undergoing phagocytosis between B6 and IL-23p19 KO mice (Fig. 11B). Interestingly, in the liver, there was a slightly lower percentage of neutrophils, but not inflammatory monocytes, that were phagocytic ($pHrodo^+$) in IL-23p19KO mice compared to B6 mice (Fig. 11C). Since this reduction in the percentages of $pHrodo^+$ neutrophils observed in the liver of IL-23p19 KO mice is very minor, and it might not be biologically significant. Similar to spleen, peritoneal macrophages from B6 and IL-23p19 KO mice displayed equivalent phagocytosis (Fig. 11D). To further analyze the potential differences in phagocytic ability on a per cell basis, the mean fluorescence intensity (MFI) was determined as shown in the representative histograms (Fig. 11E). Inflammatory monocytes, neutrophils, and macrophages from both B6 and IL-23p19 KO mice had similar MFI values (Figs. 11E, 11F, 11G, and 11H). These data suggest that the ability of inflammatory monocyte and neutrophils in the spleen, inflammatory monocytes in the liver, and peritoneal macrophages, to perform phagocytosis does not require IL-23; however, phagocytosis by liver neutrophils could be moderately regulated by IL-23.

IL-23 is not required for optimal production of ROS by inflammatory monocytes and neutrophils during LM infection

The eradication of engulfed pathogens in the phagosome is facilitated by microbicidal products including ROS and RNS. Mice defective in NADPH oxidase are unable to effectively clear LM infection (61). A recent study demonstrated that IL-23 in combination with IL-6 could enhance the production of ROS (as measured by the ROS sensitive dye, H_2DCFDA) from bone marrow neutrophils during a fungal infection (86). To examine if this combinatorial effect of IL-

6 and IL-23 occurred in our model of systemic bacterial infection, bone marrow cells were isolated from day 1 LM-infected B6 mice. We determined the percentages of H_2DCFDA^+ neutrophils upon stimulation with HKLM in the presence of 100ng/ml IL-6 and varying concentrations of IL-23. As shown in Fig. 12, stimulation with HKLM increased the percentages of neutrophils undergoing oxidative burst. Importantly, increasing the dose of IL-23 resulted in the enhancement of percentages of H_2DCFDA^+ neutrophils compared to HKLM stimulation alone. This suggested that IL-23 in combination with IL-6 could enhance ROS production from neutrophils during LM infection, and this effect occurred in an IL-23 dose-dependent fashion. We investigated this phenomenon further in the context of IL-23p19 KO mice during LM infection, by determining ROS production from inflammatory monocytes and neutrophils in the spleen and liver from day one-LM infected B6 and IL-23p19 KO mice. The IL-23p19 KO mice express GFP, which fluoresces in the same flow cytometry channel as H_2DCFDA. Therefore, it impossible to utilize H_2DCFDA for studies with IL-23p19 KO mice. To circumvent this technical issue, another ROS sensitive dye, HE was utilized to determine ROS production from IL-23p19 KO mice. As shown in Fig.13A, unstimulated cells did not undergo as much oxidative burst as compared to HKLM-stimulated cells; therefore, HKLM stimulation was used for all experiments. Inflammatory monocytes and neutrophils from both the spleen and liver of B6 and IL-23p19 KO mice were able to produce ROS during LM infection. Since neutrophils undergo tremendous oxidative burst, these cells produced more ROS than inflammatory monocytes during LM infection. However, there was no significant reduction in the

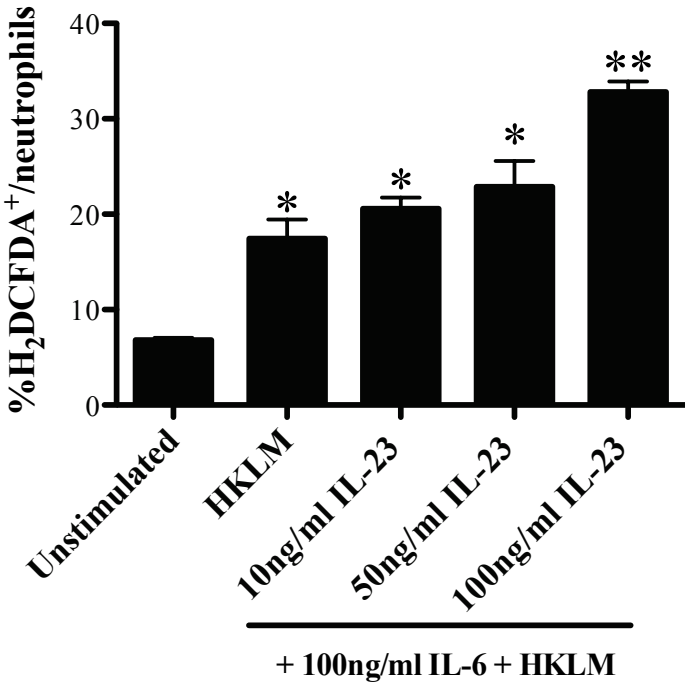


Figure 12: IL-23 in combination with IL-6 enhances the production of ROS from bone marrow neutrophils during LM infection.

B6 mice were infected with ~10^4 LM for one day. Bone marrow cells were cultured with 100ng/ml IL-6 and varying concentrations of IL-23 with or without HKLM stimulation in the presence of H_2DCFDA. Flow cytometry was performed to determine the production of ROS by neutrophils ($CD11b^+Ly6G^+$). One-way ANOVA was performed to determine statistical differences between groups. All data are expressed as mean ± SEM (n=4/group). An asterisk (*) indicates significant difference from unstimulated condition and a double asterisk (**) indicates a significant difference from HKLM stimulation. These data are representative of one independent experiment.

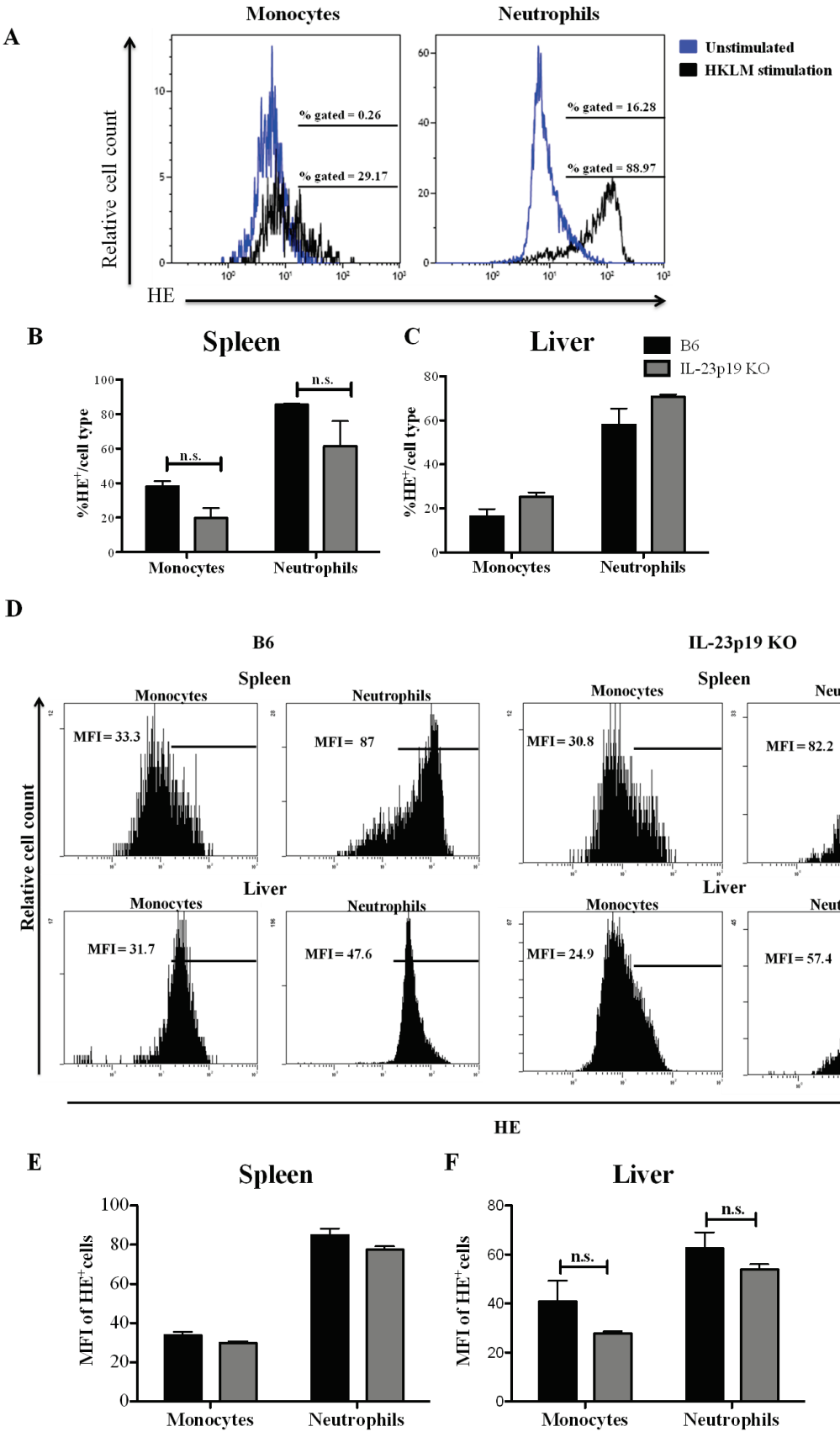

A
Monocytes
Neutrophils
Unstimulated
HKLM stimulation
Relative cell count
% gated = 0.26
% gated = 29.17
% gated = 16.28
% gated = 88.97
HE
B
Spleen
C
Liver
B6
IL-23p19 KO
%HE+/cell type
n.s.
Monocytes
Neutrophils
D
B6
IL-23p19 KO
Spleen
Liver
MFI = 33.3
MFI = 87
MFI = 30.8
MFI = 82.2
MFI = 31.7
MFI = 47.6
MFI = 24.9
MFI = 57.4
Relative cell count
HE
E
Spleen
F
Liver
MFI of HE+ cells

Figure 13: IL-23 is not required for the optimal production of ROS during LM infection.

B6 and IL-23p19 KO mice infected with ~10^4 LM for one day. Splenocytes, liver leukocytes, and peritoneal macrophages were harvested, and cultured with HE in the presence of HKLM stimulation. Representative histograms depicting ROS production from inflammatory monocytes and neutrophils in B6 splenocytes under unstimulated (blue) and HKLM stimulated (black) conditions (A). Flow cytometry was performed to determine the production of ROS by inflammatory monocytes ($Ly6G^-Ly6C^{hi}$) and neutrophils ($Ly6G^+Ly6C^{int}$) in both the spleen (B) and liver (C). Representative histograms of HE^+ cells is shown (D). The MFI of HE^+ inflammatory monocytes and HE^+ neutrophils in both the spleen (E) and liver (F). A two-way ANOVA was performed to determine statistical differences between groups. All data are expressed as mean ± SEM (n=4-5/group). These data are representative of two independent experiments.

percentages (Figs. 13B and 13C), or MFI (Figs. 13D, 13E, and 13F) of inflammatory monocytes and neutrophils producing ROS between B6 and IL-23p19 KO mice, in both the spleen and liver. These data indicate that IL-23 is not essential for optimal production of ROS from inflammatory monocytes and neutrophils, during LM infection in the spleen and liver.

IL-23 is not required for optimal expression of iNOS during LM infection

Another important molecule produced during oxidative burst is $NO^{\cdot}$**.** It has been demonstrated that the absence of iNOS renders mice moderately more susceptible to LM infection compared to B6 mice (61). To examine if the absence of IL-23 regulated the expression of iNOS during LM infection, intracellular expression of this enzyme was determined in inflammatory monocytes and neutrophils in spleen and liver at day 1 p.i. Additionally, peritoneal macrophages were isolated from B6 and IL-23p19 KO mice infected i.p. for one day with LM. Staining with iNOS antibody resulted in a distinct peak shift compared to the corresponding isotype control in both inflammatory monocytes and neutrophils (Fig. 14A). This confirmed that the observed percentages of $iNOS^{+}$ cells in the spleen and liver were not due to non-specific binding of the antibody. There were no differences in the percentages of iNOS-expressing inflammatory monocytes or iNOS-expressing neutrophils in the spleen and liver between B6 and IL-23p19 KO mice (Figs. 14B and 14C). Interestingly, there was a higher percentage of $F4/80^{hi}CD11b^{hi}$ macrophages expressing iNOS than $F4/80^{lo}CD11b^{lo}$ macrophages. However, there was no significant reduction in the percentages of iNOS-expressing macrophages between B6 and IL-23p19 KO mice (Fig. 14D). These results indicate that IL-23 does not regulate iNOS expression from inflammatory monocytes, neutrophils, and peritoneal macrophages during LM infection.

IL-23 is not required for the optimal production of MPO during LM infection

MPO is an enzyme that is required for the conversion of hydrogen peroxide to hypochlorous acid. It is produced by macrophages and neutrophils, and is widely used as an inflammatory marker to detect neutrophil activity (113). To determine if the absence of IL-23 impacted MPO production, intracellular staining was performed to detect MPO within inflammatory monocytes and neutrophils in the spleen and liver as well as in peritoneal macrophages, from B6 and IL-23p19 KO mice infected with LM for one day. Most including inflammatory monocytes, neutrophils, and macrophages expressed MPO, and the percentage of cells expressing MPO did not differ between B6 and IL-23p19 KO mice (Figs. 15A, 15B, and 15C). Since nearly ~100% of the cells expressed MPO, MFI values were determined to detect the relative expression of MPO on a per cell basis (Fig. 15D). As expected, neutrophils expressed more MPO than monocytes and macrophages. Interestingly, neutrophils in the spleen of IL-23p19 KO mice displayed slightly reduced MFI values compared to B6 mice whereas the MFI of MPO^{+} inflammatory monocytes were similar between the groups (Figs. 15E and 15F). Both the population of macrophages, $F4/80^{hi}CD11b^{hi}$ and $F4/80^{lo}CD11b^{lo}$, from B6 and IL-23p19 KO mice had equivalent MFI values (Fig. 15G). These data suggest that the expression of MPO by inflammatory monocytes and neutrophils in the liver, inflammatory monocytes in the spleen, and peritoneal macrophages, is not mediated by IL-23.

IL-23 is not required for optimal production of TNF-α from inflammatory monocytes, neutrophils, and macrophages during LM infection

TNF-α has been shown to be required for effective eradication of LM (73). Activated cells including inflammatory monocytes, neutrophils, and macrophages are predominant sources

of TNF-α during LM infection (29, 74). Importantly, TNF-α derived from neutrophils and macrophages is critical for providing protection against LM (74). To examine whether the absence of IL-23 impacts the production of TNF-α, the production of this cytokine was determined from inflammatory monocytes and neutrophils in spleen and liver as well as peritoneal macrophages from B6 and IL-23p19 KO mice at day 1 p.i. There were similar percentages of inflammatory monocytes and neutrophils producing TNF-α from the spleen and liver of B6 and IL-23p19 KO mice (Figs. 16A and 16B). Similar percentages of TNF-α-producing $F4/80^{hi}CD11b^{hi}$ macrophages were observed between B6 and IL-23p19 KO mice (Fig. 16C). However, there was a significant reduction in the percentages of $F4/80^{lo}CD11b^{lo}$ cells - producing TNF-α in IL-23p19 KO mice compared to B6 mice (Fig. 16C). This suggests IL-23 could be essential for the production of TNF-α from the $F4/80^{lo}CD11b^{lo}$ population of peritoneal macrophages. Additionally, the MFI of TNF-α-producing cell was determined as shown in the representative histograms (Fig. 16D). The MFI of TNF-α-producing cells, including inflammatory monocytes and neutrophils and peritoneal macrophages did not differ between groups. Therefore, IL-23 is not required for optimal production of TNF-α from phagocytic cells during LM infection.

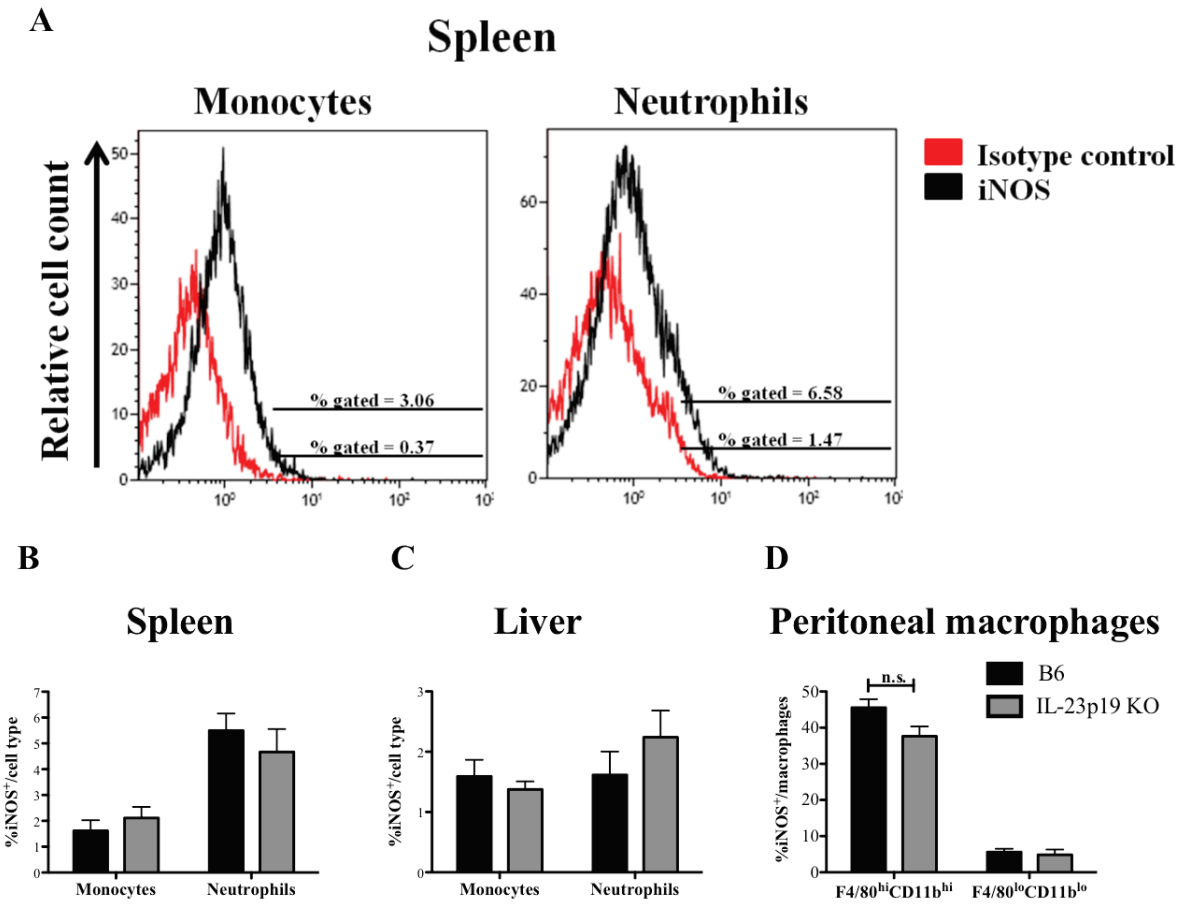


Figure 14: IL-23 is not required for the optimal expression of iNOS during LM infection.

B6 and IL-23p19 KO mice were infected with ~10^4 LM for one day. Splenocytes, liver leukocytes, and peritoneal macrophages were harvested. Flow cytometric analysis was performed to determine the expression of iNOS by inflammatory monocytes ($Ly6C^{hi}CD11b^{int}$) and neutrophils ($Ly6G^{+}CD11b^{+}$) in both the spleen and liver, and peritoneal macrophages ($F4/80^{hi}CD11b^{hi}$) and $F4/80^{lo}CD11b^{lo}$). Representative histograms depict the staining for iNOS (black) and isotype control (red) by inflammatory monocytes and neutrophils from B6 mice (A). The percentages of iNOS expressing cells were obtained after subtracting the corresponding isotype control values determined from histograms. The percentages of iNOS expressing inflammatory monocytes and neutrophils in both the spleen (B) and liver (C), and peritoneal macrophages were determined. A two-way ANOVA was performed to determine statistical differences between groups. All data are expressed as mean ± SEM (n=5/group). These data are representative of two independent experiments.

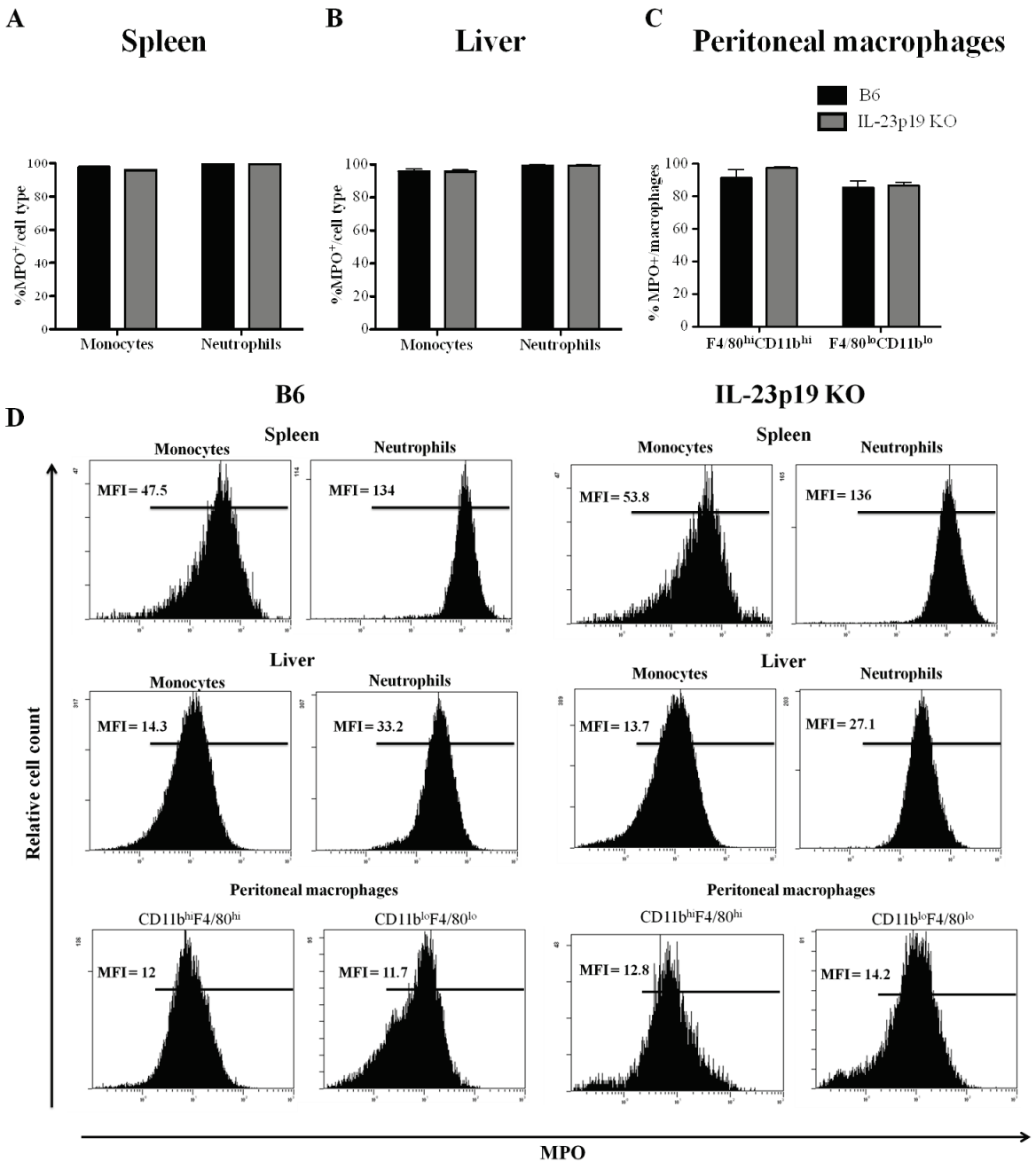

A
Spleen
B
Liver
C
Peritoneal macrophages
B6
IL-23p19 KO
% MPO+ cell type
% MPO+ macrophages
Monocytes
Neutrophils
F4/80hiCD11bhi
F4/80loCD11blo
D
B6
IL-23p19 KO
Spleen
Liver
Peritoneal macrophages
Monocytes
Neutrophils
CD11bhiF4/80hi
CD11bloF4/80lo
MFI= 47.5
MFI= 134
MFI= 53.8
MFI= 136
MFI= 14.3
MFI= 33.2
MFI= 13.7
MFI= 27.1
MFI= 12
MFI= 11.7
MFI= 12.8
MFI= 14.2
Relative cell count
MPO
E
Spleen
F
Liver
G
Peritoneal macrophages
MFI of MPO+ cell type
MFI of MPO+ macrophages
*

Figure 15: IL-23 is not required for the optimal expression of MPO during LM infection.

B6 and IL-23p19 KO mice were infected with ~10^4 LM for one day. Splenocytes, liver leukocytes, and peritoneal macrophages were harvested. Flow cytometric analysis was performed to determine the expression of MPO by inflammatory monocytes ($Ly6C^{hi}CD11b^{int}$) and neutrophils ($Ly6G^{+}CD11b^{+}$) in both the spleen and liver, and peritoneal macrophages ($F4/80^{hi}CD11b^{hi}$ and $F4/80^{lo}CD11b^{lo}$). The percentages of MPO expressing inflammatory monocytes and neutrophils in both the spleen (A) and liver (B), and peritoneal macrophages (C) were determined. Representative histograms of MPO^{+} cells are shown (D). The MFI of MPO^{+} inflammatory monocytes and MPO^{+} neutrophils in both the spleen (E) and liver (F), and MPO^{+} peritoneal macrophages (G) were also determined. A two-way ANOVA was performed to determine statistical differences between groups. An asterisk (*) indicates significant difference from B6 mice. All data are expressed as mean ± SEM (n=4-5/group). These data are representative of two independent experiments.

The absence of IL-23 reduces the overall production of TNF-α and $NO^{\cdot}$ from splenocytes during LM infection

IL-23p19 KO mice display reduced bacterial clearance in the spleen and liver compared to B6 mice (43). As mentioned previously, the effective eradication of LM is dependent upon several effector molecules, including TNF-α and $NO^{\cdot}$ (61, 73). However, it is not known how the absence of IL-23 impacts the overall levels of TNF-α and $NO^{\cdot}$ in the spleen during LM infection. Splenocytes were harvested from B6 and IL-23p19 KO mice that were infected with LM for 1 or 3 days, and quantitative assays were performed on cultured splenocyte supernatants. There was a reduction in the overall concentration of TNF-α in the culture supernatants from IL-23p19 KO mice compared to B6 mice at both days 1 and 3 p.i. (Figs. 17A and 17B). Although the concentration of $NO^{\cdot}$ did not differ between the groups at day 1 p.i. (Fig. 17C), it was significantly reduced in the supernatants from IL-23p19 KO mice compared to B6 mice at day 3 p.i. (Fig. 17D). To further examine if exogenous stimulation with IL-23 or IL-17A could directly induce or enhance the production of TNF-α and $NO^{\cdot}$, splenocytes from day 1 post-LM infected B6 mice were cultured in the presence of titrated doses of IL-23 or IL-17A. Addition of IL-23 or IL-17A did not enhance the production of TNF-α or $NO^{\cdot}$ from LM-infected B6 splenocytes (Figs. 18A and 18B). Next, we investigated whether exogenous stimulation with IL-23 or IL-17A could restore the production of TNF-α or $NO^{\cdot}$ from splenocytes of IL-23p19 KO mice to the extent of production observed in splenocytes from B6 mice. To examine this notion, splenocytes were harvested from day 1 post-LM infected B6 mice and IL-23p19 KO mice, and cultured with 10 ng/ml IL-23 or IL-17A in the presence or absence of HKLM. Since there was no dose-dependent effect of IL-23 or IL-17A on the production of TNF-α or $NO^{\cdot}$ from B6 splenocytes, a dose of 10 ng/ml was chosen. Addition of IL-23 or IL-17A did not increase the

production of TNF-α or $NO^{\cdot}$ from splenocytes of IL-23p19 KO mice compared to B6 mice during LM infection (data not shown). These results indicate that exogenous stimulation with IL-23 or IL-17 does not increase the production of TNF-α or $NO^{\cdot}$; however, the absence of IL-23 reduces the overall levels of these pro-inflammatory mediators in the spleen during LM infection.

IL-23 enhances the recruitment of TNF-α and iNOS-producing inflammatory monocytes to the spleen during LM infection

As demonstrated earlier, there is an overall reduction in the concentrations of TNF-α and $NO^{\cdot}$ in the splenocyte culture supernatants of IL-23p19 KO mice compared to B6 mice (Fig. 18). This reduction could be attributed to either inefficient production of TNF-α and $NO^{\cdot}$ by cells, or inadequate number of cells producing TNF-α and $NO^{\cdot}$ in the spleen of IL-23p19 KO mice compared to B6 mice. We have already established that the lack of IL-23 does not impact the ability of inflammatory monocytes or neutrophils to produce TNF-α or iNOS during LM infection (Figs. 14 and 17). This would suggest that the observed reduction in TNF-α and $NO^{\cdot}$ levels could be due to fewer cells making these pro-inflammatory mediators. We have shown that IL-23 is required for optimal recruitment of inflammatory monocytes during LM infection (Figs. 4 and 5). Importantly, IL-23p19 KO mice have reduced percentages and total numbers of inflammatory monocytes in the spleen compared to B6 mice at days 1 and 3 post-LM infection (Figs. 4 and 5). It is well established that LM infection induces the differentiation of TNF-α and iNOS-producing inflammatory monocytes in the spleen. Therefore, to determine if the absence of IL-23 impacts the percentages and total numbers of TNF-α and iNOS-producing inflammatory monocyte, intracellular cytokine staining for TNF-α and iNOS was performed on splenocytes isolated from B6 and IL-23p19 KO mice infected with LM for 1 or 3 days. The deficient

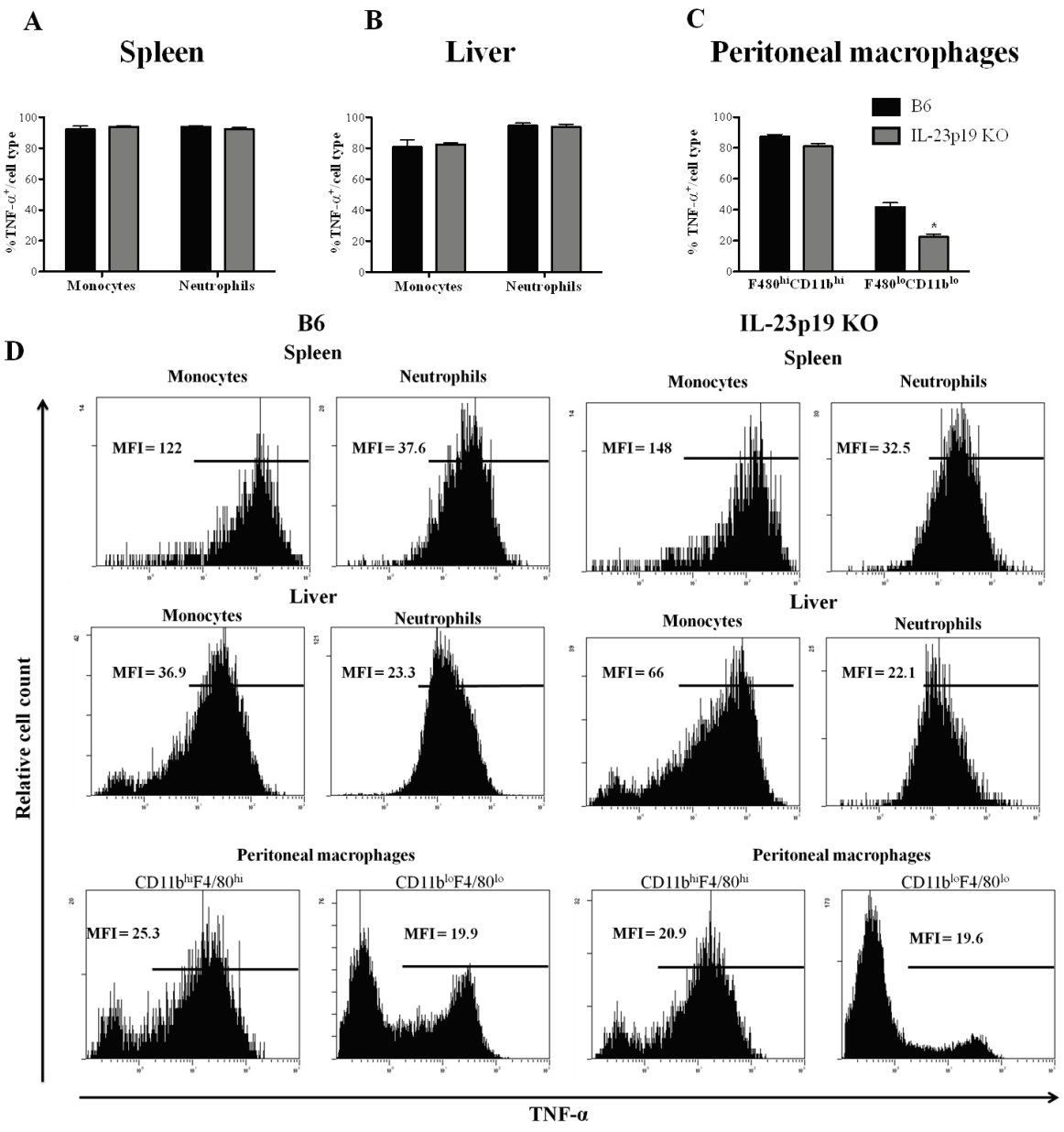

A
Spleen
% TNF-α+ / cell type
Monocytes
Neutrophils
B
Liver
C
Peritoneal macrophages
B6
IL-23p19 KO
F480hiCD11bhi
F480loCD11blo
D
B6
IL-23p19 KO
Spleen
Liver
Peritoneal macrophages
MFI= 122
MFI= 37.6
MFI= 148
MFI= 32.5
MFI= 36.9
MFI= 23.3
MFI= 66
MFI= 22.1
MFI= 25.3
MFI= 19.9
MFI= 20.9
MFI= 19.6
Relative cell count
TNF-α
E
Spleen
MFI of TNF-α+ cells
F
Liver
G
Peritoneal macrophages
n.s.
MFI of TNF-α+ macrophages

Figure 16: IL-23 is not required for the production of TNF-α during LM infection.

B6 and IL-23p19 KO mice were infected with ~10^4 LM for one day. Splenocytes, liver leukocytes, and peritoneal macrophages were harvested. Flow cytometric analysis was performed to determine the production of TNF-α by inflammatory monocytes ($Ly6C^{hi}CD11b^{int}$) and neutrophils ($Ly6G^{+}CD11b^{+}$) in both the spleen and liver, and peritoneal macrophages ($F4/80^{hi}CD11b^{hi}$ and $F4/80^{lo}CD11b^{lo}$). The percentages of TNF-α producing inflammatory monocytes and neutrophils in both the spleen (A) and liver (B), and peritoneal macrophages (C) were determined. Representative histograms of TNF-α^{+} cells are shown (D). The MFI of TNF-α^{+}inflammatory monocytes and TNF-α^{+} neutrophils in both the spleen (E) and liver (F), and TNF-α^{+} peritoneal macrophages (G) were also determined. A two-way ANOVA was performed to determine statistical differences between groups. An asterisk (*) indicates significant difference from B6 mice. All data are expressed as mean ± SEM (n=5/group). These data are representative of two independent experiments.

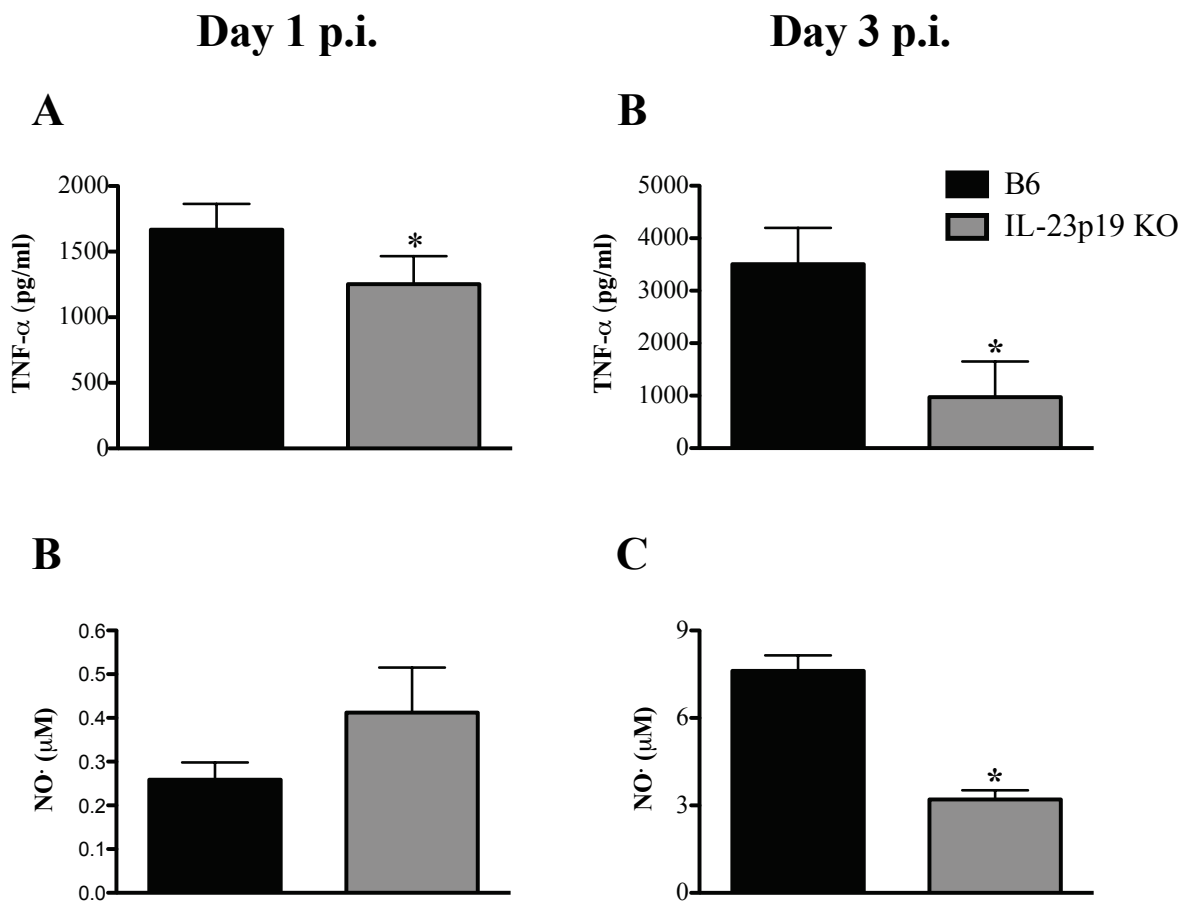


Figure 17: The absence of IL-23 reduces the overall production of TNF-α and NO· from splenocytes during LM infection.

B6 and IL-23p19 KO mice were infected with ~10^4 LM for 1 or 3 days. The overall production of TNF-α (A and B) and NO· (C and D) was determined by performing ELISAs on supernatants from overnight cultures of splenocytes stimulated with HKLM. A t-test was performed to determine statistical differences between groups. An asterisk (*) indicates a significant difference from B6 mice. All data are expressed as mean ± SEM (n=5/group). These data are representative of two independent experiments.

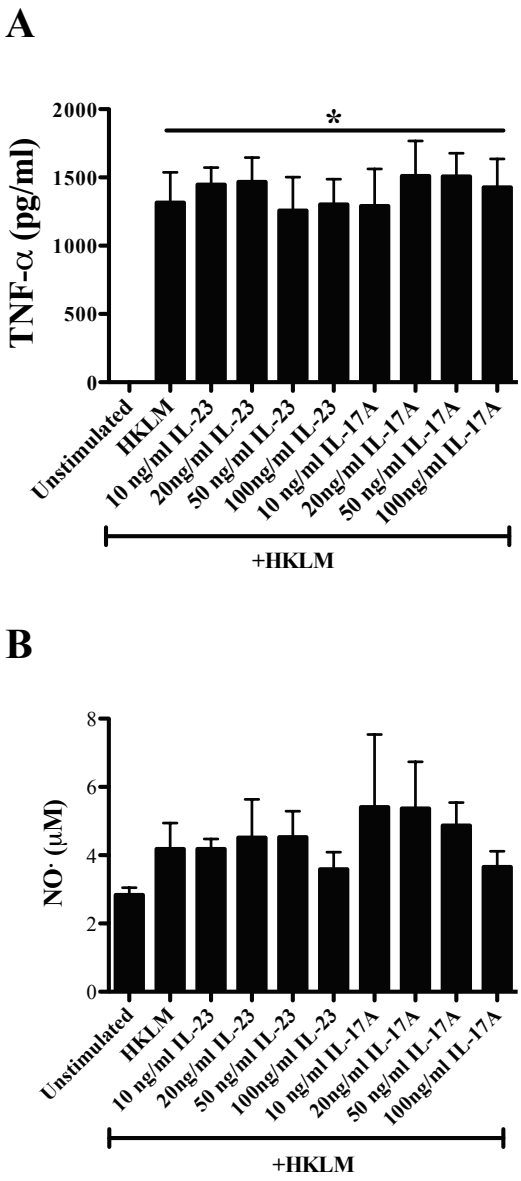
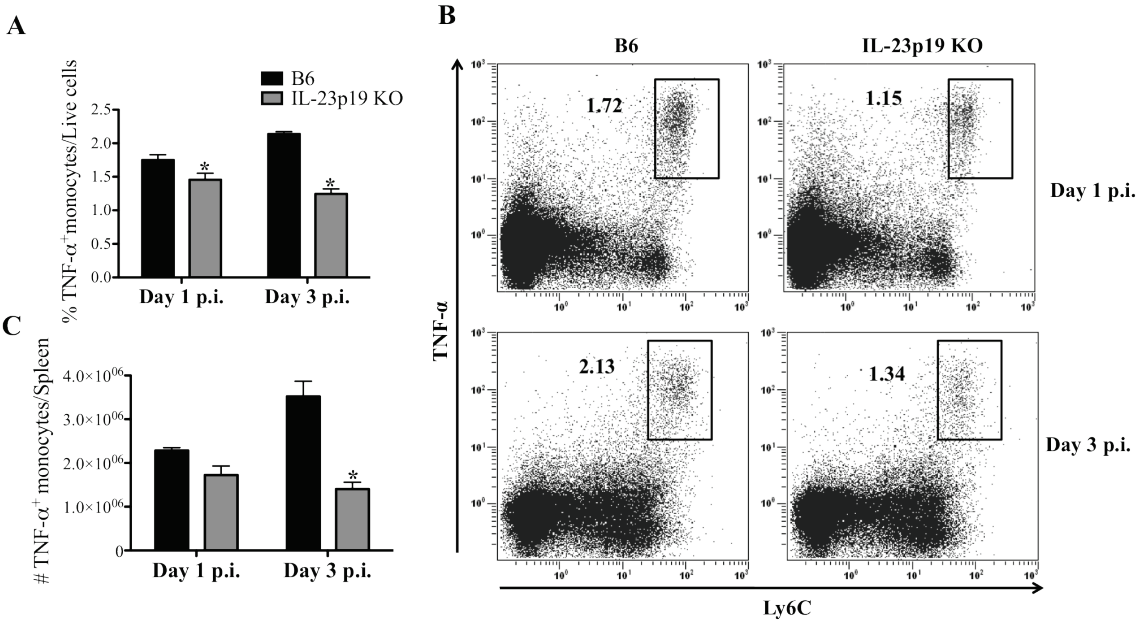


Figure 18: Exogenous stimulation with IL-23 or IL-17A does not enhance the production of TNF-α and NO· from B6 splenocytes during LM infection.

B6 mice were infected with ~10^4 LM for one day. The overall production of TNF-α (A) and NO· (B) was determined by performing assay on supernatants from overnight cultures of unstimulated splenocytes, or stimulated with varying concentrations of IL-23 or IL-17A in the presence of HKLM. A one-way ANOVA was performed to determine statistical differences between groups. An asterisk (*) indicates a significant difference from unstimulated. All data are expressed as mean ± SEM (n=4/group). These data are representative of one independent experiment.

recruitment of inflammatory monocytes to the spleen of IL-23p19 KO mice compared to B6 mice resulted in fewer inflammatory monocytes producing TNF-α, with a decrease in the percentage (Figs. 19A and 19B) and total numbers of TNF-α-producing inflammatory monocytes at both days 1 and 3 p.i. (Fig. 19C). Additionally, at day 3 p.i. there was a reduction in the percentages (Figs. 19D and 19E) and numbers of iNOS-expressing inflammatory monocytes in the IL-23p19 KO mice compared to B6 mice (Fig. 19F). These results indicate that the decreased recruitment of TNF-α and iNOS-expressing inflammatory monocytes to the spleen in IL-23p19 KO mice during LM infection causes a reduction in the overall amounts of these inflammatory mediators.

TNF-α


A
B6
IL-23p19 KO
% TNF-α+monocytes/Live cells
Day 1 p.i.
Day 3 p.i.
B
B6
IL-23p19 KO
1.72
1.15
2.13
1.34
Day 1 p.i.
Day 3 p.i.
TNF-α
Ly6C
C
TNF-α+ monocytes/Spleen
Day 1 p.i.
Day 3 p.i.

iNOS

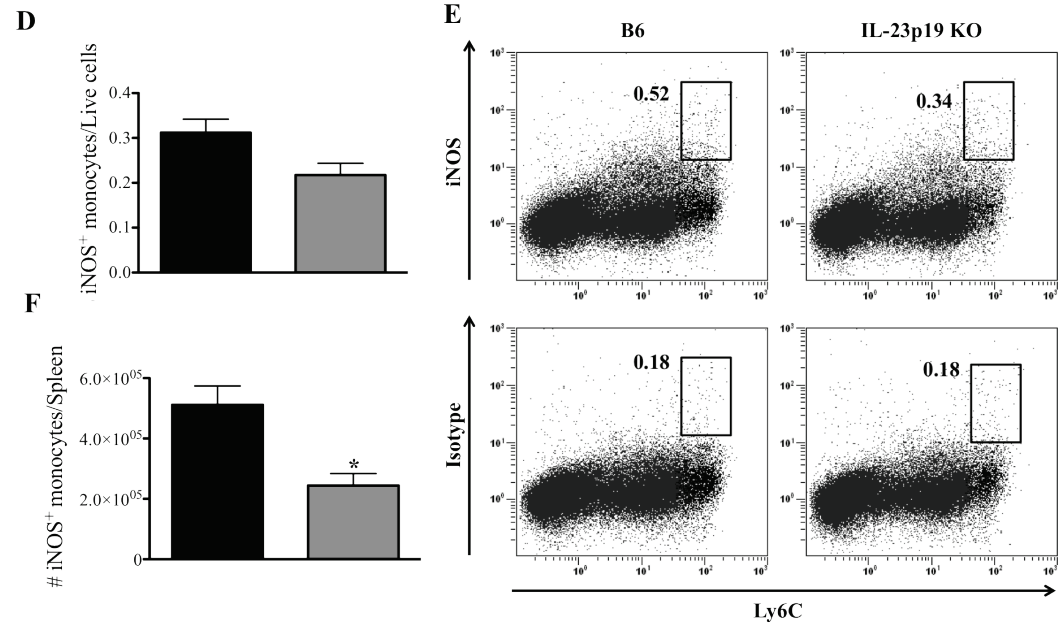

D
iNOS+ monocytes/Live cells
E
B6
IL-23p19 KO
0.52
0.34
iNOS
F
iNOS+ monocytes/Spleen
0.18
0.18
Isotype
Ly6C

Figure 19: Optimal recruitment of TNF-α- and iNOS-producing inflammatory monocytes to the spleen is dependent on IL-23 during LM infection.

B6 and IL-23p19 KO mice were infected with ~10^4 LM for 1 or 3 days. Flow cytometric analysis was performed on isolated splenocytes to determine the expression of TNF-α or iNOS by monocytes ($Ly6G^{-}Ly6C^{hi}$). The percentages (A) and numbers (C) of TNF-α-producing monocytes in the spleen at days 1 and 3 p.i. were determined from dot plots (B). The percentages (D) and numbers (F) of iNOS-expressing monocytes in the spleen at day 3 p.i. were obtained after the corresponding isotype control values determined from dot plots (E) were subtracted. A two-way ANOVA (A and C) and t tests (D and F) were performed to determine statistical differences between groups (*, significantly different from B6 mice). All data are expressed as means ± SEM (n = 5/group). These data are representative of two independent experiments.

Summary of Chapter IV

The data presented in this chapter demonstrate that IL-23 does not impact the function of inflammatory monocytes, neutrophils, and peritoneal macrophages during LM infection. The percentages of IL-17RA expressing-inflammatory monocytes and neutrophils, in the spleen and liver, increase upon LM infection. Additionally, the absence of IL-23 does not alter the expression of IL-17RA during LM infection (Fig. 10). IL-23 is not required for inflammatory monocytes, neutrophils, and peritoneal macrophages to perform phagocytosis (Fig. 11). Interestingly, IL-23 in combination with IL-6 increases the oxidative burst from bone marrow neutrophils during LM infection (Fig. 12). However, the absence of IL-23 does not impact the production of ROS, iNOS, MPO, or TNF-α, from inflammatory monocytes and neutrophils (Figs. 13, 14, 15, and 16) as well as the production of iNOS, MPO, or TNF-α from peritoneal macrophages (Figs. 14, 15, and 16). These results indicate that IL-23 is not essential for the optimal function of phagocytic cells during LM infection. Surprisingly, the lack of IL-23 results in a reduction in the overall levels of TNF-α and $NO^{\cdot}$ in the spleen of IL-23p19 KO mice compared to B6 mice (Fig. 17). However, exogenous stimulation of B6 splenocytes with IL-23 or IL-17A did not induce or enhance the production of TNF-α and $NO^{\cdot}$ during LM infection (Fig. 18). The fact that IL-23 does not impact the function of phagocytic cells establishes that the observed reduction, in TNF-α and $NO^{\cdot}$ levels in the spleen of IL-23p19 KO mice, is not due to a cell intrinsic defect. An important source of TNF-α and $NO^{\cdot}$ in the spleen is inflammatory monocytes, and the optimal recruitment of these cells to the spleen is dependent on IL-23 during LM infection. The absence of IL-23 results in a decrease in the percentages and total numbers of $TNF\text{-}\alpha^{+}$ and $iNOS^{+}$ inflammatory monocytes in the spleen during LM infection (Fig. 19), causing the observed reduction in the levels of TNF-α and $NO^{\cdot}$. Collectively, our data suggest that the

lack of efficient recruitment of TNF-α and iNOS-producing monocytes to the spleens during the early stages of LM infection could lead to increased bacterial burden, and therefore enhanced susceptibility of IL-23p19 KO mice to LM infection.

CHAPTER V

DISCUSSION

Overview of results

This study establishes that IL-23 mediates protection against LM infection in the spleen and liver by regulating the recruitment of innate immune cells in an organ-specific manner. First, we addressed whether the increased susceptibility as well as the enhanced liver bacterial burden, observed in IL-23p19 KO mice could be attributed to the deficient neutrophil recruitment to the liver during LM infection. The enhancement in the susceptibility of neutrophil-depleted IL-23p19 KO to LM infection compared to all the other groups, including isotype-treated B6 mice, neutrophil-depleted B6 mice, and isotype-treated IL-23p19 KO mice, suggests in addition to regulating neutrophil recruitment to the liver, IL-23 could be required for promoting the recruitment or function of another immune cell during LM infection. Importantly, the abrogation of differences in bacterial burden between livers of neutrophil-depleted B6 and IL-23p19 KO mice confirms that indeed, the deficient recruitment of neutrophils to the liver is causal for increased bacterial burden in this organ. As it can be seen in Fig. 3, there is no difference in susceptibility between isotype-treated B6 mice and neutrophil-depleted B6 mice. This observation is in accordance with our previous study wherein a low dose of ~10^4 LM did not impact the susceptibility of neutrophil-depleted B6 mice compared to isotype-treated B6 mice; However, a high dose of ~3.5 x 10^4 LM rendered neutrophil-depleted B6 mice susceptible to LM

infection compared to isotype-treated B6 mice. In the current study, we utilized a low dose LM infection to be able to clearly demonstrate the difference in survival between neutrophil-depleted B6 and IL-23p19KO mice. Based on our previous survival studies with a high dose LM infection, we can predict that isotype-treated B6 mice will demonstrate ~100% survival compared to neutrophil-depleted B6 mice, isotype treated-IL-23p19 KO mice, and neutrophil-depleted IL-23p19 KO mice. Additionally, neutrophil-depleted B6 and isotype-treated IL-23p19 KO mice will display similar susceptibility and succumb to LM infection between days 4 and 10 p.i. Neutrophil-depleted IL-23p19 KO mice will be very highly susceptible compared to all the other groups of mice and rapidly succumb to LM infection between days 1 and 4 post-LM infection. Thus, even at a high dose of ~3.5 x 10^4 LM, it is likely that neutrophil-depleted IL-23p19 KO mice will display enhanced susceptibility to LM infection compared to isotype-treated B6 mice, neutrophil-depleted B6 mice, and isotype-treated IL-23p19 KO mice, suggesting that our interpretation that IL-23 regulates the recruitment or function of another immune cell type will still be true at this high dose infection.

The increase in bacterial burden in the spleens of neutrophil-depleted IL-23p19 KO mice compared to neutrophil-depleted B6 mice demonstrates that IL-23 enhances the recruitment or function of another immune cell in this organ during LM infection. Accordingly, the lack of IL-23 results in reduced percentages of inflammatory monocytes in the blood, spleen, and liver during LM infection. Thus, IL-23 is essential for the optimal recruitment of inflammatory monocytes during LM infection. Importantly, this reduction in the percentages of inflammatory monocytes in IL-23p19 KO mice coincides with a decrease in the levels of the monocyte-recruiting chemokines, CCL2 and CCL7. It has been shown that emigration of inflammatory monocytes from the bone marrow requires the concerted action of CCL2 and CCL7 (22).

Additionally, IL-23 is required for the production of growth factors including GM-CSF (85). Therefore, the reduced monocyte percentages observed in the periphery of IL-23p19 KO mice could be due to the increased retention in the bone marrow or defects in generation. However, IL-23 did not impact the generation or maintenance of monocytes in the bone marrow during LM infection. Thus, IL-23 mediates the recruitment of inflammatory monocytes to the spleen by regulating the production of CCL2 and CCL7 during LM infection.

Next, we investigated the notion that IL-23 could promote the clearance of LM by regulating the function of different phagocytic cells including, inflammatory monocytes, neutrophils, and peritoneal macrophages. Many reports have demonstrated that IL-23R is expressed on macrophages and neutrophils (86, 102), supporting our notion that IL-23 could act directly to regulate phagocytic cell functions during LM infection Additionally, the percentages of IL-17RA expressing inflammatory monocytes and neutrophils in the spleen and liver increase upon LM infection, suggesting a possible indirect mechanism for IL-23 to regulate phagocytic cell functions. One of the reasons for increased LM CFUs in the infected organs of IL-23p19 KO mice could be due to inefficient phagocytosis. However, the phagocytic potential of inflammatory monocytes, neutrophils, and peritoneal macrophages, measured using pHrodo conjugated *E.coli* bioparticles, did not differ between B6 and IL-23p19 KO mice. This suggests that phagocytic cells from IL-23p19 KO mice could be as competent as B6 mice in engulfing LM.

Eradication of pathogen requires effective killing by phagocytic cells. It is possible that the absence of IL-23 impairs the ability of phagocytic cells to undergo oxidative burst or produce enzymes associated with the generation of ROS/RNS. Interestingly, exogenous stimulation with a combination of IL-23 and IL-6 increases ROS production from neutrophils in the bone marrow,

indicating a possible role for IL-23 in enhancing oxidative burst. However, the lack of IL-23 did not impact the production of ROS from inflammatory monocytes and neutrophils in the spleen and liver during LM infection. Likewise, phagocytic cells from B6 and IL-23p19 KO mice did not exhibit biologically significant differences in the production of MPO or iNOS or TNF-α during LM infection. Thus, IL-23 is not required for the optimal function of phagocytic cells during LM infection.

Interestingly, the reduction in the overall levels of TNF-α or $NO^{\cdot}$ from splenocytes of IL-23p19 KO mice indicates that IL-23 regulates the production of these important pro-inflammatory mediators during LM infection. However, the absence of IL-23 did not impact the ability of inflammatory monocytes to produce TNF-α or iNOS. Furthermore, exogenous stimulation with IL-23 or IL-17A does not increase the ability of splenocytes to produce TNF-α and $NO^{\cdot}$ during LM infection. It is known that inflammatory monocytes are an important source of TNF-α or $NO^{\cdot}$ in the spleen (29). The overall production of TNF-α and $NO^{\cdot}$ is reduced in IL-23p19 KO mice, which corresponds with reduced numbers and percentages of TNF-α and iNOS-expressing monocytes in the spleens of infected IL-23p19 KO mice. Taken together, our data suggest that the deficient recruitment of TNF-α and iNOS-producing monocytes to the spleen of IL-23p19 KO mice leads to increased bacterial burden in this organ, and enhances the susceptibility of IL-23p19 KO mice to LM infection.

Discussion and future directions

The recruitment of innate immune cells, particularly neutrophils, mediated by the IL-23/IL-17 axis has been widely demonstrated in different disease models (85, 114). IL-17 has been shown to facilitate the mobilization of neutrophils to sites of infection by inducing the

production of neutrophil chemoattractants, such as CXCL1, CXCL2, and CXCL8 (85). Moreover, IL-17RA KO mice display a reduction in the percentages and total numbers of neutrophils in the liver during LM infection (43). Our neutrophil depletion studies show that deficient neutrophil recruitment in IL-23p19 KO mice is likely causal for increased liver CFUs. These observations are in line with our previous findings that the lack of IL-23 results in diminished recruitment of neutrophils to the liver, and that neutrophils are required for protection in the liver at all doses of LM tested (36, 43). Therefore, during systemic LM infection, neutrophils confer protection in the liver, and the IL-23/IL-17 axis mediates recruitment of neutrophils to this organ.

Our finding that the depletion of neutrophils did not abrogate the differences in bacterial burden in the spleens of IL-23p19 KO mice and B6 mice supports the notion that IL-23 could regulate the recruitment of immune cells in an organ-specific manner. Importantly, our data establish that the absence of IL-23 results in deficient recruitment of inflammatory monocytes to the blood, spleen, and liver during LM infection. Inflammatory monocytes are the major responders against LM infection in the spleen (111). Seminal studies have established that the mobilization of inflammatory monocytes is dependent on chemokines, CCL2 and CCL7 (22, 23) The emigration of inflammatory monocytes from the bone marrow is driven by bone marrow mesenchymal stem cells (MSC), which produce CCL2 in response to TLR stimulation. Interestingly, targeted-deletion of CCL2 from bone marrow MSCs resulted in a modest reduction in inflammatory monocyte percentages in the circulation, compared to the complete absence of CCL2, during LM infection suggesting that cells other than MSCs could produce CCL2, and promote inflammatory monocyte recruitment (26). In our model, the lack of IL-23 reduces the production of CCL2 from bone marrow cells stimulated with HKLM; however, this reduction

did not lead to increased retention of inflammatory monocytes in the bone marrow of IL-23p19 KO mice. Even though the amount of CCL2 produced by bone marrow cells from IL-23p19 KO mice is comparatively less than that produced by bone marrow cells from B6 mice, it is sufficient to facilitate the release of inflammatory monocytes from the bone marrow. Therefore, IL-23 mediated reduction in CCL2 levels in the bone marrow does not impact the emigration of inflammatory monocytes to the periphery during LM infection.

Chemokines produced at the site of infection form a gradient to guide immune cells in the circulation or bone marrow to the foci of infection. The chemokines, CCL2 and CCL7 promote the recruitment of inflammatory monocytes during infection with several pathogens, including LM (17). Our study demonstrates that IL-23 is required for the optimal production of CCL2 and CCL7 during LM infection. Therefore, the impaired mobilization of inflammatory monocytes in LM-infected IL-23p19 KO mice compared to B6 mice could be attributed to the reduction in monocyte-recruiting chemokines in the circulation as well as in the spleen. Chemokines can be produced by both immune and non-immune cells during infection. Studies indicate that LM infection induces the production of CCL2 and CCL7 from splenic and bone marrow-derived macrophages (23, 24, 115). It is possible that the absence of IL-23 impacts the ability of macrophages in the spleen to produce CCL2 and CCL7, and causes a reduction in the overall levels of these chemokines during LM infection. Interestingly, rescue experiments performed by injecting IL-17A into IL-23p19 KO mice partially reduced LM burden in the spleen, compared to B6 mice (43), suggesting that IL-17A could enhance the recruitment of inflammatory monocytes to this organ. In a rheumatoid arthritis model, IL-17 mediates monocyte recruitment both directly, by acting as a chemoattractant, and indirectly, by inducing the production of CCL2 (19, 116). Thus, our study proposes a potential IL-23/IL-17/monocyte axis during bacterial

infections. Furthermore, this differential cell requirement in the spleen versus the liver highlights how the immune responses are tailored, according to the organ, to effectively clear the infection.

One of the emerging concepts is that the IL-23/IL-17 axis, in addition to regulating recruitment of immune cells, could enhance the function of phagocytic cells. The expression of receptors for IL-17 and IL-23 has been detected on phagocytic cells in different models. Our finding that there is an increase in the percentages of inflammatory monocytes and neutrophils that express IL-17RA during LM infection indicates a potential role for IL-23/IL-17 axis in modulating the function of phagocytic cells during LM infection.

Phagocytosis is the first step towards pathogen clearance. It was reported that IL-17A could enhance the uptake of apoptotic neutrophils by macrophages (106). We expected that the reduced levels of IL-17A and IL-17F in IL-23p19 KO mice would impact the phagocytic potential of cells from these mice. However, the absence of IL-23 did not impact the ability of inflammatory monocytes, neutrophils, and macrophages to perform phagocytosis of *E.coli* conjugated bioparticles. This assay utilizes bacteria-like bioparticles that might stimulate cells differently than a live LM infection. Therefore, our results do not preclude the possibility that phagocytic cells from IL-23p19 KO mice might be inefficient in phagocytosing live LM compared to B6 mice. Another point to consider is that in the reported research, IL-17A induced the expression of the receptor, LOX-1 that is predominantly involved with the uptake of aged or dying cells (106). Our study utilizes opsonized *E.coli* bioparticles that are predominantly internalized by Fc receptor- or complement receptor-mediated endocytosis. The observation that phagocytic cells, from both B6 and IL-23p19 KO mice, are equally able to uptake *E.coli* bioparticles suggests that IL-23 does not regulate the expression of receptors mediating phagocytosis of these bioparticles.

The absence of IL-23 did not impact the ability of inflammatory monocytes and neutrophils to make ROS during LM infection. Interestingly, exogenous stimulation with IL-23 and IL-6 increases neutrophil-derived ROS production during LM infection. Therefore, this IL-23/IL-6 induced ROS production might require the presence of these cytokines at certain concentrations at the site of infection. This could also be mediated by IL-23 and IL-6 induced IL-17 production as demonstrated with an *Aspergillus fumigatus* infection model (86). It is important to note that dectin-1 mediated recognition of *A. fumigatus* induced the production of IL-23 and IL-6, which collectively induced IL-17A production from neutrophils. This would suggest that the type of PRR i.e. TLR or dectin-1 triggered could modulate the production of pro-inflammatory mediators. It would be interesting to determine how triggering of different PRRs impacts neutrophil response by stimulating neutrophils with different PRR ligands and quantifying the production of IL-23, IL-6, IL-17A, and ROS.

Previous studies have shown that peritoneal macrophages upregulate TNF-α mRNA following *in vivo* administration of IL-23 (89), and transgenic expression of IL-23p19 results in increased serum levels of TNF-α (91). The regulation of TNF-α levels by IL-23 in the mentioned studies could be due to an increase in recruitment of TNF-α producing cells. Our results show that the absence of IL-23 does not impact the production of TNF-α from inflammatory monocytes or neutrophils during LM infection. Interestingly, there is a reduction in the percentage of TNF-α^{+}F4/80^{lo}CD11b^{lo} macrophages in IL-23p19 KO mice compared to B6 mice. It is thought that this subset of peritoneal macrophages differentiates from inflammatory monocytes during inflammation. Studies have indicated that IL-23 stimulation of human monocyte-derived macrophages increased the production of TNF-α. Therefore, it would be

interesting to determine whether exogenous stimulation of F4/80^{lo}CD11b^{lo} macrophages with IL-23 induces production of TNF-α and other pro-inflammatory mediators.

The functional ability of phagocytic cells was not altered in the absence of IL-23 as measured by phagocytosis and production of pro-inflammatory mediators during LM infection. A better way to investigate if IL-23 was required for the optimal function of phagocytic cells would be to perform *in vitro* killing assays with neutrophils and macrophages isolated from B6 and IL-23p19 KO mice. Additionally, LM co-localization studies could be performed to determine escape from the phagosome by quantitating LM associated with host actin in the host cytosol. These experiments could be technically challenging. Alternatively, a flow cytometry based killing assay can be performed by incubating GFP-tagged LM with splenocytes or liver leukocytes from B6 mice in the presence of IL-23 or IL-17 stimulation. These experiments might identify a potential role for IL-23 or IL-17 in enhancing bacterial killing.

The relative abilities of B6 and IL-23p19 KO mice to clear LM infection change as the infection progresses. At early stages of LM infection, IL-23p19 KO have reduced bacterial burden in the spleen and liver compared to B6 mice at days 1 and 3 p.i. (90). However, by day 5 p.i., the bacterial burden is higher in IL-23p19 KO mice compared to B6 mice (43). The cause for this difference in bacterial clearance by IL-23p19 KO mice, compared to B6 mice, at different time points during the course of LM infection is intriguing. It is possible that in IL-23p19 KO mice, LM are not trapped effectively in the spleen and liver, causing LM to spread systemically to other organs, such as brain and heart, leading to sepsis and enhanced susceptibility. However, there were no differences in LM CFUs, in the brain, heart, or gall bladder, between B6 and IL-23p19 KO mice at days 1 and 3 p.i suggesting that the observed reduction in bacterial burden in the spleen and liver of IL-23p19 KO mice is certainly not due to

increased dissemination (data not shown). Previous studies from our lab have shown that IL-23p19 KO mice produce more IFN-γ compared to B6 mice at days 1 and 3 p.i., and this early IFN-γ promotes LM clearance in IL-23p19 KO mice. Although localized production of IFN-γ is beneficial for defense against pathogens, increased systemic levels of IFN-γ could be detrimental to the host (117). Therefore, the production of IFN-γ could be regulated by immunomodulatory cytokines such as IL-10 or IFN-αβ. It is known that IL-10 or IFN-αβ negatively impact clearance of LM (79, 118). However, there were no differences in the production of IL-10 in the serum or spleen culture supernatants between B6 and IL-23p19 KO mice at day 3 p.i. (data not shown). Likewise, we were unable to detect differences in the expression of IFN-αβ mRNA in the spleen and liver between B6 and IL-23p19 KO mice at days 3 and 5 p.i. (data not shown). A recent study demonstrated IFN-γ could impair the mobilization of granulocytes by reducing the expression of CXCR2 (41). However, CXCR2 expression on the neutrophils and inflammatory monocytes from IL-23p19 KO mice remained unchanged compared to B6 mice during LM infection (data not shown). This would suggest that the high levels of IFN-γ in IL-23p19 KO mice do not impact CXCR2 expression on neutrophils and inflammatory monocytes, or levels of IL-10 and IFN-αβ, during LM infection. Further investigation is needed to obtain conclusive data to understand the cause for the sudden change in the infection dynamics in IL-23p19 KO mice from day 1 to day 3 p.i.

In order to identify other possible IL-23-mediated immune functions during LM infection, we performed an RT PCR array for genes associated with autoimmunity and inflammation on spleen from B6 and IL-23p19 KO mice at day 1 p.i. The genes that are upregulated over 2 fold in IL-23p19 KO mice, compared to B6 mice, are shown in Fig. 20. Notably, the lack of IL-23 resulted in the upregulation of genes for chemokines, CCL20, CCL22,

Gene	Fold regulation in IL-23p19 KO mice compared to B6 mice
CCL20	2.2723
CCL22	3.8319
CCL24	5.9898
CCR2	2.4305
CCR3	2.1791
CD40 ligand	2.4713
CXCR1	2.4009
IFN-γ	4.7064
IL-18	2.0275
Integrin β2	2.3347
Lymphotoxin α	2.8509
CCL3	-2.1798
CXCL11	-2.962
CXCL2	-2.6431
IL-10	-3.1949
IL-23p19	-24.0446

Figure 20: Genes regulated by IL-23 in the spleen during LM infection

and CCL24. These chemokines have been associated with differentiation of M2 macrophages, which display an anti-inflammatory phenotype, and are associated with wound repair. M2 macrophages are not beneficial for clearance of intracellular pathogens. It is possible that the absence of IL-23 skews the polarization of macrophages to a M2 phenotype. Therefore, IL-23p19 KO mice might have increased percentages of M2 macrophages in the spleen, compared to B6 mice, which could lead to bacterial clearance from this organ. Further studies are required understand how IL-23 impacts the differentiation of M1 and M2 macrophages during LM infection.

The role of IL-23 during LM infection

Based on this research and previous studies in our lab, a working model on how IL-23 regulates the immune response during LM infection is shown Fig. 21. Activated macrophages and DCs produce IL-23, which regulates immune response against LM via different mechanisms. IL-23 negatively regulates the immune response against LM by inhibiting IL-12 induced IFN-γ production from CD8 T and NK cells, enabling better clearance of LM from IL-23p19 KO mice, compared to B6 mice, at early time points. In contrast, IL-23 is required for the optimal production of IL-17 and IL-22 during LM infection. Interestingly, IL-22 is not necessary for the eradication of LM or protection of tissues from infection-induced tissue damage. In the LM-infected liver, IL-23 induces γδ T cells to secrete IL-17A and IL-17F that promote neutrophil recruitment to this organ, probably by inducing the secretion of neutrophil-recruiting chemokines. During LM infection, IL-23 facilitates the recruitment of inflammatory monocytes to the spleen mediated by CCL2 and CCL7 during LM infection.

Collectively, our research establishes that IL-23 provides protection against an intracellular bacterial infection by enhancing the recruitment of innate immune cells. IL-23 regulates the recruitment in an organ-specific fashion by mobilizing the recruitment of neutrophils to the liver and inflammatory monocytes to the spleen, respectively. Importantly, this study has identified a novel role for IL-23 in regulating the recruitment of inflammatory monocytes. Considering that inflammatory monocytes can be both beneficial and detrimental, depending on the disease condition, it is essential to understand the mechanisms promoting their recruitment and function. Ultimately, these studies will aid in the design and development of effective therapeutic targets in order to manipulate the immune response in order to treat disease.

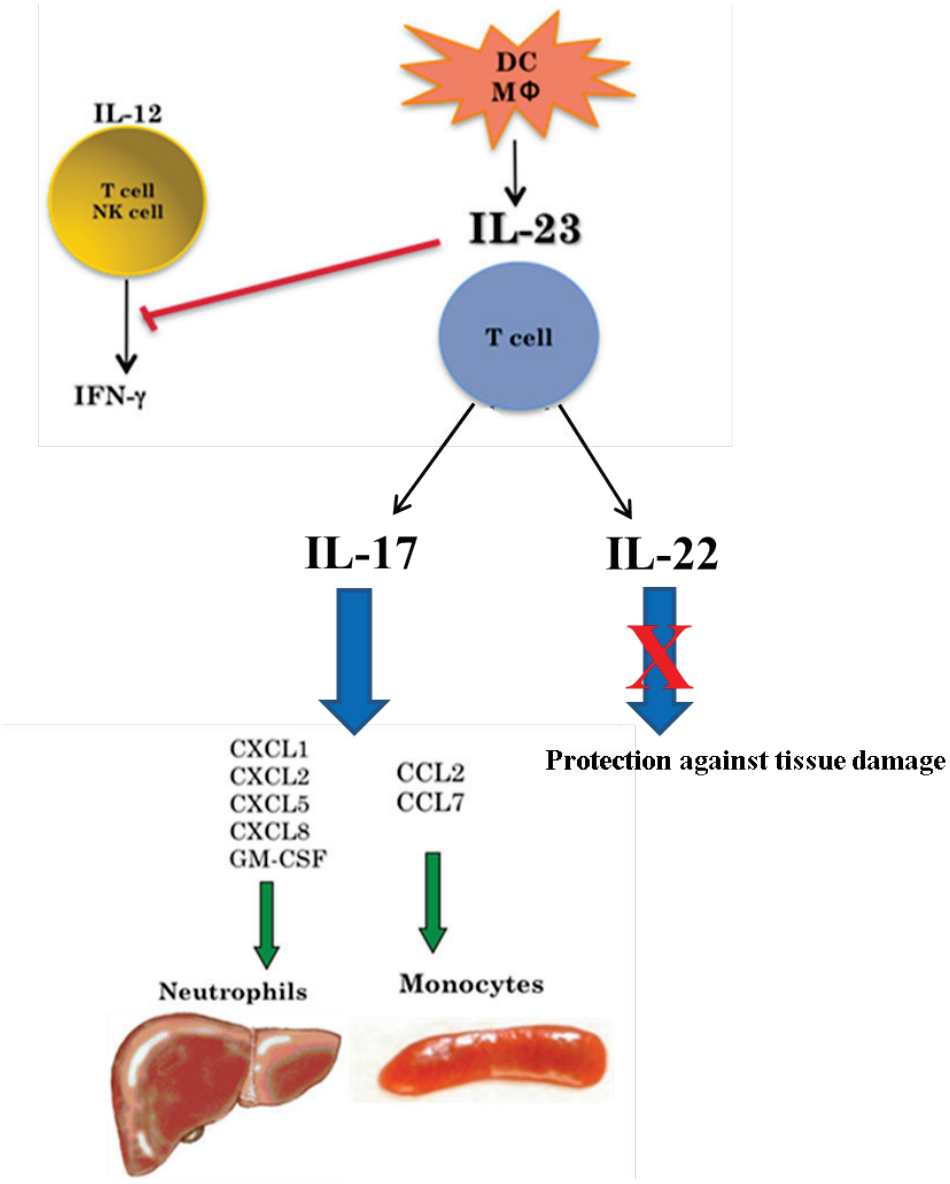


Figure 21: The role of IL-23 during LM infection

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