Okunnu, Busola M., The Divergent Roles of Neutrophils and Monocytes During Infection with the Bacterium, *Listeria monocytogenes*. Doctor of Philosophy (Biomedical Sciences), November, 2019, 95 pp., 33 figures, references, 123 titles.

Neutrophils are polymorphonuclear phagocytic cells that can play both protective and damaging roles during innate immune responses to infection and disease. Therefore, gaining a complete understanding of the functions and capabilities of neutrophils is vital. Our studies on neutrophil function were focused on their role during bacterial infections and were conducted using a *Listeria monocytogenes* (*Lm*) mouse infection model. "*Lm* is an intracellular bacterium that causes diseases such as gastroenteritis, spontaneous abortion and meningitis." *Lm* infection in mice is commonly used as a model to study cell mediated immune responses and previous studies have demonstrated that neutrophils are essential for host protection against the bacteria. A major function attributed to neutrophils is the killing of pathogens via the generation of Reactive Oxygen Species (ROS). The unintentional damage to host cells by ROS is mitigated by antioxidants such as superoxide dismutases (SODs) which convert superoxide into hydrogen peroxide.

Our studies focused on how one of the SODs, extracellular superoxide dismutase (ecSOD), modulates the function of neutrophils during *Lm* infection. Congenic mice with differing activities of ecSOD: high (ecSOD HI), normal (ecSOD WT), and no activity (ecSOD KO) were used for these studies. Previous studies from our lab have shown that the ecSOD HI mice are more susceptible to *Lm* infection in comparison to ecSOD KO mice, though the ecSOD HI mice have a higher percentage of neutrophils recruited to the sites of infection. Therefore, the purpose of these studies was to determine how ecSOD activity modifies functions such as phagocytosis, phagosomal containment and killing of *Lm* by neutrophils. EcSOD activity correlated with increased presence of extracellular bacteria in the spleen after three days of infection. High activity

of ecSOD was also observed to decrease neutrophil phagocytosis of *Lm*. However, surprisingly, ecSOD activity had no effect on phagosomal containment or killing of *Lm* by neutrophils. As a result, ecSOD activity appears to primarily modify *Lm* uptake by neutrophils during infection.

Another class of innate immune phagocytes that have also been demonstrated to be necessary for protection during *Lm* infection are monocytes. Previous studies also indicate that monocytes play important roles during infection and disease, similarly to neutrophils. Although specific functions have generally been assigned to each of the cells, similarities and differences in functions necessary for *Lm* clearance have not previously been investigated. In the present study, phagocytosis, phagosomal containment, bacterial killing and cytokine production by neutrophils and monocytes during *Lm* infection were studied. Data obtained via *in vitro* studies show that neutrophils are more effective at *Lm* uptake, phagosomal containment, and killing than monocytes. However, monocytes were found to be more effective at cytokine production during *Lm* infection, *in vivo*. Additionally, the data demonstrated that neutrophils and monocytes are also capable of producing IL-1α, a cytokine that does not yet have a clearly defined role during infection with *Lm*. Furthermore, a population of monocytes capable of producing both TNF-α and IL-1α, concurrently, were identified. Collectively, these studies highlight the impact of ecSOD activity on neutrophils, as well as the multi-functional capabilities of neutrophils and monocytes, further adding to our knowledge of these innate immune cells.

THE DIVERGENT ROLES OF NEUTROPHILS AND MONOCYTES DURING INFECTION WITH THE INTRACELLULAR BACTERIUM, *LISTERIA MONOCYTOGENES*

DISSERTATION

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Name	Abbreviation
Actin A	ActA
Actin A:*Listeria* monocytogenes Green Fluorescence Protein	actA:LMGFP
Antigen Presenting Cells	APCs
Bone Marrow Derived Macrophages	BMDMs
Colony Forming Units	CFUs
Complement Receptor 3	CR3
Complement Receptor Ig	CRIg
Damage Associated Molecular Patterns	DAMPs
Dendritic Cells	DCs
Extracellular Matrix	ECM
Green Fluorescence Protein	GFP
Gamma Interferon Lysozyme Thiolreductase	GILT
2', 7' -Dichlorodihydrofluorescein diacetate	H_2DCFDA
Extracellular Superoxide Dismutase	EcSOD
High	HI
Hanks Buffered Saline Solution	HBSS
Heat-Killed *Listeria monocytogenes*	HKLM
Human Growth Factor Receptor	HGFR
Hydrogen Peroxide	H_2O_2
Interferon Gamma	IFN-γ
Interleukin-1 Alpha	IL-1α
Interleukin-1 Beta	IL-1β
Interleukin-1 Receptor	IL-1R
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Inducible Nitric Oxide Synthase	iNOS
Knock Out	KO
Lysosyme Associated Membrane Protein	LAMP-1
Listeria monocytogenes	*Lm*
Listeriolysin O	LLO
Microtubule Associated Protein 1A/1B Light Chain	LC3
Mean Fluorescence Intensity	MFI
Multiplicity of Infection	MOI
Mycobacterium tuberculosis	*Mycobacterium*
Nicotinamide Adenine Dinucleotide Phosphate	NADPH
Natural Killer Cells	NK-cells
Nitric Oxide	NO
Neutrophil Extracellular Traps	NETs
Pathogen Associated Molecular Patterns	PAMPs
Phosphate Buffered Saline	PBS

Name	Abbreviation
Pattern Recognition Receptors	PRRs
Phospholipase C	PLC
Programmed Cell Death 1	PD-1
Reactive Oxygen Species	ROS
Reactive Nitrogen Species	RNS
Salmonella typhimurium	*S. typhi*
Superoxide	O_2^-
Superoxide Dismutase	SOD
Tris Ammonium Chloride	TAC
Toxoplasma gondii	*T. gondii*
Tumor Associated Macrophages	TAMs
Tumor Necrosis Factor Alpha	TNF-α
Tumor Necrosis Factor Receptor1	TNFR1
Wild type	WT
Wild type *Listeria monocytogenes*	WTLM

Abbreviations	Meaning
LM^+	Cells positive that have phagocytosed and/or associated with the bacteria
LM^-	Cells that have not phagocytosed and/or associated with the bacteria
GFP^+	Cells that have allowed for phagosomal escape of the bacteria
GFP^-	Cells that have not allowed for phagosomal escape of the bacteria
$\%LM^+$	Percentage of cells that have phagocytosed and/or associated with the bacteria
$\%LM^-$	Percentage of cells that have not phagocytosed and/or associated with the bacteria
LM MFI	Amount of bacteria that have been phagocytosed per cell
GFP MFI	Amount of bacteria that have escaped out of the phagosome per cell
Containment Index	The ratio of total bacteria per cell (LM MFI) to escaped bacteria per cell (GFP MFI)

CHAPTER I

INTRODUCTION

Specific Aims

Neutrophils and monocytes are innate immune cells that provide early and rapid host protection during bacterial infections. With the use of a *Listeria monocytogenes* (*Lm*) infection model, studies have been conducted to understand the functions and roles of neutrophils and monocytes during intracellular bacterial infections. Previous studies performed with this infection model have demonstrated that both neutrophils and monocytes are necessary for protection during *Lm* infection. Neutrophils have been identified as being necessary for phagocytosis and killing of *Lm* whereas monocytes, primarily, for the production of TNF-α and nitric oxide (NO) (1, 2). Both neutrophils and monocytes have been also been demonstrated to produce reactive oxygen species (ROS) and reactive nitrogen species (RNS), including, hydrogen peroxide (H_2O_2), superoxide ($O_2^{\cdot -}$) and nitric oxide, as a mechanism for killing the pathogen (2-4). ROS and RNS, though generated to damage the pathogen, are also harmful to host tissues. The host is able to subvert this damage via antioxidants, which are enzymes that convert the more severe ROS/RNS components into less potent molecules. To gain a better understanding of the role of ROS during infection with *Lm*, the effects of extracellular superoxide dismutase (ecSOD), an antioxidant that converts superoxide into hydrogen peroxide, on host resistance to bacterial infections was investigated. In our previously published studies, ecSOD activity was observed to increase neutrophil recruitment to sites of *Lm* infection but surprisingly also led to increased host susceptibility (5). As a result, it is important to understand how ecSOD modulates neutrophil functions during infection with *Lm*. Additionally, as both neutrophils and monocytes play important roles essential for host protection,

better understanding of functions specific to each cell type as well as those that overlap will contribute to general knowledge of the roles neutrophils and monocytes play during immune responses to infections, inflammation and disease. We hypothesize that although neutrophils and monocytes are capable of performing similar roles, they are each more effective at certain specific functions. Furthermore, the lack of ecSOD activity increases *Lm* uptake, containment and killing by neutrophils, resulting in effective bacterial clearance.

Specific Aim I: How does ecSOD influence the localization of Listeria monocytogenes?

Previous studies conducted in our lab demonstrated that high ecSOD activity increased neutrophil recruitment to the liver but also increased bacterial burden and host susceptibility to *Lm* infection (6). Importantly, another study conducted in our lab has also shown that neutrophils are essential for host protection during infection with *Lm* (7). Recently, a third study demonstrated that although *Lm* is a classically intracellular bacteria, a significant amount the bacteria are also located extracellularly (8). Mice were infected with the wild-type EDGe (rabbit isolate) and the mutant $InlA^m$ (murinized) strain of *Lm* and it was observed that there were more extracellular bacteria in the mesenteric lymph nodes and liver a few days after foodborne inoculation (8). It is therefore possible that the inability of the large percentage of neutrophils present to protect the ecSOD HI mice may be leading to the presence of more extracellular bacteria in the ecSOD HI mice. As ecSOD could potentially get phagocytosed into the phagosome alongside *Lm*, it is possible for ecSOD activity to have an effect on containment of the bacteria in the phagosome. We hypothesize that high ecSOD activity leads to an increase in the presence of extracellular bacteria and also inhibits efficient phagosomal containment of *Lm*. To determine how ecSOD affects localization of *Lm*, bacteria CFUs will be measured in *Lm* infected ecSOD congenic mice treated with gentamicin, an antibiotic that specifically kills extracellular bacteria. The effect of ecSOD

activity on phagosomal containment of *Lm* in neutrophils will be determined with the use of a unique reporter strain of *Lm* that emits GFP fluorescence upon escape from the phagosome.

Specific Aim II: How does ecSOD activity modulate phagocytic cellular mechanisms for clearance of Listeria monocytogenes, downstream of phagosomal escape, in neutrophils?

Following escape out of the phagosome into the cytosol, *Lm* is able to replicate and engage in cell to cell spread in order to avoid immune detection. To prevent cell to cell spread, phagocytic cells can engage in the process of macroautophagy. Macroautophagy is a cellular mechanism that involves the walling off of cytosolic bacteria into an autophagic vacuole which can be fused with a lysosome to form an autophagolysosome. In the autophagolysosome, the cell can generate ROS to kill the pathogen (9, 10). ROS have been implicated as modulators of the autophagy process in several different diseases. Specifically, increased $O_2^{\cdot -}$ via starvation has been shown to induce autophagy (11) and H_2O_2 has also been identified as a regulator of autophagy (12). Therefore, ecSOD activity may have an effect of autophagy in neutrophils. Following sequestration of the bacteria in phagosomes and autophagosomes, the pathogen has to be killed in order to prevent dissemination. Neutrophils have previously been identified as having the ability to kill *Lm*, therefore, it is necessary to determine if ecSOD activity modulates killing of *Lm*. We hypothesize that ecSOD activity inhibits neutrophil macroautophagy as well as effective bacterial killing. In order to determine the effects of ecSOD activity on neutrophil autophagy, leukocytes from the bone marrow will be infected with *Lm* and the expression of microtubule-associated protein 1A/1B-light chain 3 (LC3) in neutrophils will be measured. The effect of ecSOD on the ability of neutrophils to kill *Lm* will be determined by purifying neutrophils from uninfected ecSOD congenic mice in order to perform an *in vitro* killing assay.

Specific Aim III: How do neutrophils and monocytes functionally differ the innate immune response to Listeria monocytogenes infection?

Lm infection in mice has commonly been utilized as a model for the study of immunological processes and functions of cells that make up the immune system (13). It therefore serves as an ideal model for the study of the roles and capabilities of neutrophils and monocytes, specifically during intracellular bacterial infections. Lack of neutrophils, monocytes, or both have previously been demonstrated to lead to increased susceptibility to *Lm* infection (2, 14). Phagocytosis and bacterial killing of *Lm* are functions that have been attributed primarily to neutrophils during the early stages of infection (1, 15). Monocytes, on the other hand, are thought to differentiate into inducible nitric oxide synthase (iNOS) and TNF-α producing inflammatory monocytes ($CD11b^{+}Ly6C^{hi}$) during the course of *Lm* infection (2). Although these functions have been generally assigned to each of the cells, a direct comparison of similarities and differences in the aforementioned functions as well as others necessary for bacterial clearance have not previously been conducted or reported. We hypothesize that monocytes are more effective at cytokine production, whereas neutrophils are more effective at phagosomal containment and killing during infection with *Lm*. To investigate the differences in functions necessary for host resistance to *Lm* infection between neutrophils and monocytes, bacteria phagocytosis, phagosomal containment, killing and production of TNF-α and IL-1α will be measured.

The Immune System

The immune response is an evolutionary mechanism developed over time for protection against pathogens. It is made up of two arms, the innate and adaptive immune responses which differ due to a few characteristics. The innate immune response is not specific for a particular pathogen, has no immunological memory and is initiated almost immediately following the host's contact with a pathogen. The adaptive response, unlike the innate, is pathogen specific, leads to immunological memory and is not initiated until later on into the infection. The two arms of the immune system are also made up of different types of cells which all derive from one common cellular precursor, the pluripotent hematopoietic stem cells, which reside in the bone marrow. The pluripotent hematopoietic stem cell develops into the common lymphoid progenitor and the common myeloid progenitor cells. The common lymphoid progenitor eventually gives rise to B-cells and T-cells which mature in lymphoid organs and make up the adaptive immune system, as well as NK-cells which are part of both the innate and adaptive response. The common myeloid progenitor gives rise to neutrophils, monocytes, macrophages, dendritic cells, eosinophils, basophils and mast cells which make up the innate immune response but also play a role in the adaptive immune response.

Innate Cellular Immunity

Basophils, Eosinophils and Mast Cells

Cells that are derived from the common myeloid progenitor and make up the innate immune response perform a number of cell specific and overlapping functions. Mast cells are commonly found in the internal surfaces of the body including the airways, gastrointestinal tract and the mucosa where they protect against parasitic pathogens (16). Mast cells have granules that contain histamine which can be released following activation by IgE that is produced in response

to parasites or allergens (16). They also have the ability to produce cytokines similarly to basophils, one of the three granulocyte subsets (16, 17). Basophils are very similar to mast cells as they share similar features and cytokine profiles (16, 18). In contrast to mast cells, basophils do not reside in tissues but rather circulate in the bloodstream to be recruited into tissues. Once in the tissues, basophils augment the immune response against parasites as well as release histamine in response to allergens, similarly to mast cells (18). Another type of circulating granulocyte are the eosinophils which, similarly to mast cells and basophils, are also mediators of allergic responses (19). Eosinophils also have a number of granules that contain factors such as cationic proteins that can be released in conjunction with cytokines to induce inflammation (18). Unlike mast cells, basophils, and eosinophils, the other cells that constitute the innate immune system are not commonly involved in immune responses to parasites or allergens. This includes dendritic cells, macrophages, monocytes and neutrophils that primarily function to eliminate non-parasitic pathogens from the host.

Dendritic cells

Dendritic cells (DCs) differ from the other innate immune cells in that they function as a bridge between the innate and adaptive arms of the immune system (20, 21). Following emigration from the bone marrow into tissues, DCs remain immature until they are activated by pathogen associated molecular patterns (PAMPs) or damage associated molecular patterns (DAMPs). Activation by a PAMP or DAMP leads to maturation of the DC followed by migration to the nearest secondary lymphoid organ where it performs the function of an antigen presenting cell (APC). As the name implies, APCs present antigens to T-cells and B-cells in order to initiate an adaptive response (21, 22). Dendritic cells play a major role during infections with intracellular bacteria such as *Salmonella typhimurium* (*S. typhi*) and *Lm*. During *S. typhi* infection, inhibition

of DC trafficking via deletion of the chemokine receptor CX_3CR1 has been reported to inhibit effective clearance of the bacteria (23). During infection with *Lm*, DCs have been shown to be a major source of IL-12 which is necessary for inducing IFN-γ production, a cytokine necessary for clearance of the bacteria (24). Additionally, programmed cell death one (PD-1) deficiency in mice leads to increased production of IL-12 by DCs which increases resistance to *Lm* infection (25).

Another study conducted with the fungi, *Toxoplasma gondii* (*T. gondii*) also demonstrated that depletion of DCs led to a reduction in IL-12 which subsequently leads to a reduction of IFN-γ and results in increased susceptibility to infection (26). In regards to non-infectious immune responses, currently, cancer therapy is centered around activating the immune response in order to specifically eliminate cancer cells (27). Findings from studies on immune responses to infections can also be applied to non-infectious responses as the functions the cells perform tend to be similar and overlap. For example, IFN-γ deficient mice have been reported to have increased incidences of cancer (27) and the studies referred to above demonstrate that DC specific IL-12 induces IFN-γ production during infection. As Lm induces the production of IL-12 in dendritic cells, it is being explored as a vehicles for cancer vaccine delivery (27).

Macrophages

Another subset of innate immune cells that also function as APCs are macrophages which are phagocytic tissue resident cells. Macrophages conduct a range of functions dependent on the tissue in which they reside as well as the type of pathogen that induces their activation (28). Macrophages express a number of pattern recognition receptors (PRRs) which recognize and bind to PAMPs on the pathogen (29). Macrophages are classified into two types, M1 and M2 which differ by their effector functions as well as cytokine and receptor expression. M1 macrophages

promote a more inflammatory environment whereas M2 macrophages function to reduce inflammation and promote tissue repair (28).

Macrophages have been identified as being important for host protection during infection with intracellular bacteria such as *S. typhi*, *Mycobacterium tuberculosis* and *Lm*. During *S. typhi* infection, macrophage have been reported to be necessary for the production of IL-12 and IL-18, cytokines that drive the production of IFN-γ by NK cells (30, 31). The IFN-γ further activates the macrophages to phagocytose and kill the bacteria (32). Infection with *Mycobacterium* also results in similar cytokine production by macrophages (33). Macrophages have also been shown to kill *Mycobacterium* during acute infection, although the bacteria is able to survive and successfully replicate in the cells during chronic infection (28). During incidences of cancer, a subset of macrophages associate with tumors and are referred to as Tumor-associated macrophages (TAMs) (34). TAMs have phenotypes similar to a typical M2 macrophage and as a result promote growth of the cancer cells, thus making them detrimental rather than protective for the host (34).

Neutrophils

Neutrophils, the most abundant of the granulocytes, play a central role in the innate immune response to infections and diseases (35, 36). Following maturation in the bone marrow, neutrophils circulate in the blood to be recruited into organs during infection or inflammation (35, 37). The primary function attributed to neutrophils is the phagocytosis and killing of pathogens as they tend to be the first responders to sites of invading microbes (36). However, there aren't many reports showing neutrophils directly killing pathogen, i.e., in the form of a killing assay. The current consensus is that neutrophils kill pathogenic microorganisms via oxygen dependent respiratory burst, such as ROS/RNS, although there have been reports indicating that killing can also be achieved in an oxygen-independent manner (36, 37). Another method by which neutrophils

have been demonstrated to kill, predominantly extracellular pathogens, is via the generation of neutrophil extracellular traps (NETs) (38). NETS are formed when neutrophils expel a combination of DNA and granular proteins in the form of a web like structure which traps and leads to killing of the pathogen (38).

Neutrophils were previously viewed as more deleterious than protective during non-infectious immune responses, primarily due to their presence leading to increased inflammation and tissue damage (39). However, recent studies have been able to carefully parse out and demonstrate the protective functions of neutrophils. Studies on neutrophil functions during intracellular bacterial infections such as *S. typhi* infection, show that neutrophils produce IFN-γ and are necessary for controlling bacterial load. Depletion of neutrophils with the specific 1A8 (anti-Ly6G) antibody led to a decrease in overall cecum IFN-γ and increased bacterial load (40). It has also been reported that neutrophils kill *S. typhi*, as depletion of the cells were observed to correlate with increased bacterial burden. It is important to note, however, that these studies were conducted with the non-specific RB6-8C5 (anti-Ly6C, Ly6G) and no killing assay was conducted (41). Neutrophils have also been demonstrated to produce TNF-α and IFN-γ during infection with *T. gondii* (42-44). The role of neutrophils during cancer has not been clearly defined yet, although most studies indicate that they play a more damaging role. Inflammation due to the tissue damage caused by ROS/RNS produced by neutrophils during diseases such as colitis can lead to tumor initiation and promote tumor growth (45, 46). Recent studies have also begun to highlight protective roles of neutrophils during cancer as well. Depletion of neutrophils in a mouse breast cancer model, showed an increase in cell metastasis and tumor seeding in the lung which was linked to a reduction in H_2O_2 in the lung (46, 47).

Monocytes

Monocytes are another class of phagocytic cells that make up the innate immune system. Previously it was thought that upon existing the bone marrow, they primarily migrated to tissues where they differentiated into macrophages. However, extensive studies conducted on monocytes have revealed that they are actually cells with high plasticity (48). There are currently two well defined subsets of murine monocytes, the $Ly6C^{lo}$ subset which functionally correlates with the human $CD14^{lo}CD16^{+}$ cells and the $Ly6C^{hi}$ monocytes which are functionally similar to the human $CD14^{hi}CD16^{-}$ cells (49, 50). The $Ly6C^{hi}$ neutrophils are commonly referred to as the inflammatory monocytes and make up the majority of the overall monocyte population (51). This subset of monocytes are recruited from the bone marrow into tissues during infection, a process mediated by the signaling of the chemokine receptor, CCR2 (50). The $Ly6C^{lo}$ monocytes, on the other hand, patrol the endothelial cell surfaces and differentiate into macrophages during infection (52, 53).

Similarly to neutrophils, monocytes, specifically inflammatory monocytes, are recruited early on to sites of infection and inflammation (53). Previous studies have shown that the initial rapid response of inflammatory monocytes delays the systemic dissemination of *S. typhi* during infection (54, 55). Although, later on during infection, the inflammatory monocytes also contribute to trafficking of the bacteria to the liver (56). Lack of recruitment of inflammatory monocytes in CCR2 KO mice led to increased susceptibility and bacterial burden during infection with *T. gondii* (57). Monocytes are therefore, also necessary for early host resistance to infections. Similarly to neutrophils, inflammatory monocytes have also been reported to promote tumor growth and metastasis of cancer cells whereas the $Ly6C^{lo}$ monocytes prevent metastasis (58).

Listeria monocytogenes

Pathogenesis

"Listeria monocytogenes (*Lm*) is a gram positive intracellular bacterium that causes diseases such as gastroenteritis, spontaneous abortions as well as meningitis in immunocompromised individuals (37). Infection occurs through the ingestion of contaminated food products including deli meats, cheeses, fruits and vegetables. In the gut, *Lm* expresses InternalinA (InlA) which allows it to bind to E-cadherin, a protein expressed on the surface of the intestinal epithelial cells (59). The interaction of these two proteins leads to internalization of the bacteria into the gut epithelial cells. From the gut, *Lm* can disseminate to its target organs, the liver and spleen, via the bloodstream and lymphatics. Once in the target organs, it gains entry into hepatocytes via endocytosis driven by the binding of InternalinB (InlB) on the bacteria to the human growth factor receptor (HGFR) on the liver cells (37, 60). In the liver and spleen, *Lm* is phagocytosed by surrounding phagocytes which include neutrophils and macrophages (13).

Once in the endosome or phagosome, *Lm* secretes Listeriolysin O (LLO), a hemolysin that allows it to lyse the endosomal/phagosomal membrane and escape into the cytosol. Following its escape out of the phagosome, *Lm* expresses the *actA* gene, encoding for the ActA protein which mediates nucleation and hijacking of host actin. This allows *Lm* to replicate and engage in cell to cell spread after which it has also been reported to be capable of spreading to the brain and heart (37, 61). Approximately 90% of individuals with listeriosis end up hospitalized and about 20-30% of these cases result in death (62, 63). As a result, *Lm* is currently ranked as the third most expensive foodborne illness in the United States, resulting in a total annual cost of $26 billion dollars (64). However, as *Lm* is a well characterized bacteria, it serves as an ideal model for studying innate and adaptive immune responses" (13, 65).

Innate immune response to Lm infection

Phagocytosis of Lm

The phagocytic cells of the innate immune system which are macrophages, neutrophils and monocytes have been reported to play roles essential for protection during *Lm* infection (13, 37, 66). One of the major functions necessary for *Lm* clearance is phagocytosis of the bacteria. *Lm* has been reported to be phagocytosed primarily by neutrophils and macrophages. Macrophages, as tissue resident cells, are involved in early phagocytosis of *Lm*. A study conducted with Kupffer cells, resident liver macrophages, showed that the cells take up *Lm* as quickly as 10 minutes post i.v. infection (1). In this same study, the bacteria were found to be equally distributed in Kupffer cells, neutrophils and hepatocytes. Although the bacteria were also most likely in the recruited inflammatory monocytes since the non-specific RB6-865 (anti-Ly6C & Ly6G) which binds to both neutrophils and monocytes was used to identify the neutrophils (1).

The receptor ligand interactions that induce phagocytosis of *Lm* have mostly been attributed to complement receptors. The complement receptor that is thought to drive *Lm* phagocytosis is the complement receptor 3 (CR3, CD11b, Mac-1) which is expressed by subsets of macrophages, all neutrophils and monocytes and binds to the complement component, iC3b (67, 68). Previous studies have shown that antibody blocking of CR3 or lack of CR3 expressing cells, leads to increased bacterial burden and susceptibility to *Lm* infection (69). Macrophages recruited into the peritoneum were able to more effectively phagocytose C3 opsonized *Lm* in comparison to unopsonized *Lm* (68). Kupffer cells, unlike other macrophage subsets, do not express high levels of CR3. However, they do express the complement receptor Ig (CRIg) which also binds to iC3b (70). In a study conducted with bone marrow derived macrophages (BMDM),

CRIg was reported to enhance macrophage phagocytosis of *Lm* (71). In neutrophils and monocytes, however, complement driven phagocytosis of *Lm* has not been directly investigated.

Phagosomal containment of Lm

Phagocytosis of pathogens serves as a means of delivery to the lysosome where the pathogen can be degraded and killed (72). Delivery of the pathogen into the lysosome from the phagosome occurs via the fusion of both compartments to form a phagolysosome, a process that is referred to as phagosome maturation. This newly formed phagolysosome has an acidic environment that contains ROS/RNS, hydrolytic enzymes and other peptides that are toxic to the phagocytosed pathogen (73). However, *Lm* is able to inhibit phagosomal lysosomal fusion and escape from the phagosome, primarily through the function of LLO, although, a couple of phospholipases (PLCs) secreted by the bacteria have also been reported to facilitate escape (74-76). It is thought that LLO prevents acidification of the phagosome which in turn prevents fusion of the phagosome with the lysosome (75). Studies on phagosomal escape of *Lm* in the innate immune cells have been limited to macrophages, specifically BMDMs. The differences in the ability of neutrophils and monocytes to keep *Lm* contained has not yet been directly studied.

Killing of Lm

Lm killing is a function that is primarily attributed to neutrophils and macrophages, although during the adaptive response, monocytes have also been reported to kill the bacteria as well (1, 66, 77). A previous study was able to demonstrate direct killing of *Lm* by peritoneal cavity macrophages (78). Studies conducted in our lab, previously, also reported increased susceptibility and bacterial burden in the spleen and liver following depletion of neutrophils with the 1A8 antibody (14). Killing of *Lm* is mainly attributed to generation of ROS and RNS (13). The presence of the bacteria in the phagosome leads to the assembly of the NADPH Oxidase enzyme which

functions to convert molecular oxygen into O_2^- (36). The O_2^- can be converted into H_2O_2 which can be completely neutralized into water and oxygen via peroxidases and catalase. In instances of persistent infection, the H_2O_2 can be converted into hypochlorous acid by myeloperoxidases (37). Although O_2^- is toxic to the bacteria, it also damages the host tissues which is why it is converted into the less toxic H_2O_2, a reaction catalyzed by the superoxide dismutases (SODs) (79). There are three SODs present in mammalian cells, SOD1 in the cytosol, SOD2 in the mitochondria, and SOD3 or extracellular superoxide dismutase (ecSOD) which is found in the extracellular matrix. It has been reported that ROS components and regulators, such as SOD, can regulate and modify several cellular mechanisms and pathways including those of the innate immune response.

Lm, ROS & Autophagy

As a mechanism for prevention of bacteria dissemination following phagosomal escape, phagocytic cells can engage in the process of macroautophagy to form an autophagolysosome which contains ROS to kill the pathogen (9, 10). There is currently a lack of consensus on the role of autophagy during *Lm* infection. Studies have shown that *Lm* infection can induce the execution of autophagy via the cleavage of LC3 (Atg 8) into LC3 I and LC3 II (80, 81). However, other studies have also shown that although *Lm* co-localizes with LC3, it is able to evade killing by autophagy via the expression of ActA and PlcA which are virulence factors that also drive the process of cell to cell spread (82-84). It is imperative to note that these studies were conducted in bone marrow derived macrophages only. In addition, ROS have been implicated as modulators of the autophagy process in several different diseases. Specifically, increased $O_2^{\cdot -}$ via starvation has been shown to induce autophagy (11). Furthermore, H_2O_2 has also been indicated as a regulator of autophagy by the modulation of Atg4, a protein that controls the lipidation/conversion of LC3 into

LC3 I and LC3 II (12). Although autophagy during *Lm* infection has been studied in macrophages, its role in neutrophils or monocytes autophagy of *Lm* has not been well defined.

Lm & EcSOD Activity

EcSOD is an antioxidative enzyme that is produced by both immune and non-immune cells in multiple different tissues including the liver, heart and lungs (85). It localizes in the extracellular matrix (ECM) where it functions to prevent degradation of the ECM during inflammation (86). Due to its protective role during non-infectious inflammatory disease models (87, 88), our lab conducted studies to determine if ecSOD also plays a role in the innate immune response to *Lm* infection. Three groups of congenic mice expressing varying levels of ecSOD activity were generated: the ecSOD HI mice with high activity of ecSOD; WT mice that express normal activity of ecSOD; and ecSOD KO mice which have no ecSOD activity (89).

The ecSOD HI mice were observed to be more susceptible to *Lm* infection in comparison to the ecSOD WT and KO. Analysis of the innate immune response to *Lm* infection in the congenic mice revealed differences in the recruitment of neutrophils. A higher percentage and number of neutrophils are recruited into the liver and blood of the ecSOD HI mice in comparison to the ecSOD KO mice (6). There was also more TNF-α produced in the liver of the ecSOD KO mice and a higher percentage of neutrophils from the ecSOD KO livers produce TNF-α (6). Depletion of neutrophils in the congenic mice after infection with *Lm* led to an increase in bacterial burden in the ecSOD KO mice but a decrease in the ecSOD HI mice (6). The effects of ecSOD were found to be not intrinsic to the cells but rather dependent on the host environment. Transfer of the ecSOD HI and KO neutrophils into ecSOD WT mice led to similar recruitments of the respective neutrophils into the liver (3). Therefore, a better understanding of how ecSOD activity modulates neutrophil function is needed.

TNF-α and IL-1α production during Lm infection

Cytokines are major components of the immune response as they induce cell signaling, activation and recruitment of not only immune cells, but also parenchymal cells. During the innate immune response to *Lm* infection, cytokine production has also been identified as being necessary for host resistance. One such cytokine is TNF-α, a protein that further activates phagocytic cells and induces increased phagocytosis and killing of the bacteria (90). Following infection with *Lm*, TNF-α is found in the liver and spleen as early as day 1 post infection (14, 90). TNF-α production has been attributed to macrophages, neutrophils and the inflammatory monocytes. IFN-γ from NK cells have been shown to induce production of TNF-α by macrophages (91). Mice lacking expression of the TNFR1 were found to be more susceptible to *Lm* infection (92). Additionally, specific deletion of TNF-α in neutrophils, monocytes and macrophages also led to increased host susceptibility following infection with *Lm* (93). Production of TNF-α by neutrophils and monocytes has also been previously reported. Neutrophils in the liver and spleen have been shown to produce TNF-α during *Lm* infection. There was also an observed production of TNF-α by the nitric oxide producing inflammatory monocytes (2). However, differences in TNF-α production by neutrophils and monocytes has not been thoroughly dissected.

IL-1α and IL-1β have also been thought to play important roles during *Lm* infection, although most of the studies have been centered around the role of IL-1β (66). IL-1α and IL-1β both signal by binding to the IL-1R and blockage of the receptor led to increased susceptibility to *Lm* infection (94). However, neutralization of IL-1β had no observable effects on mice infected *Lm* (95). Therefore, the deleterious effects on host resistance by the receptor neutralization may be due to IL-1α binding and downstream signaling. Unlike TNF-α, the role of neutrophils and

monocytes in IL-1α production has not yet been studied. Knowledge on IL-1α production during *Lm* infection and its effect on neutrophil and monocyte function would aid in a better understanding of the role of IL-1α during innate immune responses.

Significance

Neutrophils play a role in most, if not all inflammatory responses though more so during infections (35, 36). Similarly to neutrophils, monocytes have also been implicated as contributing to early and rapid inflammatory responses (53). Therefore, a thorough understanding of the functional abilities of the cells adds to the knowledge of immune responses which can aid in targeting treatments for different diseases and infections. The findings made in the *Lm* mouse infection model can be applied to other types of immune responses in which neutrophils and monocytes are recruited and activated. Additionally, understanding how regulators of ROS, such as ecSOD, modulate the function of neutrophils will contribute to a better understanding of the role of ROS in immune cell function. Recently, antioxidants have been explored as treatment options to combat tissue damages induced by ROS/RNS. As ecSOD is an antioxidant, a thorough understanding of its role in modifications of immune responses is a necessity if it's to be considered as a type of treatment. Finally, *Lm* is currently being developed as a vector for cancer vaccine delivery (96). As this involves activation of the immune system, it is important to gain a complete understanding of the different immune cell responses that *Lm* elucidates in order to avoid unintentional collateral damage, and harness the unique capabilities of monocytes and neutrophils.

CHAPTER II

MATERIALS AND METHODS

Mice

"C57BL/6J (B6) mice were originally obtained from The Jackson Laboratory (Bar Harbor, ME, USA) and bred in house at the University of North Texas Health Science Center with accreditation from the American Association for the Accreditation of Laboratory Animal Care. Mice used in these studies were housed with sterile bedding in sterile microisolator cages and all studies were performed with approval from the University of North Texas Health Science Center Institutional Animal Care and Use Committee. Mice used in all experiments ranged from 2-4 months in age and were gender matched.

Bacteria

Lm strain 10403s (WTLM) was grown on brain heart infusion (BHI) agar plates. The *Lm* reporter strain, actA:LMGFP (DH-L1245), was generously provided by Dr. Elizabeth H. Schwartz. GFP expression is driven by the *actA* promoter as previously described (97). Bacterial virulence was maintained via constant passage in B6 mice. For all experiments, both strains of bacteria were grown to stationary phase in BHI broth at 37ºC and washed twice in PBS, prior to infecting cells or mice.

For *in vitro* studies, the bacteria were diluted to the desired Multiplicity of Infection (MOI) in phenol red free DMEM lacking antibiotics and supplemented with L-glutamine, 10% FBS and vitamins (Invitrogen). Bacteria were opsonized by adding mouse serum to the solution at a final concentration of 10% and placed on ice for 30 minutes before being added to the cells (15). Mice

were inoculated i.v, for *in vivo* infections, with bacteria diluted in PBS to the desired concentration. Dilutions of bacteria were plated to calculate actual dosage and MOI" (65).

Gentamicin treatment assay

EcSOD congenic mice were infected with $1x10^4$ WTLM. Mice were then treated, i.p, with either 3mg of gentamicin or 1X PBS at 12 hours post-infection followed by harvesting of liver and spleen at day 1 post-infection. For day 3 post-infection studies, mice were treated with 3mg of gentamicin or 1X PBS at 2 days post-infection and organs harvested at day 3. Whole organs were diluted and then plated for CFUs.

Organ harvest and preparation

"Whole spleens were harvested and ground in Tris Ammonium Chloride (TAC) to lyse red blood cells and obtain splenocytes. Whole livers were harvested following liver perfusion as previously described (98), then homogenized followed by layering on a percoll gradient and isolating liver leukocytes from the interface of the gradient as previously reported (5). Bone marrow leukocytes were harvested as previously described but briefly, the femur and tibia bones were flushed with Hanks Buffered Saline Solution (HBSS) containing 2% FCS and 2mM EDTA, followed by lysing of red blood cells with 0.2% and 1.6% NaCl solutions (99).

In vitro cell culture

Splenocytes, bone marrow and liver leukocytes were cultured for 1 hour with DMEM lacking antibiotics at 37^oC in 5% CO_2. Splenocytes and bone marrow leukocytes were cultured at a concentration of $1x10^6$ per well and liver leukocytes at $5x10^5$ per well in 48 well plates. For the IFN-γ studies, recombinant IFN-γ (Biolegend) was added to the wells during the 1 hour culture. Bacteria, opsonized or un-opsonized were added to the wells at the desired MOIs and allowed to culture for 1, 2 or 4 hours. For the chloroquine assay, the cells were treated with chloroquine

(Sigma) at the same time the bacteria were added. After culturing, gentamicin (Sigma) was added at a final concentration of 50μg/mL for 30 minutes after which the cells were washed and stained for flow cytometry analysis. Culturing to stain for intracellular cytokines included stimulation with heat-killed *Lm* (HKLM) at a 50:1 MOI and treatment with Golgi Plug (Fisher Scientific) to prevent cytokine secretion for 3.5 hours.

Cell Sorting, Flow Cytometry and Imaging Cytometry

Anti-Ly6G (1A8) PECy7, anti-Ly6C (HK1.4) AF647, PerCP, or BV421 (Biolegend), anti-CD11b (M1/70.15) PE-TR (Invitrogen, CA, USA) were used to stain for surface antigens and anti-CD16/CD32 (Biolegend) was used to block non-specific binding. Staining for *Lm* was performed with the *Listeria* O polyserum antibody (BD Biosciences) and visualized with anti-rabbit PE (Biolegend). Staining for LAMP-1 was conducted using the anti-LAMP-1 (1D4B) BV711 antibody (Biolegend). Cells were stained for flow cytometry and imaging cytometry as previously described (5). Anti-TNF-α (MP6-XT22) BV605 and IL-1α (ALF-161) PE (Biolegend) were used to stain for intracellular cytokines according to a previously described protocol (5). Flow cytometry was conducted using the BD LSRII flow cytometer and data were analyzed with Beckman Coulter Kaluza software. Cell sorting was conducted with the Sony SH800S and imaging cytometry with the Amnis Imagestream MKII Imaging flow cytometer, and images were analyzed with the IDEAS software.

Confocal Microscopy

Bone marrow leukocytes were isolated and cultured as described above with either no bacteria, WTLM or actA:LMGFP for 2 hours and then prepared for confocal microscopy using a previously published protocol (100). The cells were suspended in 2% BSA, centrifuged onto positively charged poly-L-lysine superfrost slides (Fisher Scientific), and then briefly allowed to dry.

Following fixation with formalin (Sigma) for 10 minutes at room temperature, the cells were washed with PBS, and then permeabilized with TBS & 0.1% Triton X-100. The cells were then stained with the *Listeria* O polyserum antibody (BD Biosciences), anti-rabbit AF647 (Biolegend), and DAPI in TBS & 0.1% Triton X-100 with 1% BSA. Visualization of the cells was conducted with the use of a Zeiss LSM 880 confocal microscope with AiryScan and a 63x oil immersion objective. Data were analyzed using the Zen black software.

ROS measurement

ROS was measured using the 2'7' -dichlorodihydrofluorescein diacetate (H_2DCFDA) fluorescent dye. Bone marrow leukocytes were cultured as previously described above with HKLM at an MOI of 50:1. After 1 hour of culturing, H_2DCFDA was added at a final concentration of 10μm. The cells were then cultured for another hour after which gentamicin was added. The cells were then cultured for an additional 30 minutes. The reaction was stopped by placing the cells in -20^{0}C for 2 minutes after which they were stained for neutrophil and monocyte cell surface markers for flow cytometry analysis.

Listeria killing Assay

The *in vitro Lm* killing assay was performed as previously described (101). Briefly, after isolating bone marrow leukocytes, the cells were stained to identify neutrophils and monocytes (described above) and then sorted using a Sony SH800S sorter. Sort purity was conducted and was routinely greater than 95% for both neutrophils and monocytes. The sort purified bone marrow neutrophils and monocytes were cultured for 2 hours with WTLM that had been opsonized with 20% mouse serum. Wells containing either bacteria only or both bacteria and cells were diluted in water and plated on BHI agar plates (Fisher Scientific). Percent killing was calculated by first subtracting the mean CFUs of the triplicate bacteria + cells wells from the mean CFUs of the triplicate bacteria

only wells. The generated number was then divided by the mean CFUs of the triplicate bacteria only wells and then multiplied by 100 to obtain a percentage.

Statistical analysis

T-tests were used to analyze data containing only two groups while one way or two way ANOVAs, as appropriate, were used for data containing multiple groups. A Bonferroni multiple comparison *post-hoc* test was performed following ANOVA tests. *P*-values less than or equal to 0.05 were indicated as significant, with "*", "**", or "***" representing a p-value of ≤ 0.05, 0.01, or 0.001, respectively." (65)

CHAPTER III

MODULATION OF NEUTROPHIL FUNCTION BY ECSOD DURING INFECTION WITH THE INTRACELLULAR BACTERIA, *LISTERIA MONOCYTOGENES*

EcSOD activity leads to extracellular localization of Lm in the spleen.

Previous studies conducted in our lab demonstrated that ecSOD activity is detrimental to the host during infection with *Lm* (1). Mice with high ecSOD activity were shown to have a high percentage of neutrophils recruited into the liver and blood during *Lm* infection in comparison to the ecSOD KO mice. However, the ecSOD HI mice were more susceptible the ecSOD KO mice to *Lm* infection and had higher bacterial burden in the spleen and liver as well (1). The high bacterial burden and increased susceptibility to infection contrasts with the high percentage of neutrophils present in the ecSOD HI mice as neutrophils have been demonstrated to be essential for protection (2). Furthermore, depletion of neutrophils led to an increase in bacterial burden in the ecSOD HI mice but a decrease in ecSOD KO mice (1). The higher bacteria burden coupled with the contradictorily high percentage of neutrophils recruited to sites of infection highlighted the possibility that ecSOD activity modulates neutrophil function during *Lm* infection. Therefore, it was hypothesized that the ecSOD HI neutrophils are functionally detrimental to host resistance.

To gain a better understanding of neutrophil functions during intracellular bacterial infections, the modulations during *Lm* infection by ecSOD activity was investigated. It is possible that ineffective phagocytosis and clearance of the bacteria may be leading to the presence of extracellular bacteria in the target organs of the ecSOD HI mice. Increased extracellular bacterial burden may be leading to continuous invasion of cells by *Lm* which further prevents proper

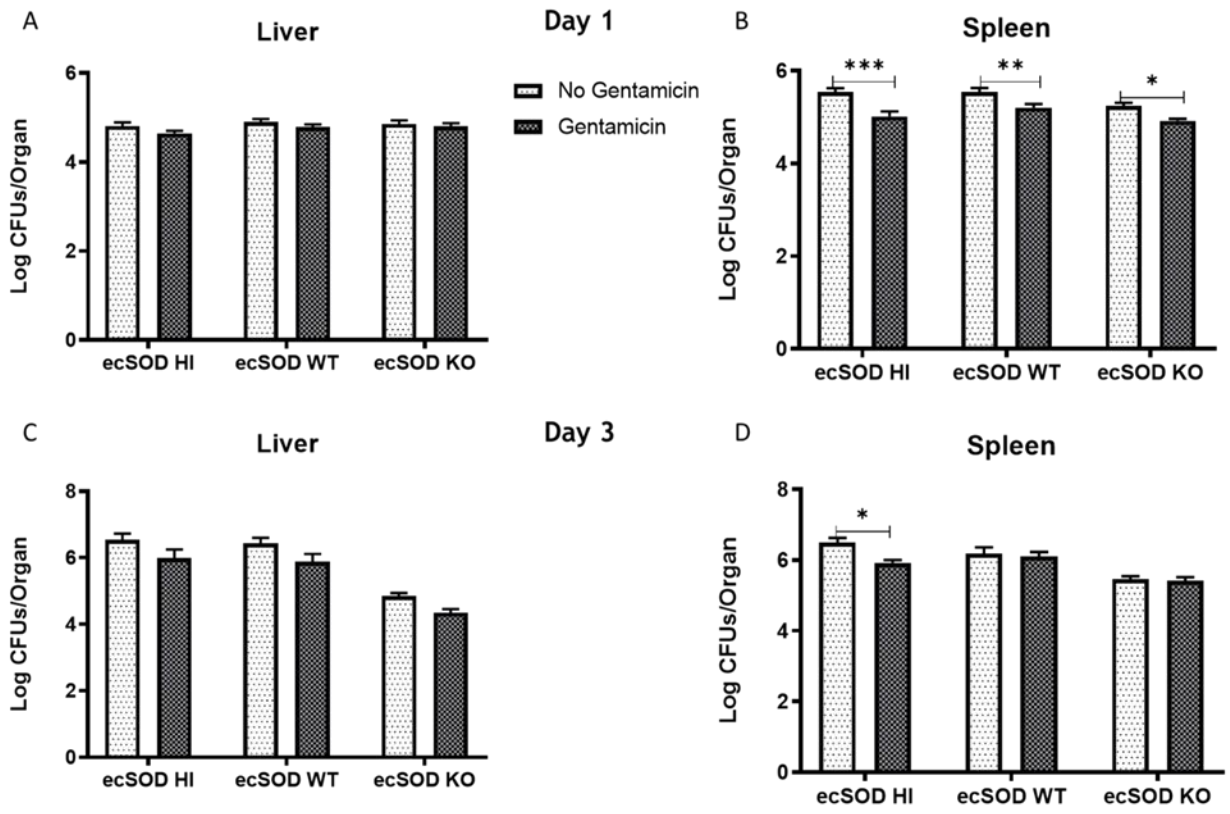

Figure 1: EcSOD activity leads to extracellular bacteria in the spleen

EcSOD congenic mice were infected with ~1x10^4 WTLM and treated with either gentamicin or PBS at 10 hours (A-B) or 2 days, post infection (C-D). At day 1 (A-B) or day 3 post infection (C-D), livers and spleens were harvested and organ homogenates plated for CFUs. Data are representative of 2 independent experiments and n $\geq$3 mice per group. 2-way ANOVA with Bonferroni multiple comparison *post-hoc* test.

resolution of the infection. To test this hypothesis, ecSOD congenic mice were infected with *Lm* and treated with gentamicin, which is an antibiotic that only kills extracellular bacteria because it is unable to cross the plasma membrane of mammalian cells (102). Therefore, a reduction in bacterial burden in the gentamicin treated mice would be indicative of the presence of extracellular bacteria. At one-day post infection, there were similar CFUs in the livers of gentamicin treated and PBS treated mice (Figure 1A). In the spleens of gentamicin treated mice, there was a reduction in *Lm* CFUs in the ecSOD HI, WT and KO mice in comparison to the PBS treated mice (Figure 1B). However, there were no differences in the spleen CFUs of the gentamicin treated mice from all three congenic mice groups. At day three post infection, although there are differences in bacterial burden between the ecSOD groups, independent of gentamicin treatment (as previously published), there are no observed differences in CFUs between the gentamicin treated and PBS treated mice livers (Figure 1C). There was, however, a reduction in bacterial burden in the gentamicin treated ecSOD HI mice spleens, indicating the presence of extracellular bacteria (Figure 1D). In the ecSOD WT and KO spleens, there were no differences in CFUs between the gentamicin treated and untreated mice, therefore, there are no extracellular bacteria present (Figure 1D). These data indicate that there are extracellular bacteria present in the spleen, but not in the liver, early on during infection that remain persistent in the mice with high ecSOD activity.

Characterizing the actA:LMGFP strain of Lm

The primary function attributed to neutrophils during bacterial infections is phagocytosis of pathogens (1). In the case of *Lm*, following phagocytosis, the bacteria have the ability to escape out of the phagosome by secreting the hemolysin, LLO. During phagocytosis of the bacteria by neutrophils, it is possible that ecSOD, as it is present in the extracellular matrix, may be phagocytosed along with *Lm*. In the ecSOD HI mice, high activity of ecSOD could then potentially

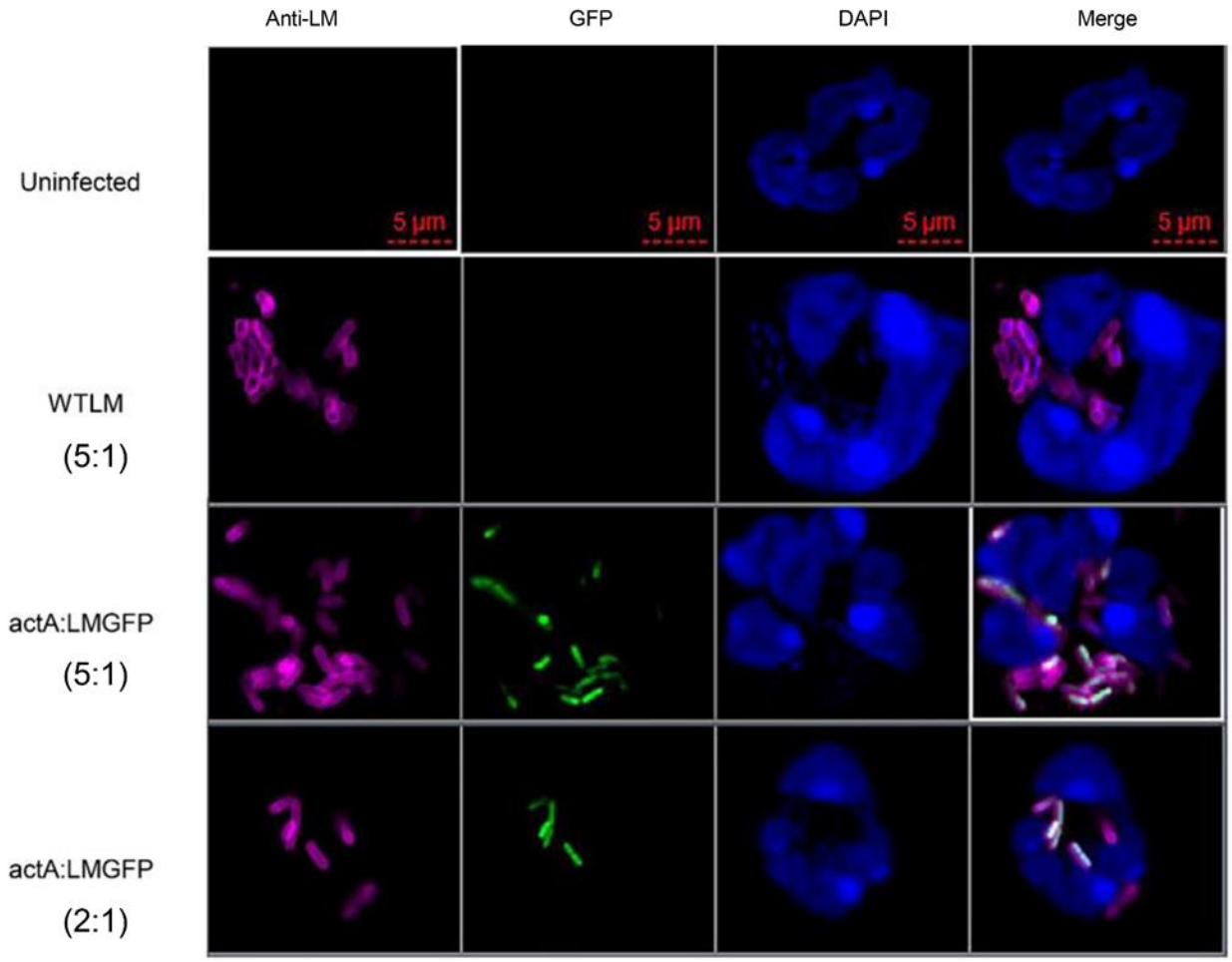

Figure 2: Imaging of actA:LMGFP in neutrophils

Bone marrow leukocytes isolated from B6 mice were either left uninfected or infected with either opsonized WTLM (MOI 5:1) or actA:LMGFP (MOI 5:1 and 2:1) for 2 hours. The cells were spun onto slides using a Cytospin and then stained with *Listeria* O polyserum antibody, anti-rabbit AF647, and DAPI. Individual stains are shown as well as the merge of all stains. Neutrophils were identified by their unique nucleus.

lead to inhibition of effective ROS generation and result in increased phagosomal escape of *Lm* into the cytosol. To investigate phagosomal containment, "a reporter strain of *Lm*, termed actA:LMGFP (Strain DH-L1245), was utilized. ActA:LMGFP emits GFP fluorescence following escape of the bacteria from the phagosome into the cytosol due to GFP expression being driven by the *Lm* actA promoter (97). Confocal microscopy was conducted to validate the GFP fluorescence emitted from the actA:LMGFP bacteria following phagosomal escape. Bone marrow leukocytes were either uninfected, infected with WTLM, or infected with actA:LMGFP at MOIs of 2:1 or 5:1. Neutrophils were identified based on their unique nucleus via DAPI staining. As demonstrated in Figure 2, there is no GFP or Anti-LM fluorescence in the uninfected cell. In the second panel where cells are infected with WTLM, there is Anti-LM fluorescence but no GFP fluorescence as expected. In the neutrophils infected with actA:LMGFP, there is both Anti-LM and GFP fluorescence. The GFP expression is also rod shaped and from the escaped bacteria as all the bacteria are Anti-LM^+ but not all of the bacteria express GFP. Importantly, all of the GFP positive bacteria stain positive for Anti-LM (Figure 2)." (65)

Next, "a dose response study was conducted with bone marrow neutrophils in order to delineate the relationship between phagocytosed and escaped bacteria. Bone marrow leukocytes were infected with actA:LMGFP at different MOIs ranging from 1:1 to 20:1. The cells were stained with neutrophil markers and the antibody against *Lm* as an indicator of total bacteria phagocytosed by the cells." (65) After gating on live cells using forward and side scatter, neutrophils were gated on as $CD11b^+$ $Ly6G^+$ (Figure 3A) and monocytes were gated on as $CD11b^+$ $Ly6C^{hi}$ $Ly6G^-$ (Figure 3B). *Lm* positive neutrophils and monocytes were gated on anti-LM PE^+ populations and cells that allowed for escape were gated on the GFP^+ cells out of the Anti-LM PE^+ population (Figures 3A-C). "Mean fluorescence intensity (MFI) of PE was used an indicator of the amount of total bacteria

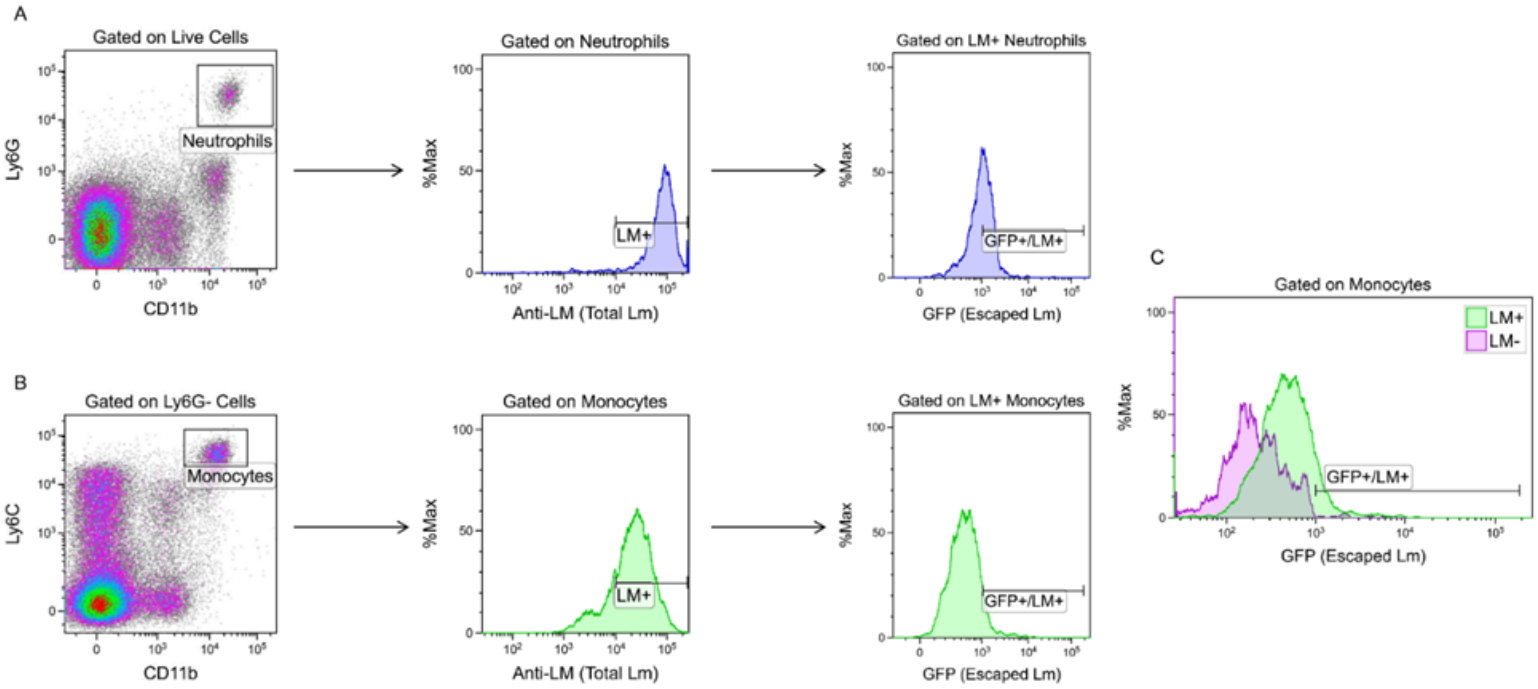

Figure 3: Flow Cytometry gating strategies:

To identify neutrophils and monocytes, a live cell gate was set on forward and side scatter. From the live cell gate, neutrophils were identified as $CD11b^{+}$ $Ly6G^{+}$ and a gate was set on the LM^{+} neutrophils to identify cells that are *Lm* positive. The GFP^{+} neutrophils were gated on out of the LM^{+} neutrophils to determine the percent of cells that had allowed for phagosomal escape (A). Monocytes were gated on as $CD11b^{+}Ly6C^{hi}Ly6G^{-}$ and the monocytes positive for LM were determined and then gated on for GFP expression (B). Also shown is a representative overlay showing GFP gating out of LM^{-} and LM^{+} neutrophils (C).

per cell and the GFP MFI for the amount of escaped bacteria per cell. The containment index was calculated by dividing the anti-LM PE MFI by the GFP MFI. As hypothesized, an increase in MOI led to an increase in the percentage of neutrophils positive for *Lm*, i.e., cells that had phagocytosed bacteria (Figures 4A&B)."(65)

"To identify the cells that had allowed for phagosomal escape, the GFP^+ cells out of the LM^+ cells (GFP^+/LM^+) were analyzed (Figures 3A-C). As the MOI increased, the percentage of cells that took up *Lm* increased, however, the percentage of cells that allowed for phagosomal escape of *Lm* plateaued after the 5:1 MOI (Figure 4B). The mean fluorescence intensity (MFI) of the LM^+ and the GFP^+ (after gating on the LM^+) cells were studied so as to compare the total amount of bacteria taken up (LM^+) against the amount of escaped bacteria (GFP^+). An increase in *Lm* MOI resulted in an increase in the total amount of bacteria phagocytosed by the neutrophils (Figure 4C). However, the amount of bacteria that escaped out of the phagosome remained relatively stagnant with the increase in MOI (Figure 4C). Therefore, although there is an increase in the amount of bacteria taken up by the cells, the amount of bacteria escaping from the phagosome remains the same. The containment index, a numerical indicator of phagosomal containment, was generated by calculating the ratio of the MFI of the total phagocytosed bacteria to the MFI of the total escaped bacteria. The increase in MOI also led to an increase in the containment index of the cells (Figure 4D) which shows that, although the cells are taking up more bacteria overall, the amount of bacteria that is escaping stays relatively the same. This essentially means the neutrophils are able to effectively keep the bacteria contained even as the amount of total bacteria present in the cell increases. These data suggest that there is a positive correlation between total bacteria present, the percentage of cells that take up bacteria and the percentage of

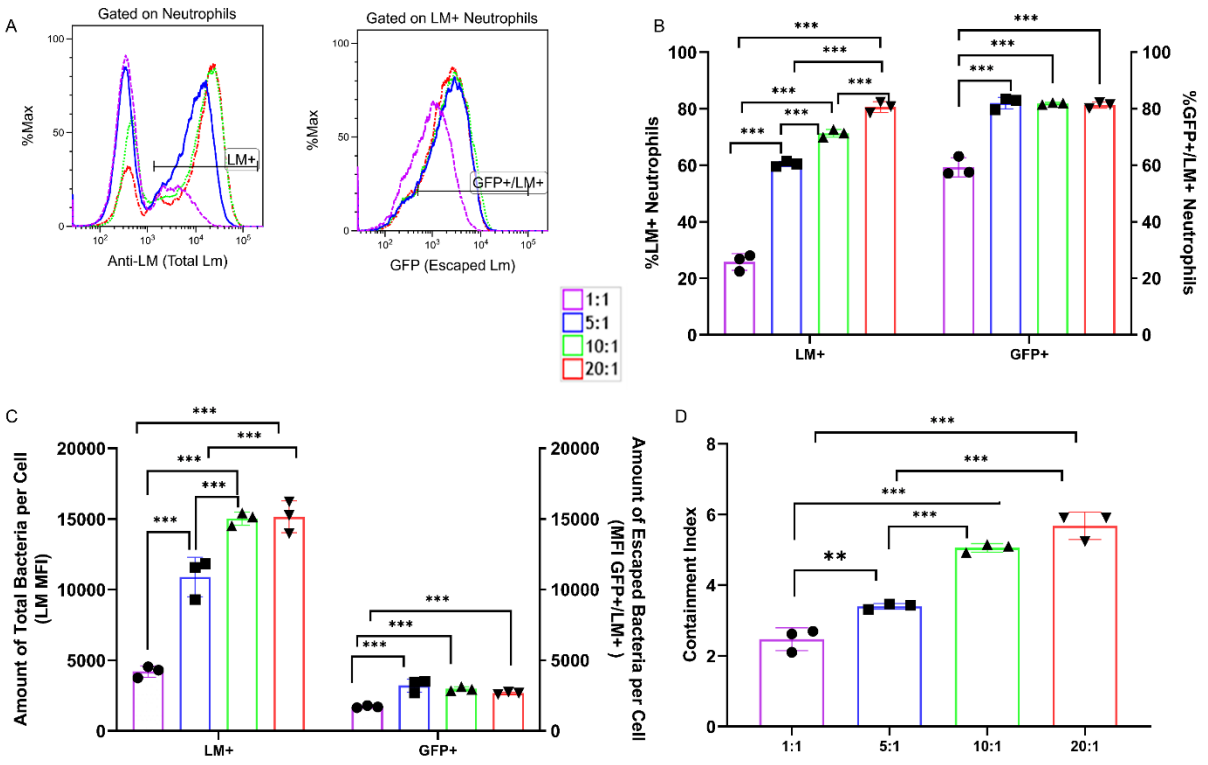

Figure 4: actA:LMGFP as a reporter for the measurement of phagosomal escape:

Bone marrow leukocytes harvested from B6 mice were infected, *in vitro*, with actA:LMGFP at different MOIs ranging from 1:1 to 20:1 for 2 hours. Extracellular bacteria were killed with gentamicin followed by staining of cells for flow cytometry with antibodies against *Lm* and neutrophil cell surface markers. Shown are representative histograms depicting neutrophils that phagocytosed (LM^+) and allowed for phagosomal escape (GFP^+) of *Lm* (A). The percentage of neutrophils that have phagocytosed and allowed for phagosomal escape of *Lm* were calculated (B). The MFI of LM^+ cells which is representative of the amount of total bacteria taken up as well as the GFP^+ MFI, representative of the amount of escaped bacteria were also analyzed (C). The containment index was calculated as a ratio the MFI of total to escaped bacteria (D). Data are representative of 2 independent experiments with n = 3. cells that allow *Lm* escape. 2-way (B-C) and 1-way ANOVA (D) with Bonferroni multiple comparison *post-hoc* test.

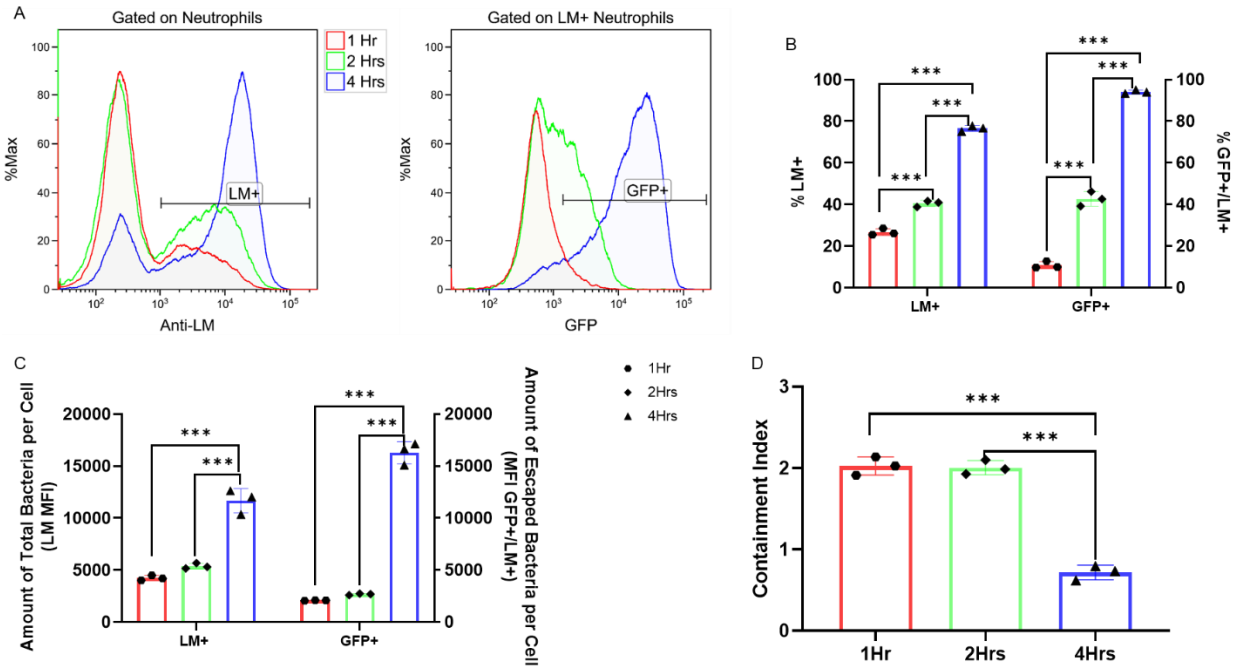

Figure 5: Kinetics of actA:LMGFP infection in neutrophils:

Bone marrow leukocytes were harvested from B6 mice and infected with opsonized actA:LMGFP at an MOI of 5:1 for 1, 2 or 4 hours. Extracellular bacteria were killed with gentamicin. Shown are representative histograms of LM^+ and GFP^+ neutrophils (A) and the percentage of LM^+ neutrophils following 1, 2 and 4 hours of infection (B). The MFI of LM^+ and GFP^+ (C) neutrophils were analyzed as well as the containment index at the different time points (D). Data are representative of 2 independent experiments with n = 3. 2-way (B-C) and 1-way ANOVA (D) with Bonferroni multiple comparison *post-hoc* test.

cells that allow *Lm* escape. It also demonstrates that even at high doses, neutrophils are able to effectively contain *Lm* in the phagosome." (65)

"So as to assess the appropriate amount of time to infect the cells, a kinetics assay was conducted. Bone marrow leukocytes from ecSOD WT mice were infected with actA:LMGFP at an MOI of 5:1 for 1, 2 or 4 hours. There was a significant increase in the percentage of LM^+ neutrophils at the 2 and 4 hour time points in comparison to the 1 hour time point (Figures 5A&B). The percentage of GFP+ neutrophils also increased significantly following 2 and 4 hours of infection (Figures 5A&B). At 1 and 2 hours post infection, there were no significant differences the LM^+ MFI and GFP^+ MFI of the cells (Figure 5C), which indicates that at those time points the neutrophils are taking up equal amounts of bacteria and allowing for similar amounts to escape out of the phagosome. However, there is a significant increase in both the LM^+ MFI and the GFP^+ MFI following 4 hours of infection (Figure 5C). Additionally, although the containment index for the 1 and 2 hour infections are similar, a significant reduction occurs following 4 hours of infection (Figure 5D). These data suggest that after 4 hours of infection, the neutrophils take up an excessive amount of *Lm* which hinders their ability to effectively keep the bacteria contained in the phagosome. Therefore, we chose the 2 hour time point for the continuation of these studies."(65)

EcSOD activity leads to a decrease in phagocytosis of Lm

In order to determine how ecSOD activity modifies bacterial containment by neutrophils, phagocytosis of bacteria was first investigated. Liver leukocytes, splenocytes and bone marrow leukocytes isolated from ecSOD congenic mice were infected with actA:LMGFP and the percentage of neutrophils that took up *Lm* (LM^+) was measured. There were no statistical differences in the percentage of liver neutrophils that phagocytosed *Lm*, though, it does appear that

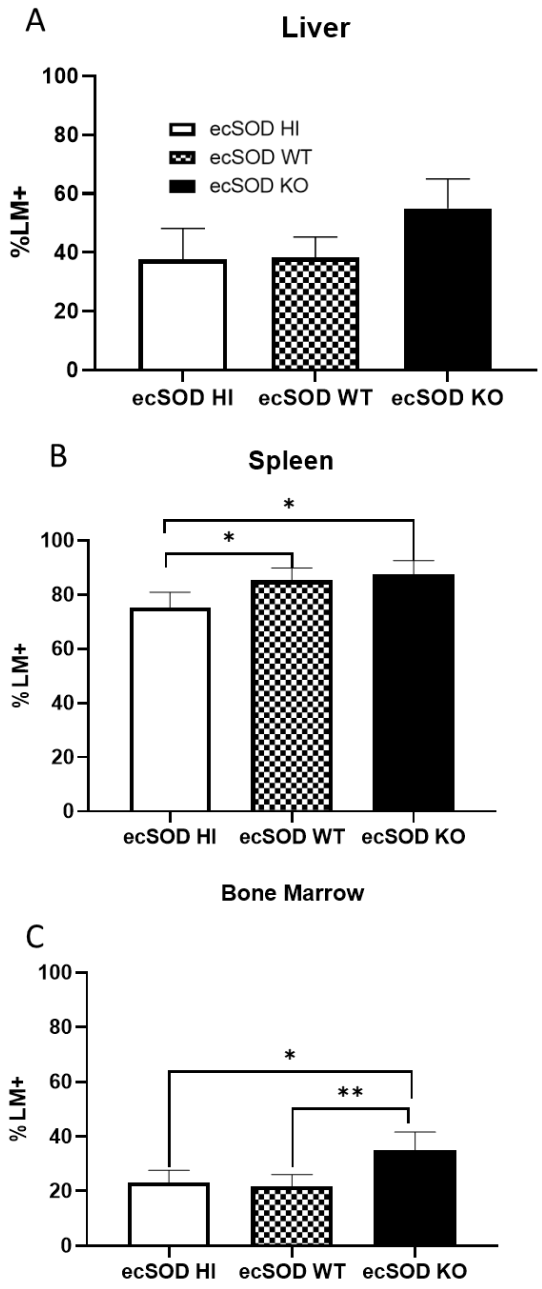

Figure 6: EcSOD activity decreases neutrophil phagocytosis of *Lm*:

Liver leukocytes, splenocytes and bone marrow leukocytes were harvested from the ecSOD congenic mice. Cells were infected with actA:LMGFP at an MOI of 5:1 for 2 hours and extracellular bacteria killed with gentamicin. Cells were stained for flow cytometry with neutrophil markers and the antibody against *Lm*. The percentage of cells from the liver (A), spleen (B) and bone marrow (C) that are LM^+ were measured. Data are representative of 2 independent experiments and $n \geq 4$ mice per group. 1-way ANOVA with Bonferroni multiple comparison *post-hoc* test.

a higher percentage of the ecSOD KO cells took up the bacteria (Figure 6A). A higher percentage of neutrophils from the spleen and bone marrow of ecSOD KO mice also took up *Lm* in comparison to the ecSOD HI and ecSOD WT cells (Figures 6B&C). Therefore, ecSOD activity decreases neutrophil phagocytosis of *Lm*.

EcSOD activity has no effect on phagosomal containment of Lm

The method by which ecSOD may be modulating containment of bacteria by neutrophils was next investigated. First, the amount of *Lm* taken up was measured in cells from the ecSOD congenic mice. The MFI of the LM^+ cells was calculated as an indicator of the amount of bacteria taken up per neutrophil. The ecSOD KO neutrophils from the liver and spleen took up more bacteria in comparison to the ecSOD HI and WT cells (Figures 7A&B). This indicates that ecSOD activity leads to a decrease in the amount of bacteria phagocytosed by neutrophils. However, there were no significant differences in the amount of *Lm* taken up by bone marrow neutrophils (Figure 7C). The GFP^+ MFI was also evaluated to determine the amount of bacteria that escaped per cell. There were no statistically significant differences in the amount of bacteria that escaped out of the phagosome between the ecSOD congenic mice neutrophils from the liver, spleen and bone marrow (Figures 7A-C). Therefore, although the ecSOD KO neutrophils from the liver and spleen are taking up more *Lm* (Figures 7A&B), these cells are allowing for similar amount of bacteria to escape out of the phagosome in comparison to the ecSOD HI neutrophils. From these results, it could be inferred that the ecSOD KO neutrophils are more effective at phagosomal containment of *Lm*, however, this proved to be incorrect. The *Lm* containment index of liver (Figure 7D), spleen (Figure 7E) and bone marrow (Figure 7F) of ecSOD HI, WT and KO mice was assessed. There were no differences in the containment index of the neutrophils from all three organs (Figures 7D-F).

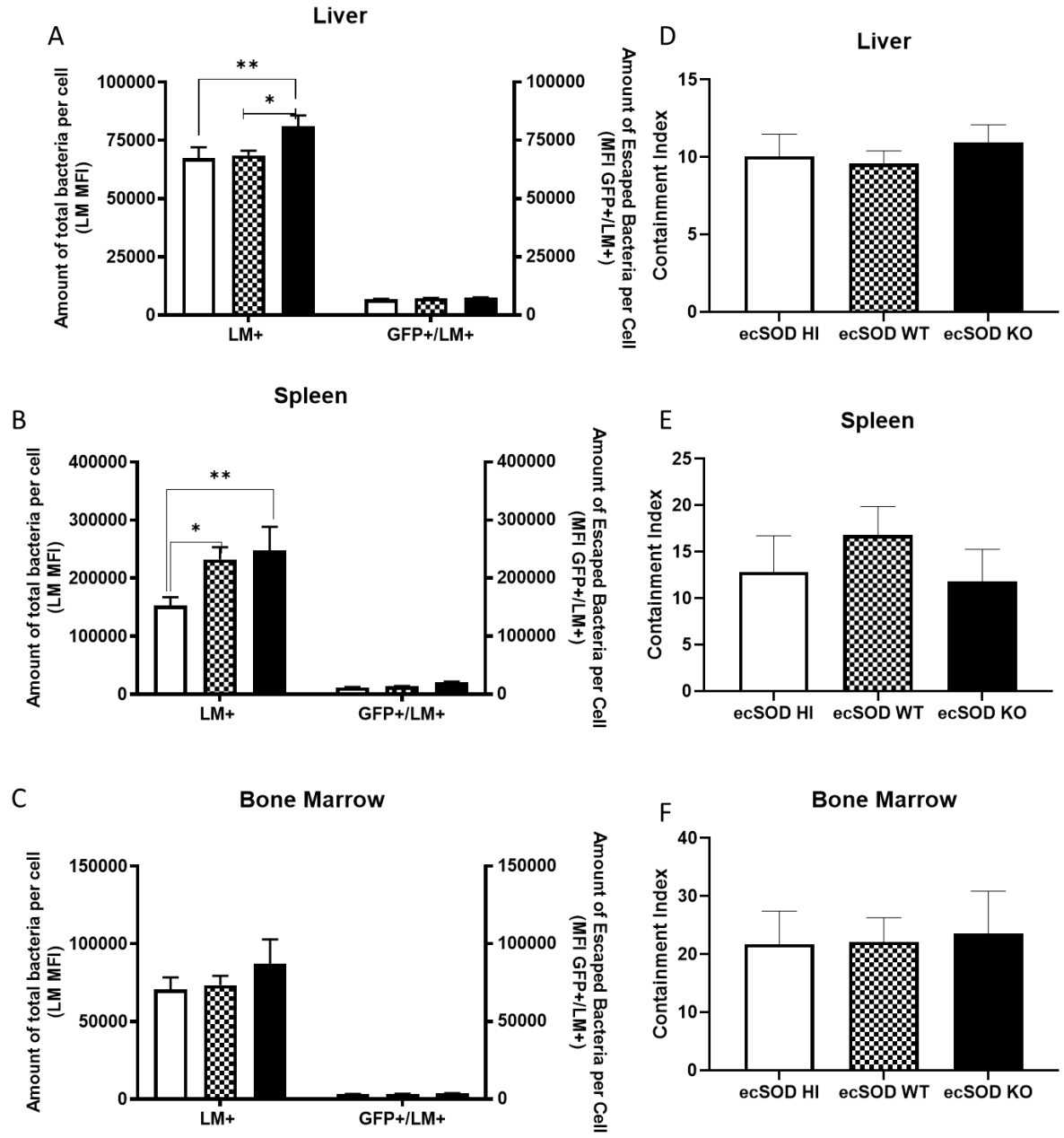

Figure 7: EcSOD activity has no effect on phagosomal containment of *Lm* by neutrophils:

Liver leukocytes, splenocytes and bone marrow leukocytes were harvested from the ecSOD congenic mice. Cells were infected with actA:LMGFP for 2 hours at an MOI of 5:1 and extracellular bacteria killed with gentamicin. Cells were stained for flow cytometry with neutrophil markers and the antibody against *Lm*. The LM^+ and GFP^+ MFI of liver (A), spleen (B) and bone marrow (C) neutrophils were measured. The containment index of neutrophils from the liver (D), spleen (E) and bone marrow (F) were also calculated. Data are representative of 2 independent experiments and $n \geq 4$ mice per group. 2-way (A-B) and 1-way (D-E) ANOVAs with Bonferroni multiple comparison *post-hoc* test.

Although the ecSOD KO neutrophils took up more bacteria and allowed for similar amounts of *Lm* to escape, calculation of the ratio of total to escaped bacteria indicated that neutrophils from all three groups of mice contain *Lm* in the phagosome similarly. Therefore, EcSOD activity has no effect on the ability of neutrophils to contain *Lm*.

Pre-activation increases Lm phagocytosis by bone marrow neutrophils

The lack of differences in *Lm* containment between the neutrophils from the three congenic groups was an unexpected finding. It was hypothesized that these results may be due to the cells not being primed prior to infection. To determine if pre-activation could lead to more effective phagosomal containment, the cells were primed prior to infection with IFN-γ. IFN-γ is a cytokine that has been identified as necessary for protection during *Lm* infection as it promotes effective clearance of bacteria (103, 104). During *Lm* infection, IFN-γ has been demonstrated to induce increased phagocytosis, specifically in macrophages (105). Leukocytes from the liver, spleen and bone marrow of ecSOD WT mice were incubated with or without IFN-γ prior to infection with actA:LMGFP. There were no differences in the percentage of liver neutrophils that were LM^+, which are cells that phagocytosed *Lm* (Figure 8A). Similarly to the liver neutrophils, there were also no differences in the percentage of splenic cells that phagocytosed *Lm* (Figure 8B). These results indicate that IFN-γ does not enhance the percentage of neutrophils from the spleen and liver that phagocytose *Lm*. However, pre-activation of bone marrow neutrophils led to an increase in the percentage of neutrophils that phagocytosed *Lm* (Figure 8C). These data suggest that pre-activation of neutrophils with IFN-γ has no effect on liver and splenic neutrophils but increases phagocytosis of *Lm* by bone marrow neutrophils.

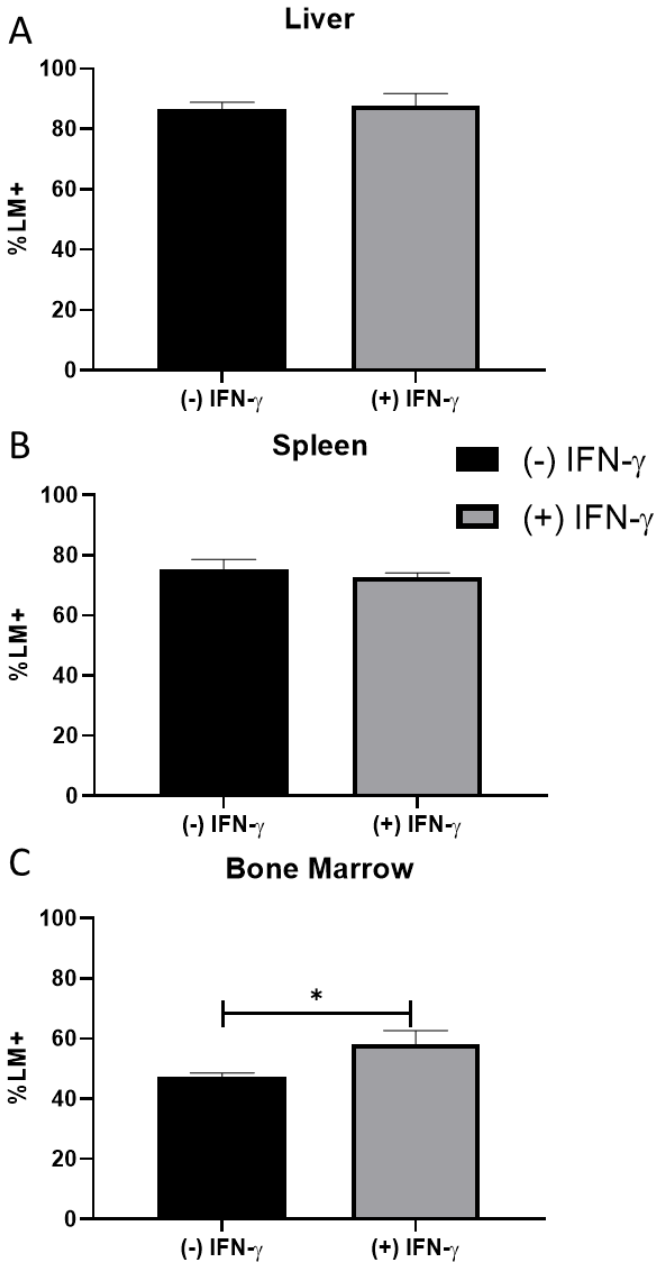

Figure 8: IFN-γ increases *Lm* phagocytosis by bone marrow neutrophils:

Liver leukocytes, splenocytes and bone marrow leukocytes were harvested from the ecSOD WT mice. Cells were treated with IFN-γ for an hour and then infected with actA:LMGFP at an MOI of 5:1. Extracellular bacteria were killed with gentamicin. Cells were stained for flow cytometry with neutrophil markers and the antibody against *Lm*. The percentage of LM^+ neutrophils from the liver (A), spleen (B) and bone marrow (C) were measured. Data are representative of 2 independent experiments and n = 3 mice per group. 2-tailed t-tests.

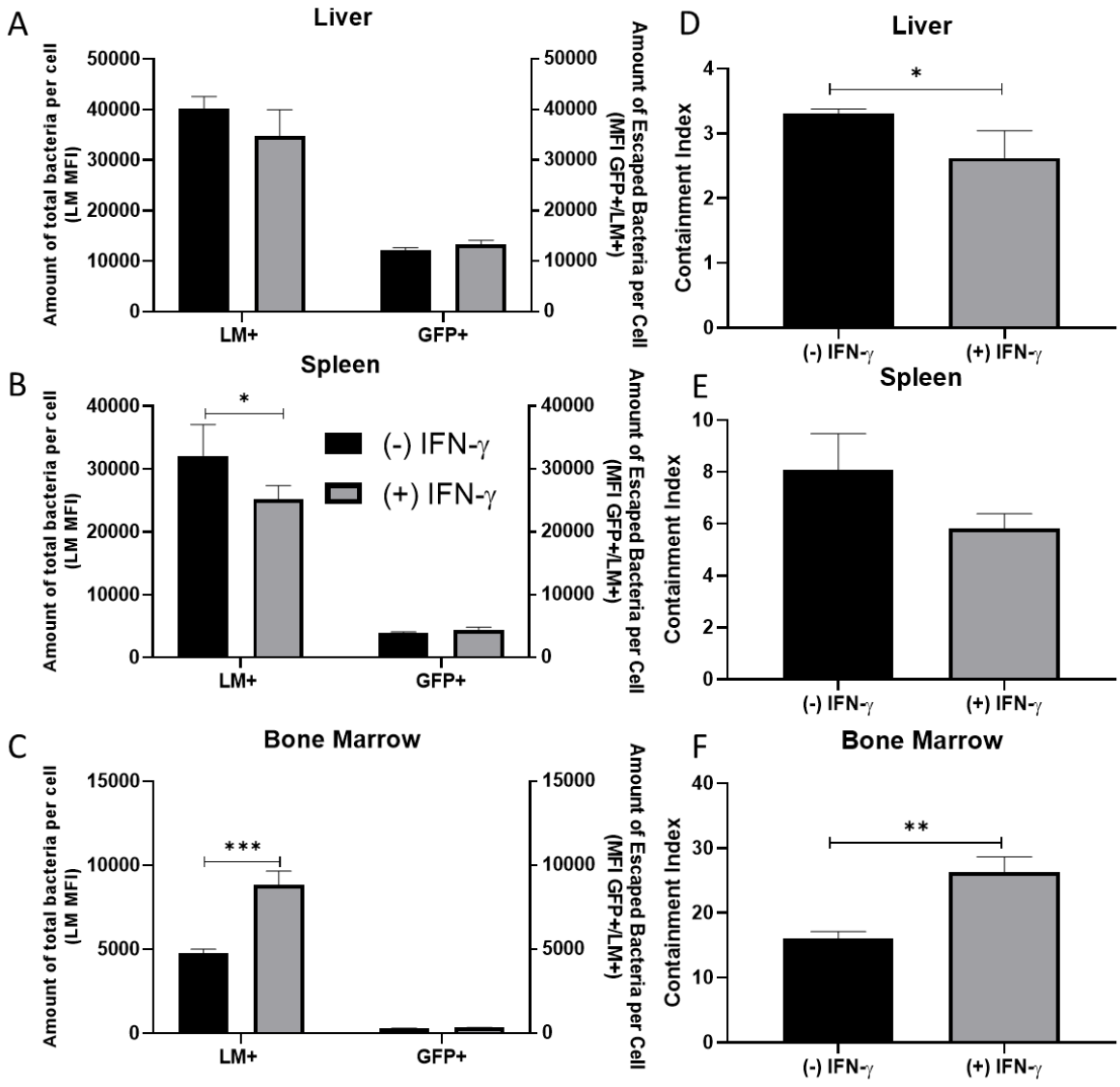

Figure 9: IFN-γ increases containment of *Lm* by bone marrow neutrophils:

Liver leukocytes, splenocytes and bone marrow leukocytes were harvested from the ecSOD WT mice. Cells were treated with IFN-γ and then infected with actA:LMGFP for 2 hours at an MOI of 5:1. Extracellular bacteria were killed with gentamicin. Cells were stained for flow cytometry with neutrophil markers and the antibody against *Lm*. The LM and GFP MFI of neutrophils from the liver (A), spleen (B) and bone marrow (C) were measured. The containment index of liver (D), spleen (E) and bone marrow (F) neutrophils were also calculated. Data are representative of 2 independent experiments and n = 3 mice per group. 2-way with Bonferroni multiple comparison *post-hoc* test (A-C) and 2 tailed t-tests (D-F).

Pre-activation with IFN-γ increases phagosomal containment of Lm in bone marrow neutrophils

To determine the effects of pre-activation of neutrophils on phagosomal containment of *Lm*, the MFI of LM^+ and GFP^+ cells were measured with and without the presence of IFN-γ. Pre-activation did not increase the amount of *Lm* that were phagocytosed by liver neutrophils nor did it have any effect on the amount of bacteria that escaped out of the phagosome (Figure 9A). However, calculation of the containment index showed that pre-activation with IFN-γ leads to a decrease in liver neutrophil containment of *Lm* (Figure 9D). There was an unexpected reduction in the amount of *Lm* taken up splenic neutrophils following pre-activation with IFN-γ (Figure 9B). There were also no differences in the amount of bacteria that escaped out of the phagosome nor in the containment index of the splenic neutrophils (Figure 9B&E). Neutrophils from the bone marrow, however, phagocytosed more *Lm* following pre-activation with IFN-γ but there were no differences in the amount of bacteria that escaped out of the phagosome (Figure 9C). Pre-activation of the bone marrow leukocytes led to an increase in the containment index which indicated that treatment with IFN-γ leads to more effective containment of *Lm* by bone marrow neutrophils (Figure 9F). However, pre-activation of liver and splenic neutrophils does not lead to more effective phagosomal containment. Additionally, there were no observed differences in phagocytosis and phagosomal containment of *Lm* between neutrophils from the congenic mice pre-treated in IFN-γ (data not shown).

The presence of SOD has no effect on phagocytosis of Lm by ecSOD KO neutrophils.

The observed lack of differences in the ability of neutrophils from the three groups of ecSOD congenic mice to contain *Lm* was unexpected. We hypothesized that lack of differences may be due to the stymied effects of ecSOD as a result of the assay being conducted *in vitro*.

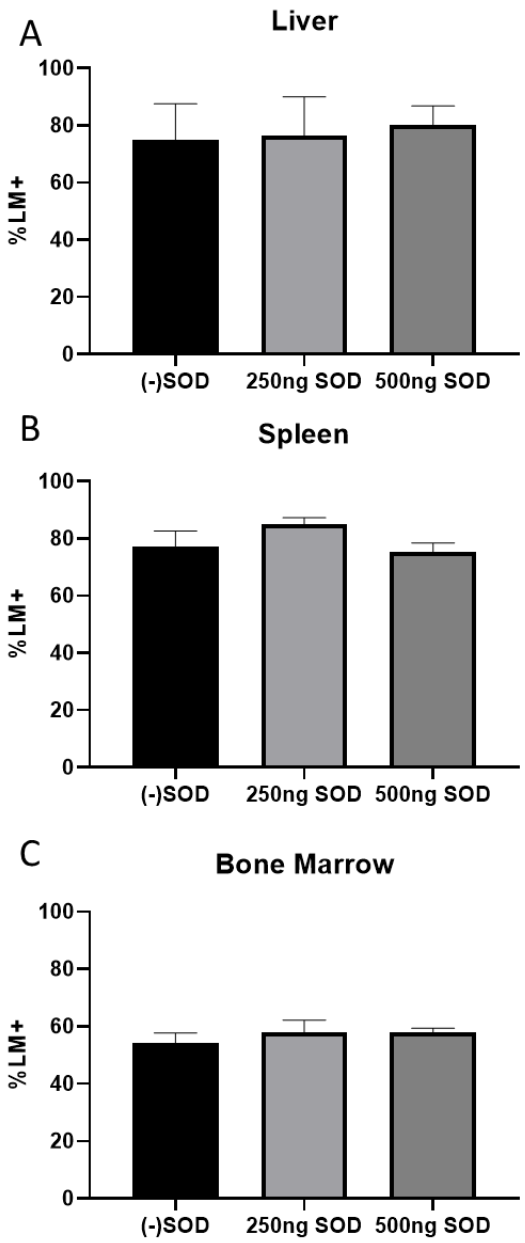

Figure 10: Treatment with recombinant SOD2 does not impair *Lm* phagocytosis by ecSOD KO neutrophils:

Liver leukocytes, splenocytes and bone marrow leukocytes were harvested from ecSOD KO mice. Cells were treated with different concentrations of recombinant SOD2 and infected with actA:LMGFP for 2 hours at an MOI of 5:1. Extracellular bacteria were killed with gentamicin. Cells were stained for flow cytometry with neutrophil markers and the antibody against *Lm*. The percentage of LM^+ neutrophils from the liver (A), spleen (B) and bone marrow (C) were measured. Data are representative of 2 independent experiments and n = 3 mice per group. 1-way ANOVA with Bonferroni multiple comparison *post-hoc* test.

As ecSOD is localized extracellularly, *in vivo*, it is likely phagocytosed with *Lm* and present in the phagosome with the bacteria. To replicate this scenario, liver leukocytes, splenocytes and bone marrow leukocytes from ecSOD KO mice were treated with recombinant SOD while being infected with actA:LMGFP. As recombinant mouse ecSOD is unavailable, SOD2 which is normally localized in the mitochondria, but has the same enzymatic activity as ecSOD, was used for these studies. First, the percentage of LM^+ cells was measured to determine if the presence of SOD would decrease phagocytosis of the bacteria by neutrophils from ecSOD KO mice. SOD2 was added at either a dosage of 250ng/mL or 500ng/mL to liver leukocytes, splenocytes and bone marrow leukocytes of ecSOD KO mice. Theoretically, the added recombinant SOD2 would also be present in the phagosome alongside the bacteria.

Bacterial phagocytosis by non-treated or SOD2 treated neutrophils was measured. There were no differences in the percentage of liver cells that phagocytosed *Lm* when treated with either 250ng/mL or 500ng/mL of SOD (Figure 10A). Similarly to the liver, the presence of exogenous SOD2 had no effect on the percentage of splenic (Figure 10B) or bone marrow neutrophils (Figure 10C) from ecSOD KO mice that took up *Lm*. The lack of an increase or decrease in bacteria phagocytosis following treatment of the cells with exogenous SOD indicates that ecSOD might not directly influence the process of phagocytosis by neutrophils. Treatment of ecSOD KO neutrophils with the two different doses of recombinant SOD2 did not change the amount of bacteria taken up by liver (Figure 11A), spleen (Figure 11B) or bone marrow neutrophils (Figure 11C). There were also no significant differences in the amount of *Lm* that escaped out of the phagosome of liver (Figure 11A), spleen (Figure 11B), and bone marrow neutrophils (Figure 11C) following treatment with SOD2.

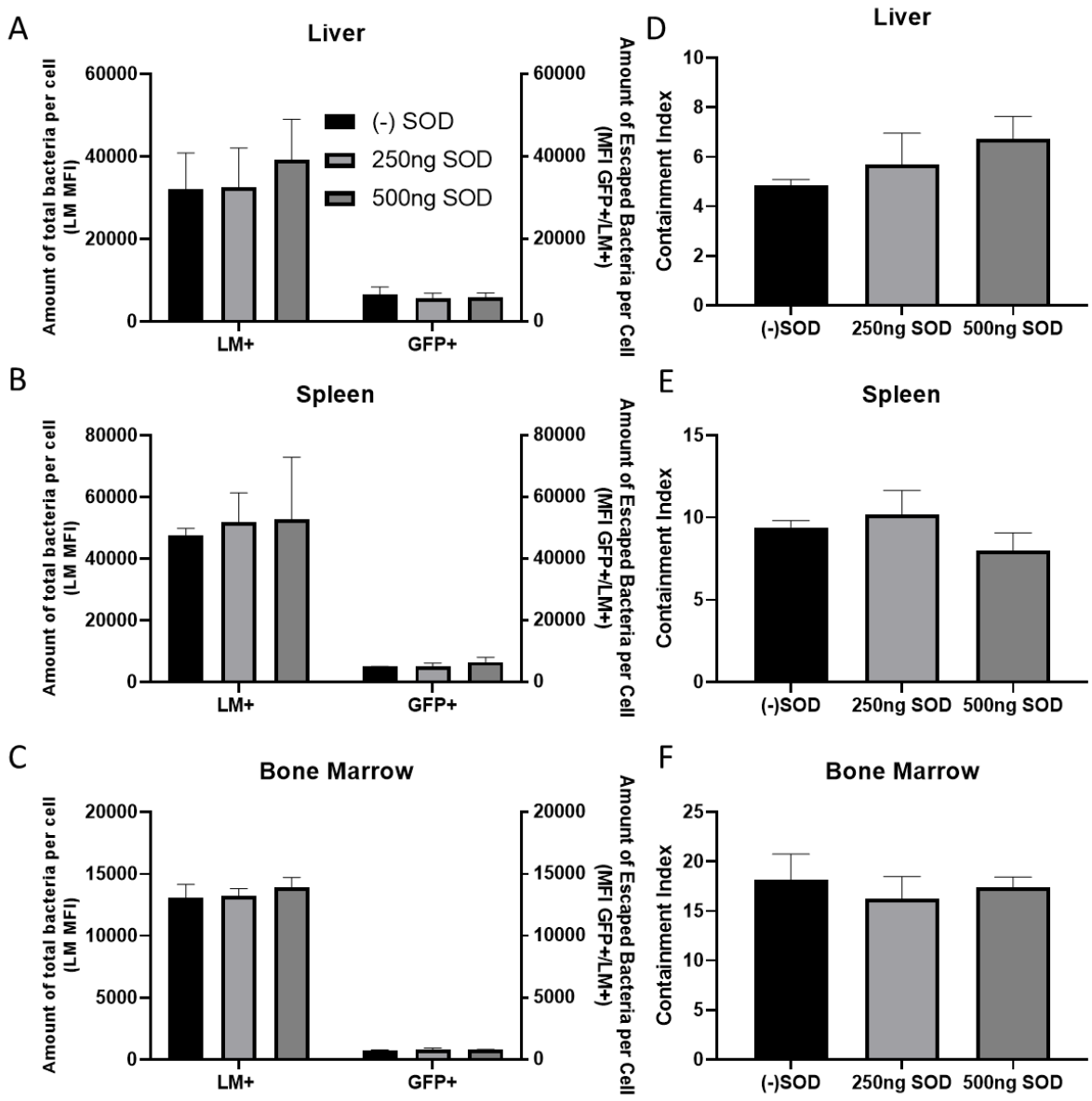

Figure 11: Treatment with recombinant SOD2 does not impair phagosomal containment of *Lm* by ecSOD KO neutrophils:

Liver leukocytes, splenocytes and bone marrow leukocytes were harvested from ecSOD KO mice. Cells were treated with recombinant SOD2 and infected with actA:LMGFP for 2 hours at an MOI of 5:1. Extracellular bacteria were killed with gentamicin. Cells were stained for flow cytometry with neutrophil markers and the antibody against *Lm*. The LM MFI, GFP MFI and containment index of neutrophils from the liver (A), spleen (B) and bone marrow (C) were measured. Data are representative of 2 independent experiments and n = 3 mice per group. 2-way (A-C) and 1-way (D-F) ANOVAs with Bonferroni multiple comparison *post-hoc* test.

In addition, calculation of the containment index showed that treatment with SOD2 did not change the containment index of *Lm* in neutrophils from the liver (Figure 11D), spleen (Figure 11E) and bone marrow (Figure 11F) of ecSOD KO mice. Therefore, the presence of SOD2 during infection does not modulate phagosomal containment of *Lm* by ecSOD KO neutrophils.

EcSOD activity decreases expression of LAMP-1 in neutrophils during Lm infection.

Bacteria are taken up into phagosomes as a means of delivery to the lysosome where the pathogen can be degraded and killed (72). Delivery of the pathogen into the lysosome from the phagosome occurs via fusion of the phagosome with the lysosome to form a phagolysosome, a process that is referred to as phagosome maturation. This newly formed phagolysosome contains ROS, hydrolytic enzymes and other peptides that are toxic to the phagocytosed pathogen (73). The mature phagolysosome is characterized by expression of lysosome associated membrane protein-1 (LAMP-1). LAMP-1 is expressed constitutively in lysosomes but has also been deemed necessary for the formation of the mature phagosome (73). As formation of the phagolysosome is necessary for bacteria killing, the effects of ecSOD activity on LAMP-1 was investigated. For these studies, ecSOD congenic mice were infected with WTLM and splenocytes were harvested following three days of infection. The cells were stained with the antibody against *Lm* as well as the anti-LAMP-1 antibody. First, the percentage of LAMP-1 positive cells out of the LM^- and LM^+ cells was then assessed. . A higher percentage of LM^- neutrophils from ecSOD HI mice were positive for LAMP-1 in comparison to the ecSOD KO LM^- cells (Figure 12B). However, when the cells are positive for LM, there is no difference in the percentage of neutrophils that are positive for LAMP-1 between the ecSOD congenic groups (Figure 12B). The MFI of LAMP-1 was also calculated to determine if ecSOD affected the relative amount of LAMP-1 protein being expressed in the cells.

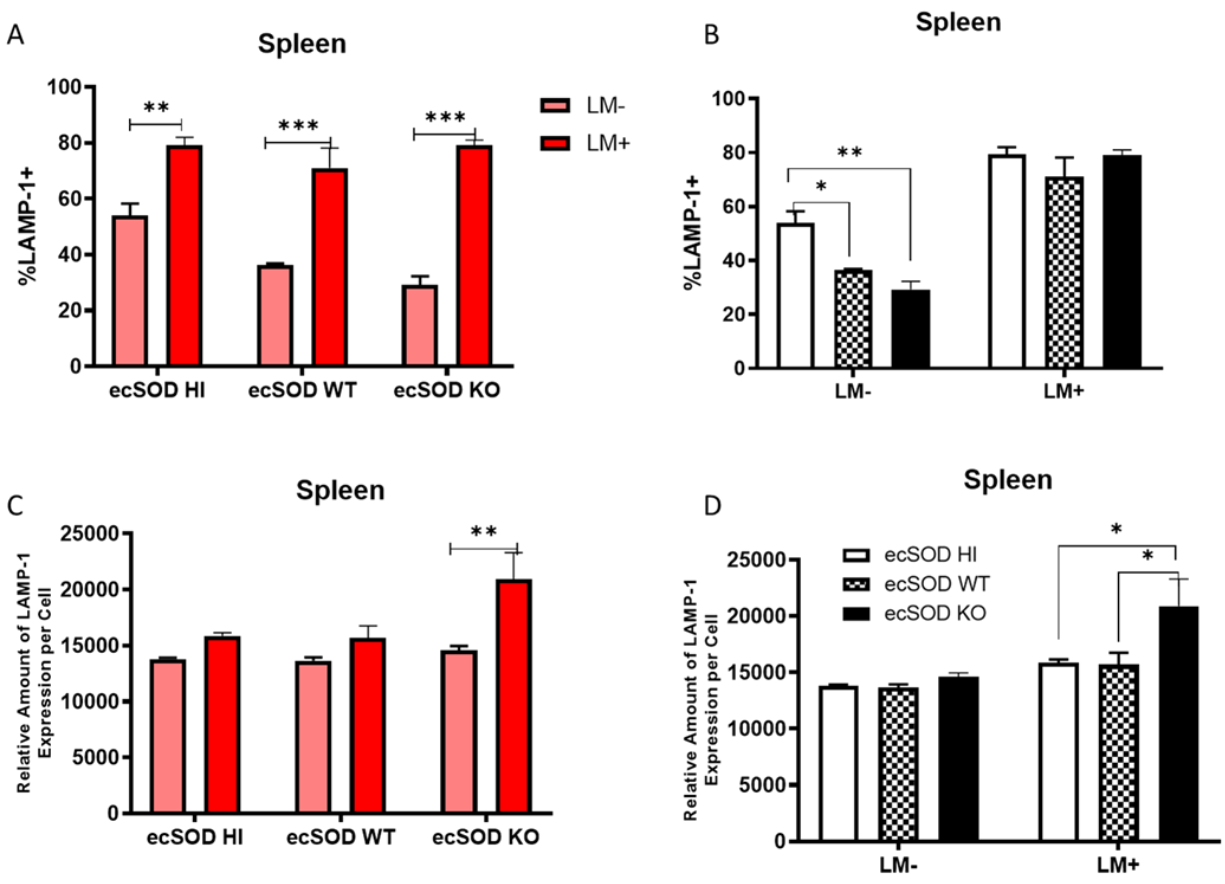

Figure 12: ecSOD decreases LAMP-1 expression by neutrophils during *Lm* infection:

EcSOD congenic mice were infected with $1x10^4$ WTLM and at day 3 post-infection splenocytes were harvested. The cells were stained for flow cytometry with neutrophil specific markers and the anti-*Lm* antibody. The percentage of LAMP-1^+ cells out of the LM^- and LM^+ cells were measured (A&B) and the LAMP-1 MFI was also calculated (C&D). Data are representative of 2 independent experiments and $n \geq 4$ mice per group. 2-way ANOVAs with Bonferroni multiple comparison *post-hoc* test.

In the neutrophils from ecSOD HI and WT mice, there were no differences in the MFI of LAMP-1 between the LM^- and LM^+ cells (Figure 12C). In the neutrophils from ecSOD KO mice, however, the LM^+ cells have a higher LAMP-1 MFI in comparison to the LM^- cells (Figure 12C). Additionally, when neutrophils have not phagocytosed the bacteria (LM^-), there are no differences in LAMP-1 expression between the ecSOD congenic groups (Figure 12D). In the event that the neutrophils take up *Lm* (LM^+), the cells from the ecSOD KO mice express more LAMP-I in comparison to the neutrophils from the ecSOD HI mice (Figure 12D). As a result, lack of ecSOD activity leads to increased expression of LAMP-1 in neutrophils which could possibly drive more efficient killing of *Lm*.

EcSOD activity has no effect on Lm killing by neutrophils.

Following formation of the phagolysosome, the ultimate goal of the neutrophils is to kill the phagocytosed pathogen. As neutrophils have been identified as being necessary for elimination and clearance of the bacteria (1, 14), the effect of ecSOD activity on *Lm* killing was investigated. Neutrophils were sort-purified from the isolated bone marrow leukocytes of ecSOD HI, ecSOD WT and ecSOD KO mice. The cells were then infected with WTLM in order to conduct a killing assay. The CFUs from wells containing neutrophils from each ecSOD congenic group were compared to the CFUs from wells containing the bacterial only and a percentage was calculated in order to report percent killing. There were no observed differences in the percentage of *Lm* killed by neutrophils from all three congenic groups (Figure 13). Neutrophils from the ecSOD HI, WT and KO mice killed approximately 40% of the bacteria present. Therefore, although neutrophils have the ability to kill *Lm*, ecSOD activity does not impact *Lm* killing.

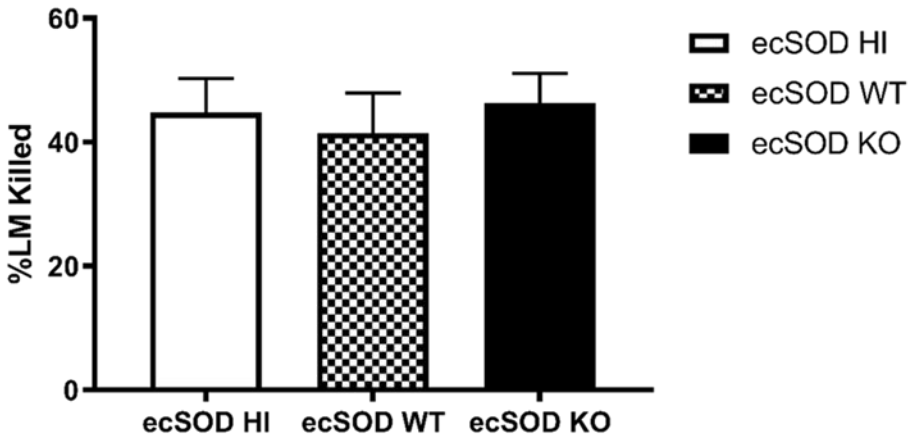

Figure 13: EcSOD activity does not modify neutrophil killing of *Lm*:

Neutrophils were sorted out of the bone marrow of ecSOD congenic mice. The cells were then infected with WTLM at an MOI of 1:1 for a killing assay. The percentage of *Lm* killed were calculated. Data is representative of 2 independent experiments and n = 3 mice per group. 1-way ANOVA with Bonferroni multiple comparison *post-hoc* test.

Phagosomal escape increases the induction of autophagy, independent of ecSOD activity.

Our lab has previously reported that there is more ROS generated in the liver leukocytes of ecSOD KO. A higher percentage of the ecSOD KO neutrophils were found to be positive for Hydroethidine, a dye used for the measurement of ROS generation (106). Additionally, measurement of H_2DCFDA, another ROS dye, also showed that ecSOD KO bone marrow neutrophils generate more ROS. Since ROS can upregulate autophagy, hypothetically, induction and execution of autophagy could be more prevalent in the ecSOD KO phagocytes which would aid in more effective bacterial clearance. To test the above stated hypothesis, splenocytes were infected with the actA:LMGFP strain of *Lm*. As observed in the dose response experiment, an increase in the percentage of LM^+ neutrophils correlate with an increase in the percentage GFP^+ neutrophils. As a higher percentage of ecSOD KO neutrophils usually take up *Lm*, a higher percentage allowed for phagosomal escape in comparison to the ecSOD HI cells (Figure 14A). There were, however, no differences in the percentage of $LC3^+$ neutrophils between the ecSOD groups (Figure 14A).

To gain a better understanding of the effects of phagosomal escape on LC3 expression, the percentage of GFP^- and GFP^+ neutrophils that are double positive for LC3 was measured first. A higher percentage of the GFP^+ ecSOD HI, WT and KO neutrophils were positive for LC3 in comparison to the GFP^- neutrophils (Figure 14B), suggesting that phagosomal escape results in upregulation of the autophagy process. Second, the MFI of LC3, indicative of the amount of LC3 being expressed was also measured. There were no observed differences in LC3 MFI between the GFP^+ ecSOD HI, WT and KO neutrophils (Figure 14C). These sets of data indicate that although phagosomal escape correlates with the induction of autophagy, ecSOD activity does not play a role in the regulation of autophagy in neutrophils.

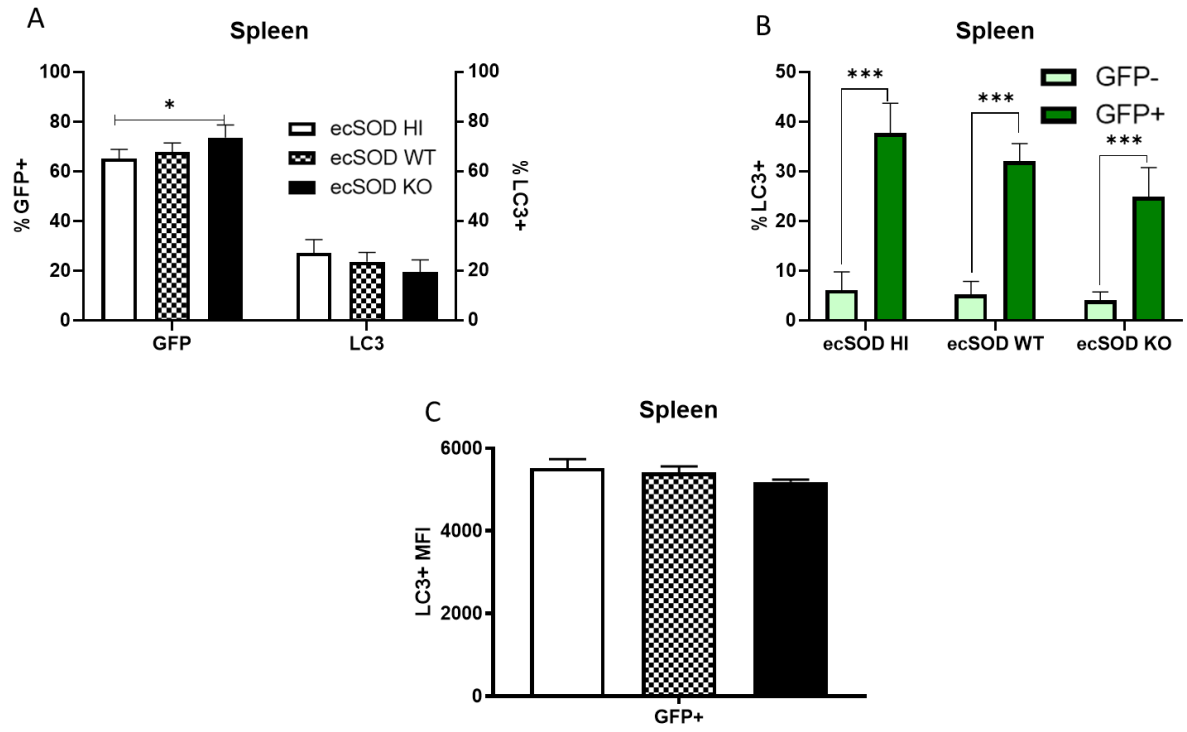

Figure 14: Phagosomal escape increases neutrophil expression of LC3, independent of ecSOD activity:

Splenocytes were harvested from ecSOD congenic mice and infected with actA:LMGFP at an MOI of 5:1. Extracellular bacteria were killed with gentamicin and the cells stained for flow cytometry with neutrophil markers and the anti-LC3 antibody. The percentage of GFP^+ and $LC3^+$ cells was determined (A). The percentage of GFP^- and GFP^+ cells that were $LC3^+$ was also measured (B). The MFI of the $LC3^+$ cells out of the GFP^+ neutrophils was also calculated (C). Data are representative of 2 independent experiments and $n \geq 3$ mice per group. 2-way (A-B) and 1-way ANOVAs with Bonferroni multiple comparison *post-hoc* test.

Inhibition of autophagy leads to reduction in Lm phagocytosis by neutrophils.

Although the above sets of data indicate that ecSOD has no effect on the induction of autophagy, they do demonstrate that phagosomal escape does influence the process. Therefore, to determine the relationship between phagosomal escape and autophagy in neutrophils, the effect of autophagy inhibition was investigated. Inhibition of autophagy was conducted with the use of chloroquine. Chloroquine is a pharmacological agent that prevents lysosomal acidification by preventing autophagosome fusion with the lysosome which subsequently prevents effective autophagy (107, 108). Chloroquine was administered to splenocytes and bone marrow leukocytes at three different does of 10μM, 50μM and 100μM and the cells infected with *Lm*. The percentage of splenic and bone marrow neutrophils that were LM^+, which are the cells that phagocytosed bacteria, significantly decreased when treated with 50μM and 100μM of chloroquine (Figures 15A&B). These data indicate that inhibition of autophagy actually leads to decreased uptake of *Lm* by neutrophils.

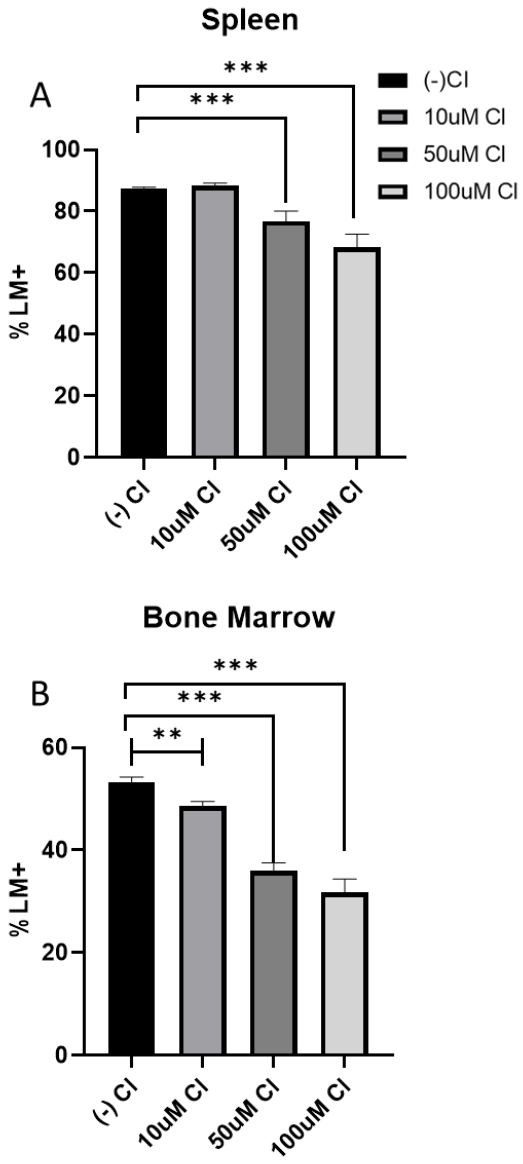

Figure 15: Treatment with chloroquine reduces phagocytosis of *Lm* by ecSOD HI neutrophils:

Splenocytes and bone marrow leukocytes were harvested from ecSOD HI mice and treated with differing concentrations of chloroquine and then infected with actA:LMGFP for 2 hours at a MOI of 5:1. Extracellular bacteria were killed with gentamicin and the cells stained for flow cytometry with neutrophil markers and the anti-*Lm* antibody. The percentage of LM^+ neutrophils from the spleen (A) and bone marrow (B) were measured. Data are representative of 2 independent experiments and n = 3 mice per group. 1-way ANOVA with Bonferroni multiple comparison *post-hoc* test.

Inhibition of autophagy reduces phagosomal containment of Lm by neutrophils.

Following the investigation of the effects of inhibition of autophagy on the phagocytosis of *Lm* by neutrophils, the effects on phagosomal containment were also studied. After infection of splenocytes and bone marrow leukocytes with the actA:LMGFP bacteria, the MFI of LM^+ and GFP^+ neutrophils was analyzed. In comparison to the non-chloroquine treated cells, neutrophils treated with 50μM or 100μM of chloroquine had a lower LM^+ MFI in both the spleen and bone marrow (Figures 16A&B). Essentially, chloroquine treatment led to a decrease in the amount of total bacteria taken up by neutrophils from the bone marrow and spleen. In splenic neutrophils, chloroquine treatment also led to a decrease in the GFP^+ MFI (Figure 16A). Less bacteria escaped out of the phagosomes of the chloroquine treated neutrophils in comparison to the non-chloroquine treated cells. In bone marrow neutrophils there were no differences in the GFP^+ MFI of chloroquine treated neutrophils in comparison to the non-treated (Figure 16B). Quantification of bacterial containment via calculation of the containment index showed that the splenic neutrophils treated with 100μM of chloroquine have a lower containment index in comparison to the untreated cells (Figure 16C). Bone marrow neutrophils treated with either 50μM or 100μM of chloroquine also had a lower containment index in comparison to the untreated neutrophils (Figure 16D). In summation, inhibition of autophagy with chloroquine limits the amount of *Lm* taken up and reduces effective phagosomal containment of the bacteria by neutrophils.

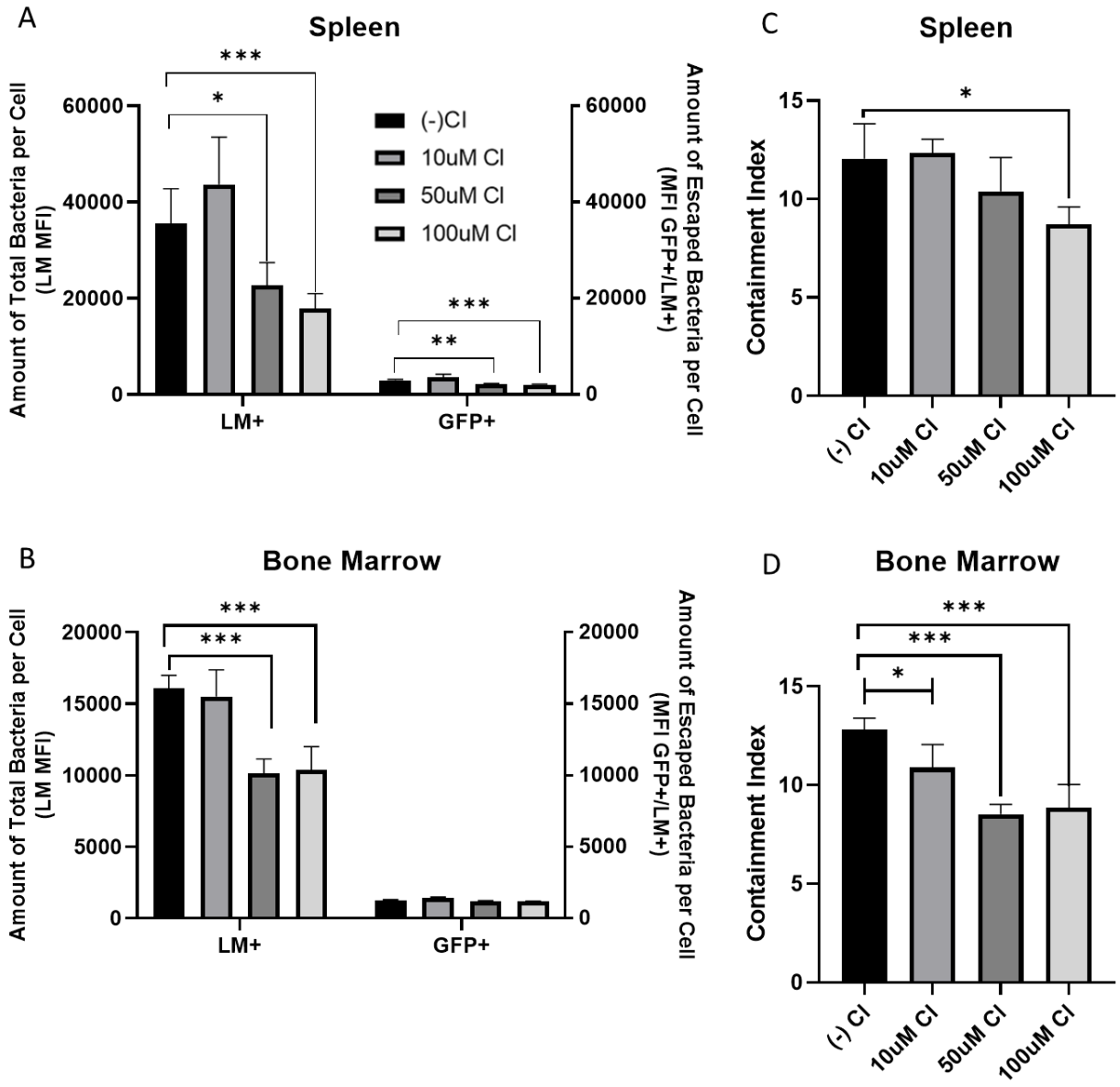

Figure 16: Treatment with chloroquine reduces phagosomal containment of Lm by neutrophils:

Splenocytes and bone marrow leukocytes were harvested from ecSOD HI mice and treated with differing concentrations of chloroquine and then infected with actA:LMGFP at a MOI of 5:1. Extracellular bacteria were killed with gentamicin and the cells stained for flow cytometry with neutrophil markers and the anti-LM antibody. The LM and GFP MFI of neutrophils from the spleen (A) and bone marrow (B) were measured. The containment index of the splenic (C) and bone marrow (D) neutrophils were also calculated. Data are representative of 2 independent experiments and n = 3 mice per group. 2-way (A-B) and 1-way (C-D) ANOVAs with Bonferroni multiple comparison *pot-hoc* test.

Summary of Chapter III

The above sets of data highlight the effects of ecSOD on *Lm* phagocytosis by neutrophils. The presence of extracellular bacteria in the spleen, though independent of ecSOD activity following one day of infection, is dependent on HI activity of ecSOD at day 3 post infection. The effect of ecSOD activity on phagocytosis of *Lm* was also investigated and neutrophils from the ecSOD KO mice more effectively phagocytosed the bacteria in comparison to neutrophils from ecSOD WT and ecSOD HI mice. However, ecSOD activity did not have an effect on phagosomal containment of *Lm* by liver, splenic or bone marrow neutrophils. Additionally, pre-activation of the neutrophils with IFN-γ increased phagosomal containment of *Lm* by neutrophils from the bone marrow but not the spleen or liver. The ability of the neutrophils to kill the bacteria was also investigated. EcSOD activity decreased LAMP-1 expression in splenic neutrophils following infection with *Lm* which led to the inference that ecSOD activity reduces phagosomal maturation in neutrophils. An *in vitro* killing assay was performed to determine differences in bacteria killing by neutrophils from the bone marrow of the ecSOD congenic mice. However, there were no differences in *Lm* killing by neutrophils from all three congenic groups. The role of ecSOD activity on autophagy was also investigated. Although infection led to an increase in the percentage of neutrophils inducing autophagy, there were no differences in LC3 expression between neutrophils from the ecSOD congenic mice. Additionally, inhibition of autophagy with the drug chloroquine, led to decreased phagocytosis and phagosomal containment of *Lm* by neutrophils. This suggests that ecSOD indirectly regulates uptake of *Lm* but does not modulate phagosomal escape, killing or autophagy in neutrophils.

CHAPTER IV

FUNCTIONAL DIFFERENCES BETWEEN NEUTROPHILS AND MONOCYTES DURING *LISTERIA MONOCYTOGENES* INFECTION

Serum opsonization of Lm increases phagocytosis by neutrophils

The lack of neutrophils and monocytes during infection with *Lm* has been proven to be detrimental to host resistance (13). Previous studies conducted with both or either cell types have assigned specific functions to neutrophils and monocytes. However, a comprehensive understanding of their similarities and differences in function necessary for clearance of *Lm* will likely provide more knowledge about functional capabilities of neutrophils and monocytes during other diseases and inflammatory responses, especially other bacterial infections. To gain a better understanding of differences and similarities in function, the ability of neutrophils and monocytes to phagocytose and contain *Lm* was investigated. A previous study on phagocytosis of *Lm* by macrophages demonstrated that complement receptor signaling, specifically the receptor CR3 which binds to iC3b, drives increased phagocytosis of the bacteria (68). In addition, neutralization of CR3 in mice led to increased susceptibility to *Lm* infection (69). Therefore, it is also possible that complement signaling plays a role in phagocytosis of *Lm* by neutrophils and monocytes. To investigate this, splenocytes and bone marrow leukocytes were infected with either unopsonized or serum opsonized actA:LMGFP *Lm* and the percentage of LM^+ neutrophils and monocytes were measured. A higher percentage of splenic and bone marrow neutrophils phagocytosed *Lm* when the bacteria is opsonized compared to when it is unopsonized (Figures 17A-C). In contrast, serum opsonization of *Lm* led to a decrease in the percentage of LM^+ splenic and bone marrow monocytes (Figures 17B&C). These data indicate that serum opsonization of *Lm* leads to increased phagocytosis of the bacteria by neutrophils but decreases *Lm* phagocytosis by monocytes.

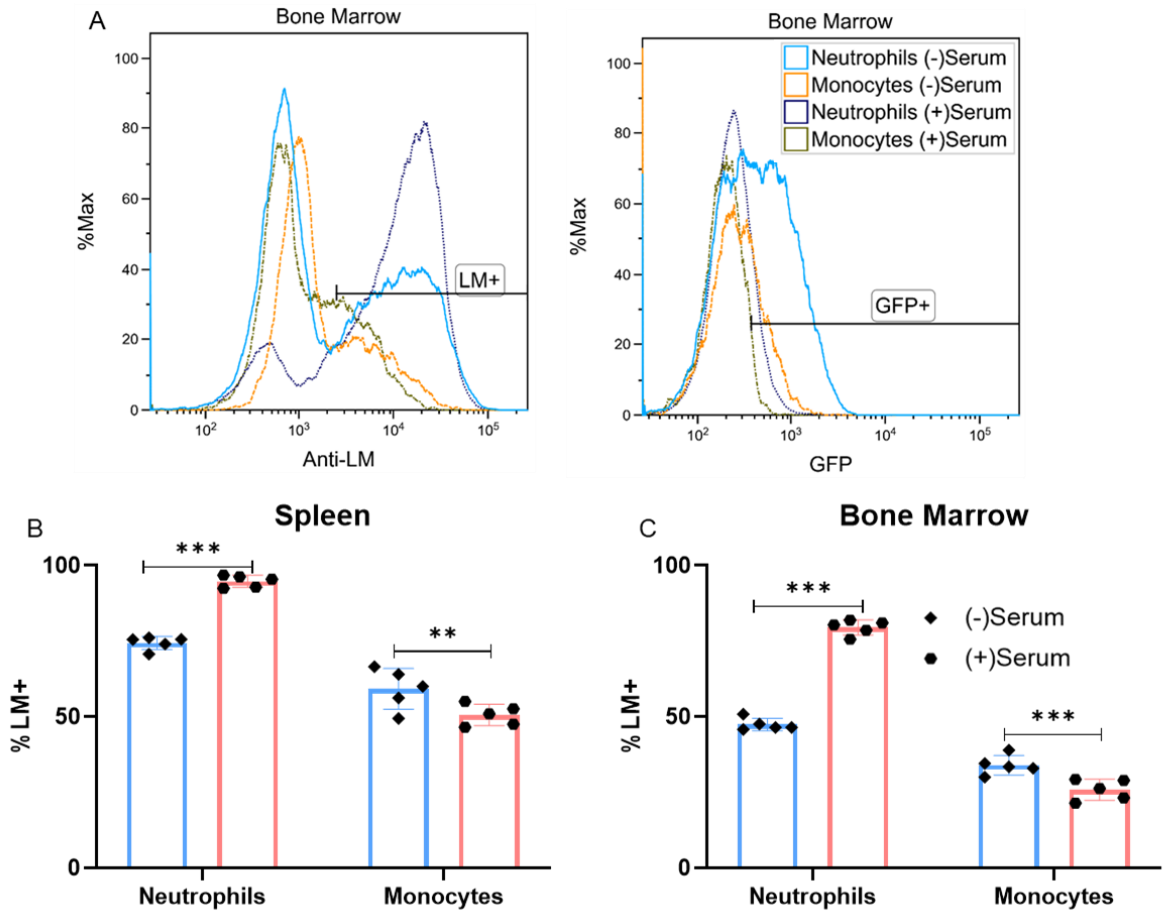

Figure 17: Serum opsonization increases neutrophil phagocytosis of *Lm*:

Splenocytes and bone marrow leukocytes were harvested from B6 mice and infected with opsonized or unopsonized actA:LMGFP for 2 hours at an MOI of 5:1. Extracellular bacteria was killed with gentamicin and cells stained for *Lm* as well as neutrophils and monocyte markers. Shown are representative histograms of LM^+ and GFP^+ neutrophils infected with unopsonized or serum opsonized *Lm* (A). The percentage of LM^+ neutrophils and monocytes infected with unopsonized or serum opsonized *Lm* from the spleen (B) and bone marrow (C) was measured via flow cytometry. Data are representative of 2 independent experiments with n = 5. 2-way ANOVA with Bonferroni multiple comparison *post-hoc* test.

Serum opsonization increases phagosomal containment of Lm by neutrophils

Next, the effects of serum opsonization on phagosomal containment of *Lm* by neutrophils and monocytes was studied. Splenocytes and bone marrow leukocytes were infected with actA:LMGFP and the MFI of LM^+ cells was measured as an indicator of the total amount of bacteria taken up by the cells. Opsonization of *Lm* with serum led to a higher LM^+ MFI in splenic neutrophils, (Figure 18A) but not in bone marrow neutrophils (Figure 18B). There were no differences in the LM^+ MFI of splenic monocytes that took up either opsonized or unopsonized *Lm* (Figure 18A). However, bacteria opsonization led to a decrease in the LM^+ MFI of bone marrow monocytes (Figure 18B). To determine the effects of serum opsonization on *Lm* escape from the phagosome, the GFP^+ MFI was analyzed after infection of splenocytes and liver leukocytes with either unopsonized or opsonized actA:LMGFP.

There was an observed lower GFP^+ MFI in the splenic (Figure 18C) and bone marrow neutrophils (Figure 18D) infected with serum opsonized *Lm* in comparison to the cells infected with unopsonized bacteria. Splenic monocytes infected with opsonized *Lm* had similar GFP^+ MFI in comparison to monocytes infected with unopsonized bacteria (Figure 18C). In the bone marrow, however, there was a reduction in the GFP^+ MFI of the monocytes infected with opsonized *Lm* in comparison to unopsonized *Lm* infected cells (Figure 18D). Phagosomal containment of *Lm* by the cells was measured by calculating the containment index, and serum opsonization yielded a higher containment index in both splenic (figure 18E) and bone marrow neutrophils (Figure 18F). However, *Lm* opsonization had no effect on the containment index of monocytes from the spleen (Figure 18E) and bone marrow (Figure 18F). In summation, serum opsonization leads to phagocytosis of more bacteria and increased containment of *Lm* by neutrophils.

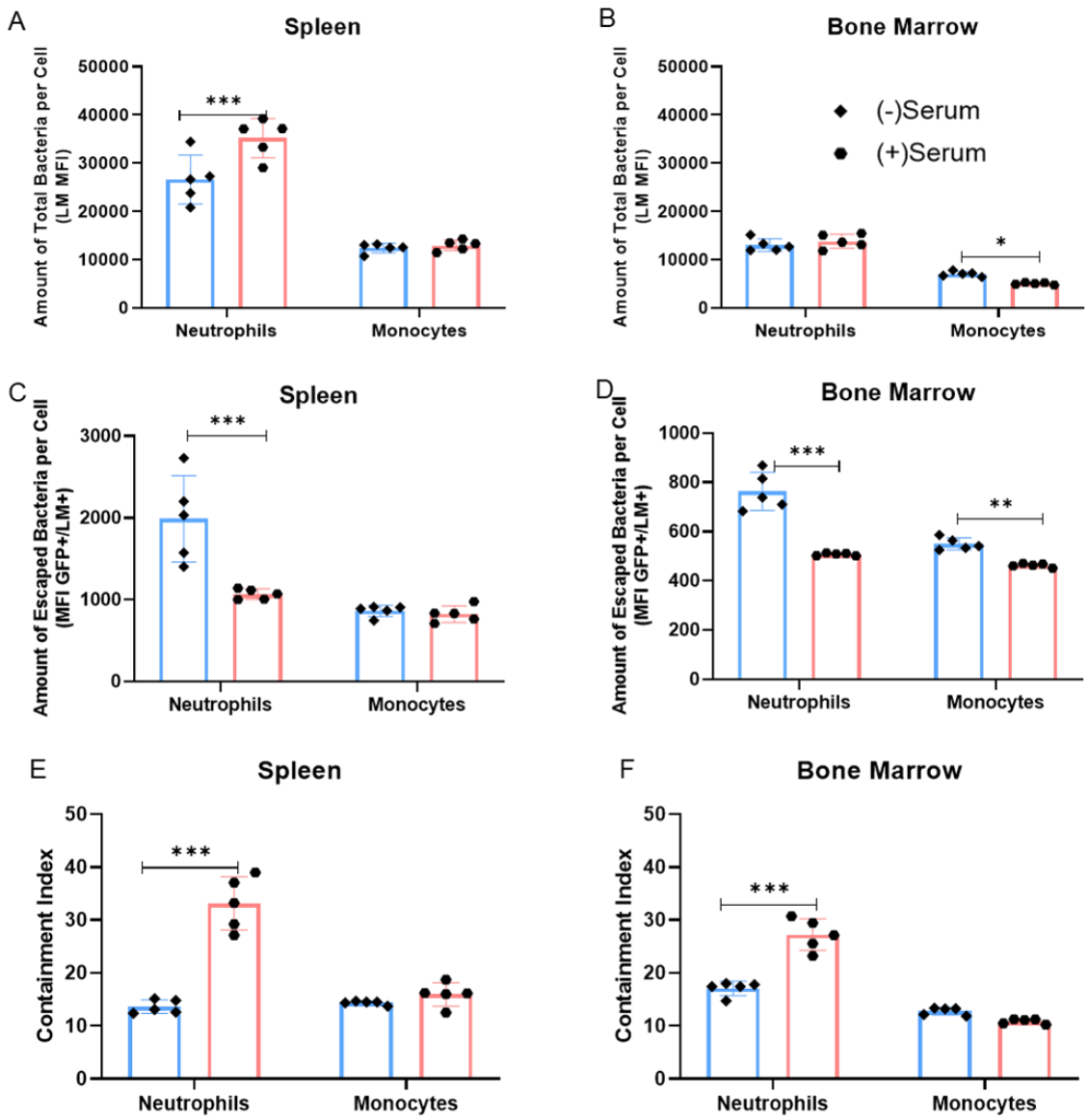

Figure 18: Serum opsonization increases phagosomal containment of Lm by neutrophils:

Splenocytes and bone marrow leukocytes were harvested from B6 mice and infected with opsonized or unopsonized actA:LMGFP at an MOI of 5:1. Extracellular bacteria was killed with gentamicin and cells stained for *Lm* as well as neutrophils and monocyte markers. The LM^+ MFI of splenic (A) and bone marrow (B) serum opsonized or unopsonized neutrophils and monocytes were measured. The GFP^+ MFI of splenic (C) and bone marrow (D) serum opsonized or unopsonized neutrophils and monocytes were also measured. The containment index of splenic (E) and bone marrow (F) serum opsonized or unopsonized neutrophils and monocytes were calculated. Data are representative of 2 independent experiments with n = 5. 2-way ANOVA with Bonferroni multiple comparison *post-hoc* test.

In monocytes, although serum opsonization decreased the amount of bacteria take up by bone marrow cells, it had no effect on phagosomal containment of *Lm* by splenic or bone marrow cells.

Kinetics of actA:LMGFP infection in neutrophils and monocytes

Following the observed effects of opsonization on neutrophil uptake and containment of *Lm*, the effects of the duration of infection on phagocytosis and containment of *Lm* by neutrophils and monocytes was studied. "A kinetics assay was conducted whereby bone marrow leukocytes were infected with serum opsonized actA:LMGFP for 1, 2, and 4 hours, and then stained with the antibody against neutrophils, monocytes and *Lm*. At all time points post infection, a higher percentage of neutrophils were LM^+ in comparison to monocytes (Figures 19A&B). There was also an observed increase in the percentage of both LM^+ neutrophils and monocytes that phagocytosed the bacteria (Figure 19B). Analysis of the LM^+ MFI showed that over time, there is an increase in the neutrophil LM^+ MFI with a massive increase occurring between the 2 and 4 hour time points (Figure 19C). In the monocytes, however, an increase is only observed between the 2 and 4 hour time points in monocytes (Figure 19C). Additionally, at all time points post infection analyzed, neutrophils also had a higher LM^+ MFI in comparison to monocytes (Figure 19C).

Next, the GFP^+ MFI was measured as a determinant of bacterial escape from the phagosome and there were no observed differences between neutrophils and monocytes at 1 and 2 hours post infection (Figure 19D). At 4 hours post infection, however, the GFP^+ MFI of neutrophils increases significantly in comparison to the 1 and 2 hour time points and in comparison to the monocytes, the neutrophils also had a higher MFI (Figure 19D). At 1 and 2 hours post-infection, the neutrophils had a higher containment index in comparison to the monocytes, however there were no differences in the neutrophil and monocyte containment index following 4 hours of infection (Figure 19E).

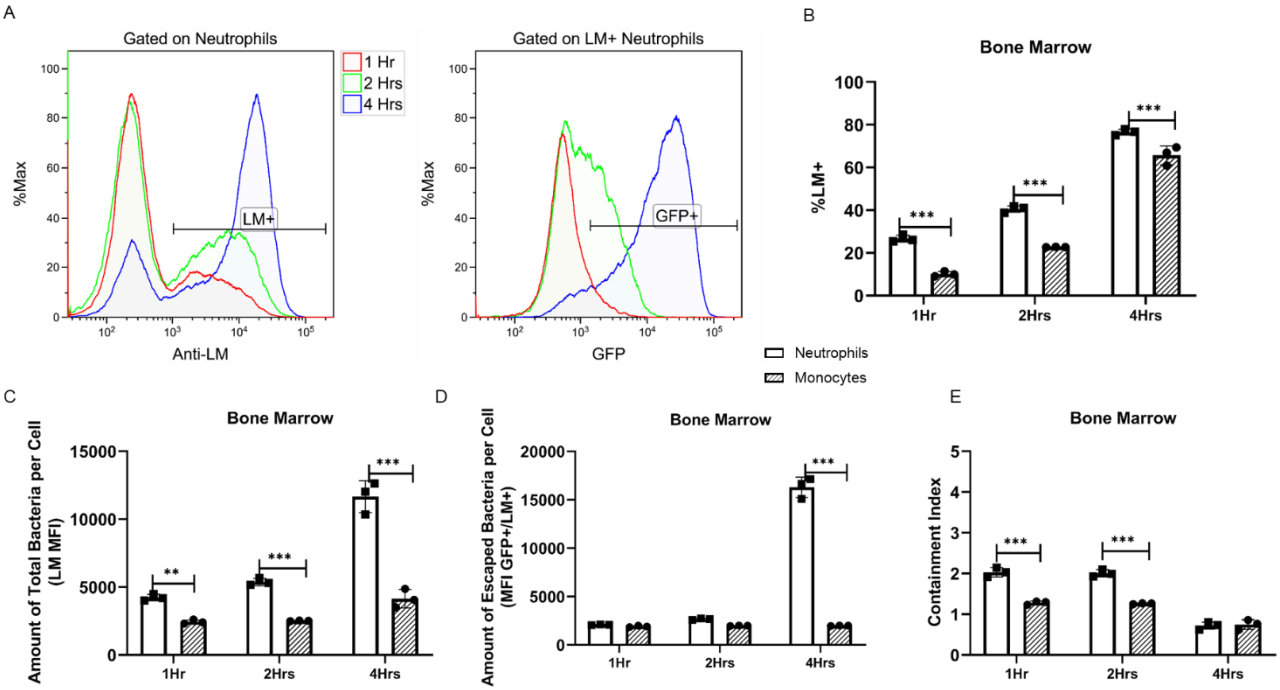

Figure 19: Kinetics of actA:LMGFP infection in neutrophils and monocytes:

Bone marrow leukocytes were harvested from B6 mice and infected with opsonized actA:LMGFP at an MOI of 5:1 for 1, 2 or 4 hours. Extracellular bacteria were killed with gentamicin. Shown are representative histograms of LM^+ and GFP^+ neutrophils (A) and the percentage of LM^+ neutrophils and monocytes following 1, 2 or 4 hours of infection (B). The MFI of LM^+ (C) and GFP^+ (D) neutrophils and monocytes were analyzed as well as the containment index at the different time points (E). Data are representative of 2 independent experiments with n = 3. 2-way ANOVA with Bonferroni multiple comparison *post-hoc* test.

These data suggest that neutrophils are more efficient at bacterial containment than monocytes until the neutrophils take up an excessive amount of *Lm*, and essentially become exhausted.

Neutrophils are more effective at phagocytosis and containment of Lm than monocytes

To directly investigate and compare *Lm* phagocytosis by neutrophils and monocytes from the organs *Lm* normally targets, in addition to bone marrow leukocytes, liver leukocytes and splenocytes were infected, *in vitro*, with serum opsonized actA:LMGFP. Extracellular bacteria were killed with gentamicin and the percentage of cells that associated with the bacteria was measured with flow cytometry. A higher percentage of neutrophils obtained from all three organs were positive for *Lm* in comparison to monocytes (Figures 20A-D). It is also important to note that although a higher percentage of the neutrophils associated with the bacteria, approximately 80% percent of the monocytes from the spleen and 60% from the liver associated with *Lm* (Figures 20C&D). This suggests that although neutrophils take up *Lm* more effectively, monocytes also have the ability to phagocytose the bacteria.

To further investigate differences in *Lm* phagosomal containment by neutrophils and inflammatory monocytes, bone marrow, liver leukocytes and splenocytes were infected with actA:LMGFP and stained with the antibody against *Lm*. The MFI of the LM^+ cells was measured and it was observed that neutrophils from all three organs had a higher LM MFI (Figure 21A). Essentially, neutrophils took up more bacteria in comparison to monocytes (Figure 21A). Analysis of the GFP^+ MFI, a measurement of the amount of escaped bacteria, showed that there are no differences in the amount of bacteria that escape out of the neutrophil and monocyte phagosomes (Figure 21B). However, the neutrophils from the bone marrow, liver and spleen have a higher containment index in comparison to monocytes from the same organs (Figure 21C).

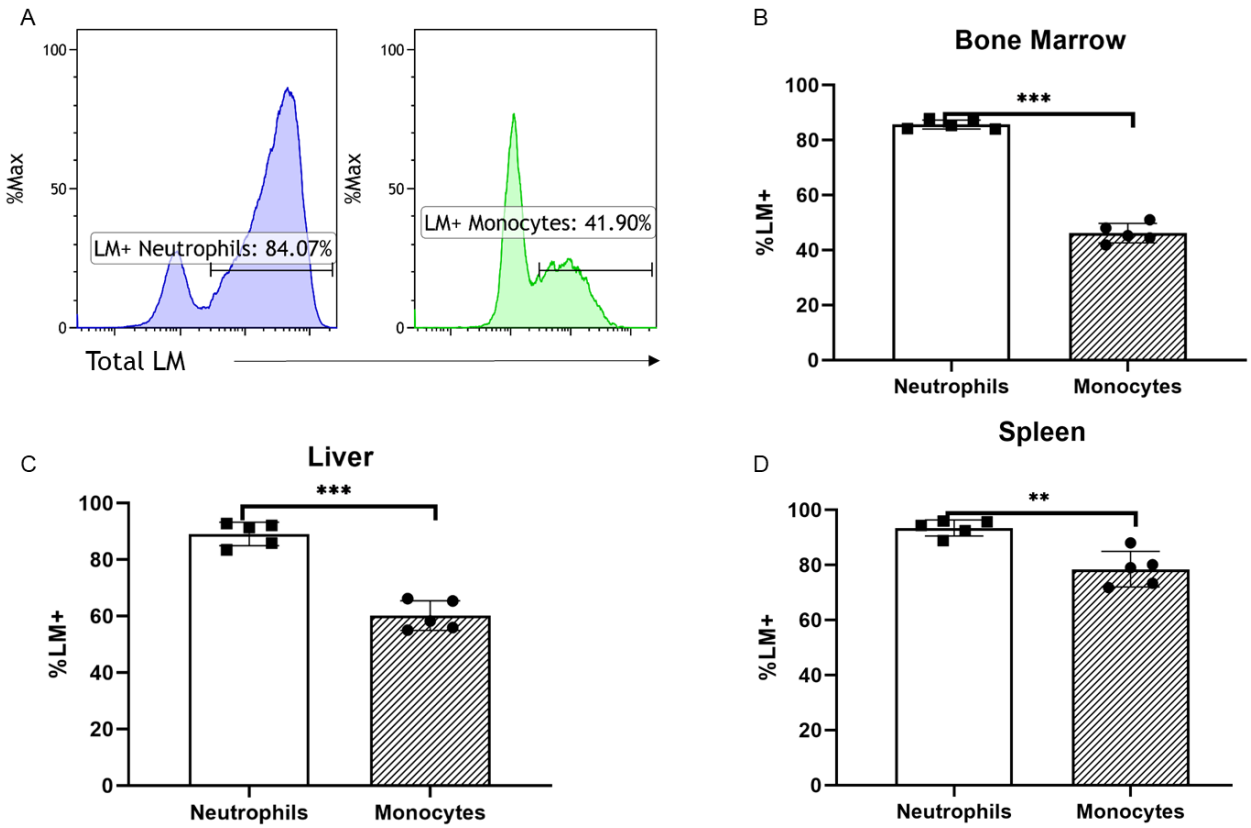

Figure 20: Neutrophils are more effective at phagocytosis of *Lm* than monocytes:

Bone marrow leukocytes, liver leukocytes and splenocytes harvested from B6 mice were infected, with opsonized actA:LMGFP for 2 hours at an MOI of 5:1. This was followed by killing of extracellular *Lm* with gentamicin after which the cells were stained for neutrophil and monocyte surface markers as well as *Lm*. Representative flow cytometry histogram showing the percent max of *Lm* positive neutrophils and monocytes and the gating for LM^+ cells (A) The percentage of LM^+ neutrophils and monocytes from the bone marrow (B), liver (C), and spleen (D) were used to determine the percentage of cells that phagocytosed the bacteria. Data are representative of 2 independent experiments with $n \geq 4$. Two-tailed t-test.

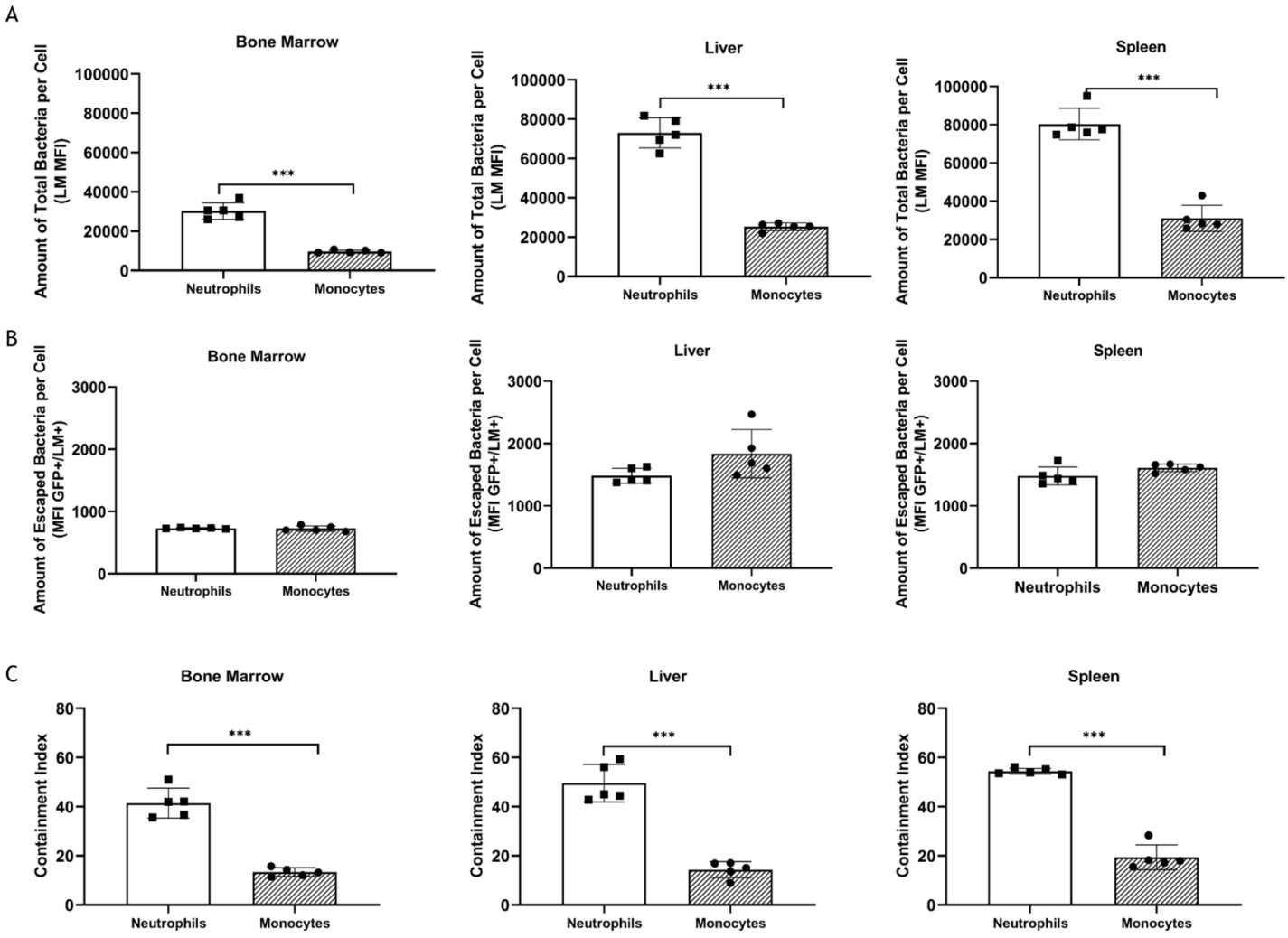

Figure 21: Neutrophils are more effective at the phagosomal containment of *Lm* than monocytes:

Bone marrow, liver leukocytes and splenocytes were harvested from B6 mice and infected with opsonized actA:LMGFP at an MOI of 5:1 followed by killing of extracellular bacteria with gentamicin. Flow cytometry was used to measure the neutrophil and monocyte MFI of LM^+, representative of the total amount of bacteria taken up (A). The GFP^+ MFI, representative of the amount of escaped bacteria per cell, was also measured (B). The ratio of total to escaped bacteria was calculated to yield the containment index of neutrophils and monocytes (C). Data are representative of 2 independent experiments with $n \geq 4$. Two-tailed t-test.

Collectively, these data demonstrate that although neutrophils are taking up more *Lm* overall, they are allowing. for equal amounts of bacteria to escape in comparison to the monocytes. Therefore, neutrophils are more effective at *Lm* phagosomal containment than monocytes.

Lm uptake increases expression of LAMP-1 in neutrophils but not in monocytes

Upon uptake of the bacteria into the phagosome, phagocytic cells attempt to fuse it with the lysosome in order to form a phagolysosome which contains products toxic to the pathogen. Due to the formation of the phagolysosome being a necessity for bacterial killing, it serves as an indicator of the cells attempt to kill the pathogen. A common marker for the phagolysosome is LAMP-1, a molecule necessary for the formation of the mature phagosome. Therefore, LAMP-1 expression is used as a measurement of phagolysosme formation and possible subsequent killing of *Lm*. To determine differences in the ability of neutrophils to form phagolysosomes and/or autophagolysosomes, expression of LAMP-1 was measured via flow cytometry.

Bone marrow leukocytes were infected with *Lm*, *in vitro*, and the MFI of LAMP-1 was calculated as an indicator of the expression of the protein on a per cell basis. In the LM^- neutrophils and monocytes, which are cells that did not take up *Lm*, there were no differences in LAMP-1 expression between the two cell types (Figures 22A&B). This indicates that when the cells have not taken up *Lm*, neutrophils and monocytes express similar levels of LAMP-1. The expression of LAMP-1 was also analyzed in the LM^+ neutrophils and monocytes, which are cells that have taken up the bacteria. The LM^+ neutrophils express a higher LAMP-1 MFI in comparison to the LM^+ monocytes (Figures 22A&C). These data suggest that phagocytosis of *Lm* increases LAMP-1 expression in neutrophils which is indicative of more effective phagosomal maturation in comparison to monocytes.

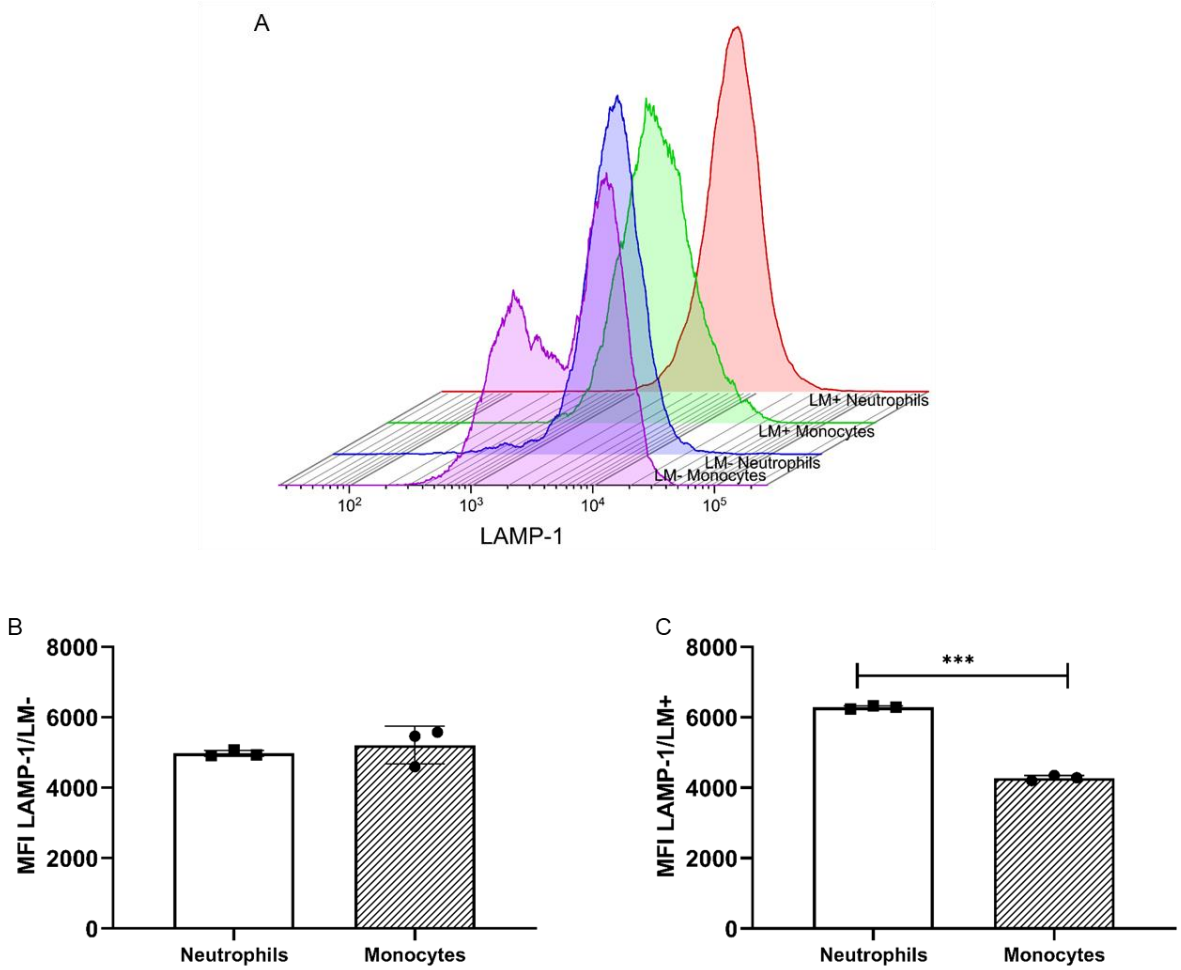

Figure 22: Neutrophils express higher levels of LAMP-1 following *Lm* infection:

Bone marrow leukocytes were harvested from B6 mice and infected with WTLM for 2 hours at an MOI of 5:1, extracellular bacteria were killed with gentamicin and cells stained for *Lm*, LAMP-1, and neutrophil and monocyte markers. Shown are representative LAMP-1 histograms of LM^+ and LM^- neutrophils and monocytes (A). The MFI of the LAMP-1^+ cells out of the LM^- (B) and LM^+ (C) neutrophils and monocytes were measured. Data are representative of 2 independent experiments with n = 3. Two-tailed t-test.

Neutrophils are more effective than monocytes at killing of Lm

The environment of the mature phagolysosome is one that is geared towards killing of the pathogen. Killing of the bacteria is a necessity for prevention of dissemination as well as clearance of the pathogen. Previously, *Lm* killing is a function that has been primarily assigned to neutrophils. However, a study also demonstrated that monocytes are capable of killing *Lm* during a secondary response. Specifically, during the memory response to *Lm*, monocytes and neutrophils were producing more ROS and had an increase in phagosomal pH levels in comparison to the primary response (77). However, a direct killing assay was not performed with these cells. ROS generation has been the main method by which phagocytic cells, including neutrophils and macrophages, are thought to kill not just *Lm* but other pathogens as well (109). However, interestingly, a recent study found that dysfunction of the NADPH oxidase does not impede killing of *Lm* by neutrophils (15).

To gain a better understanding of differences in ROS generation in neutrophils and monocytes, bone marrow leukocytes from B6 mice were stimulated with HKLM and treated with H_2DCFDA, which is a dye that emits a green fluorescence following interaction with ROS components. The MFI of the cells positive for H_2DCFDA was calculated. In comparison to monocytes, neutrophils expressed a higher H_2DCFDA MFI (Figure 23A) which indicates they are producing more ROS. The ultimate outcome after phagocytosis, phagosomal containment, phagosome maturation and generation of ROS is killing of the pathogen. Therefore, differences in *Lm* killing by neutrophils and monocytes was investigated. Neutrophils and monocytes were sort purified from bone marrow leukocytes and infected with WTLM.

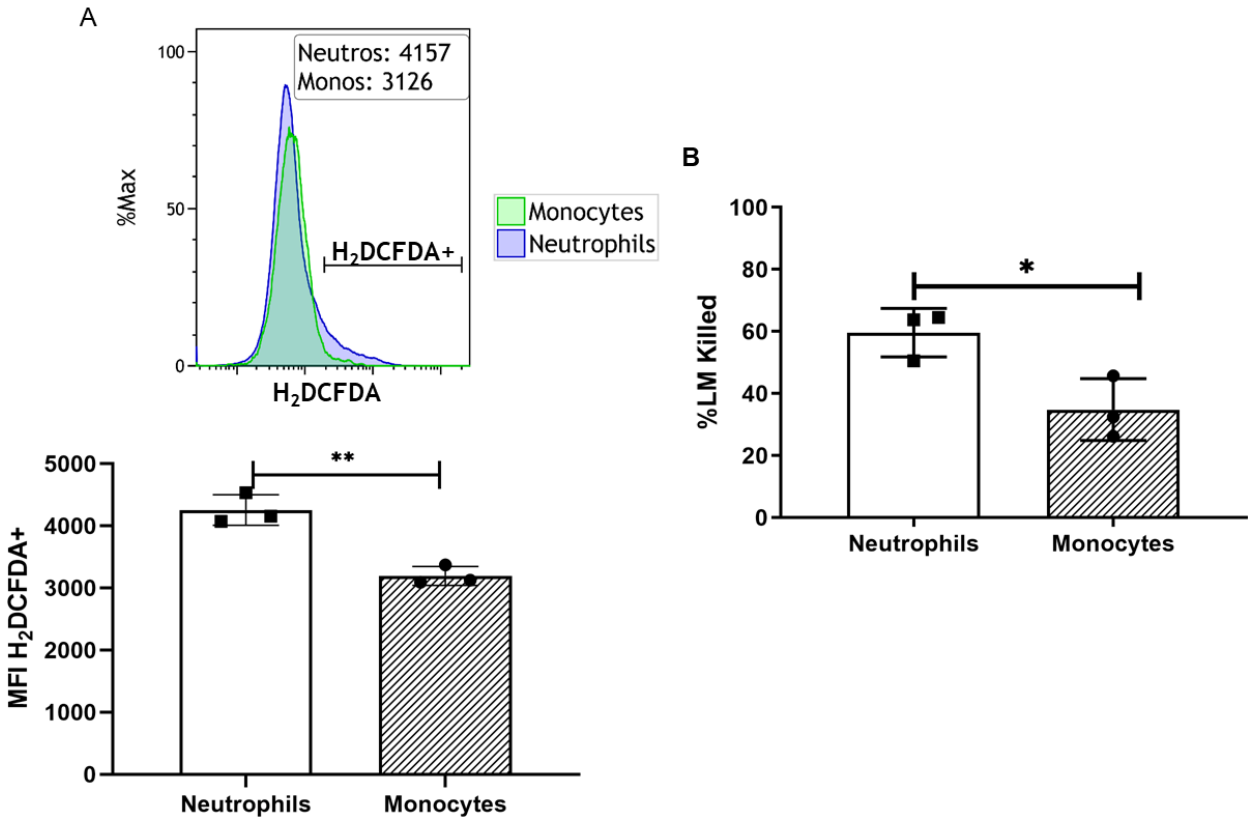

Figure 23: Neutrophils are more effective than monocytes at *Lm* killing:

Bone marrow leukocytes were harvested from B6 mice and infected for 2 hours with opsonized WTLM and H2DCFDA was then added to the cells during the second hour of infection. extracellular bacteria were killed with gentamicin. Generation of ROS was measured via the MFI of the H_2DCFDA^+ cells (A). Neutrophils and monocytes were sorted from the bone marrow and a killing assay was performed to determine the percent of *Lm* killed by the cells (B). Data are representative of 2 independent experiments with n = 3. Two-tailed t-test.

The percent bacteria killed was calculated and neutrophils killed a higher percentage of *Lm* in comparison to monocytes (Figure 23B). These sets of data indicate that although monocytes have the ability to produce ROS and kill *Lm*, neutrophils are more effective at both of these functions.

Monocytes are more effective at TNF-α production during Lm infection

Another major function of neutrophils and monocytes necessary for effective host response to *Lm* infection is cytokine production. Deletion of the TNF-α gene, specifically in neutrophils, monocytes and macrophages, or lack of the TNFR1 in mice led to increased susceptibility to *Lm* infection (93, 110). In addition, depletion of both neutrophils and monocytes with the anti-GR1 antibody led to a decrease in TNF-α production in both the spleen and liver (14). TNF-α production by either neutrophils or monocytes during *Lm* infection has previously been investigated (2, 14) although direct comparisons in the ability of both cell types to produce TNF-α is yet to be reported. In order to determine differences in the ability of neutrophil and monocytes to produce TNF-α, B6 mice were infected with *Lm* for either 1 or 3 days. Splenocytes and liver leukocytes were harvested from the infected mice and stimulated with HKLM. The percentage, MFI and total number of TNF-α+ cells was then measured with the use of flow cytometry.

Following 1 day of infection, a higher percentage of splenic monocytes (~50%) were positive for TNF-α in comparison to neutrophils (~20%) (Figures 24A&B). Analysis of the TNF-α+ MFI also showed that monocytes had a higher TNF-α MFI in comparison to neutrophils in the spleen (Figures 24A&C). However, calculation of the number of TNF-α producing neutrophils and monocytes in the spleen showed that there were no differences in the number of neutrophils and monocytes producing the cytokine following 1 day of infection with *Lm* (Figure 24C).

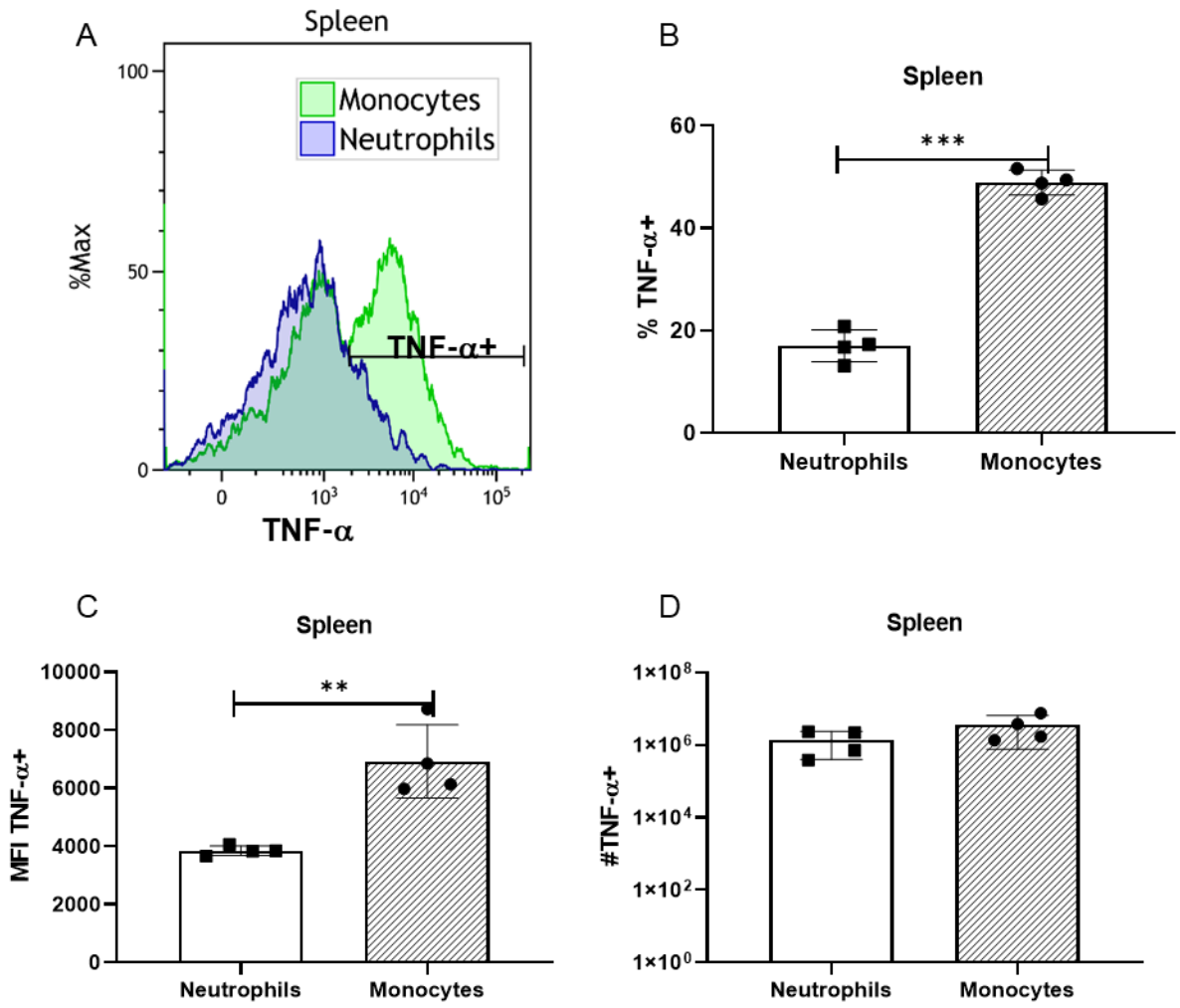

Figure 24: Splenic monocytes are more effective at TNF-α production at 1 day post *Lm* infection:

B6 mice were infected with $1x10^4$ *Lm* and at day 1 post-infection spleens were harvested. Splenocytes were stimulated with HKLM and then stained for neutrophil and monocyte markers as well as intracellular expression of TNF-α. The percent of cells positive for TNF-α (A&B), the MFI (C) and the number of TNF-α+ cells (D) were determined. Data are representative of 2 independent experiments with $n \geq 4$. Two-tailed t-test.

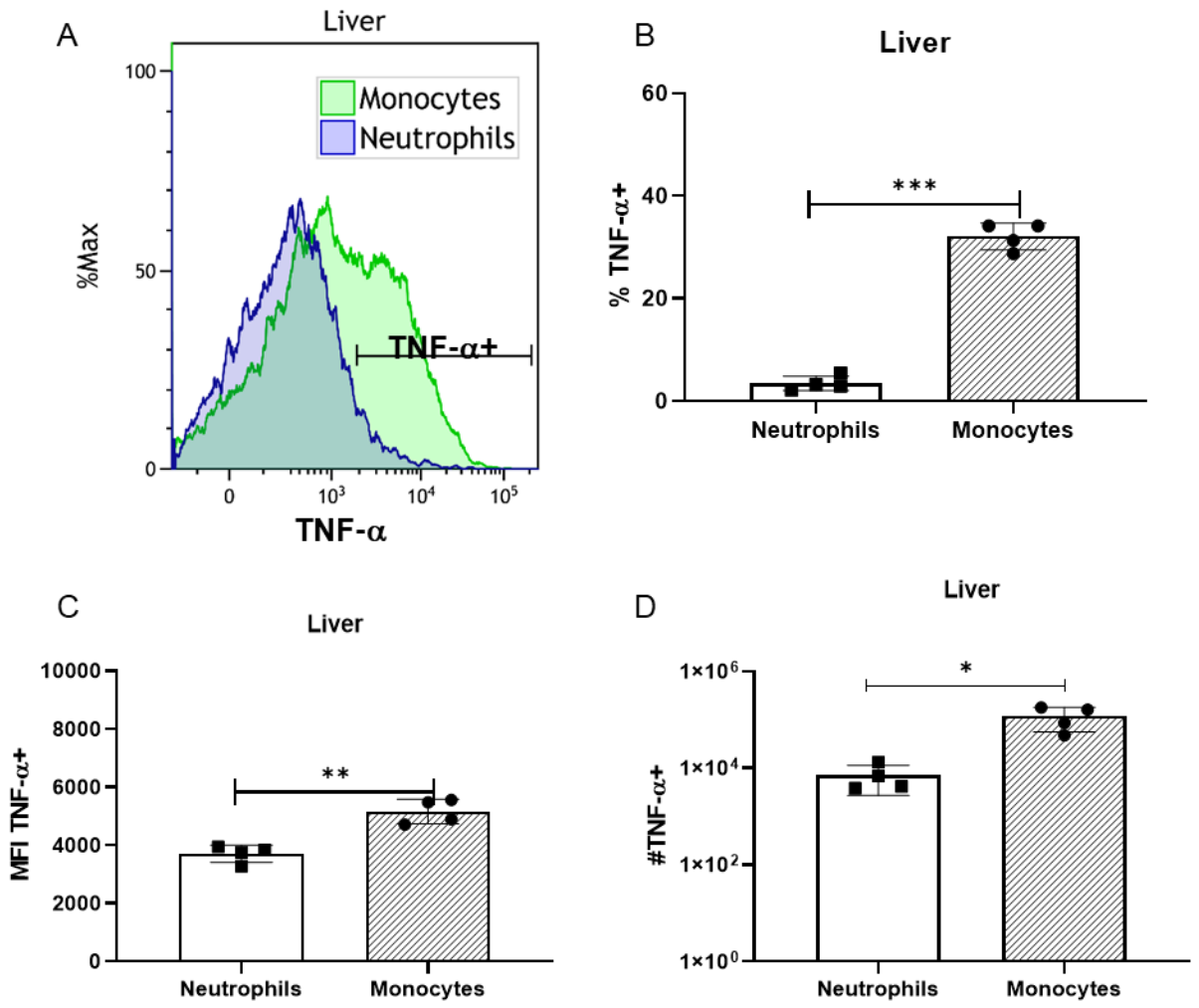

Figure 25: Liver monocytes are more effective at TNF-α production at 1 day post *Lm* infection:

B6 mice were infected with $1x10^4$ *Lm* and at day 1 post-infection livers were harvested. Liver leukocytes were stimulated with HKLM and then stained for neutrophil and monocyte markers as well as intracellular expression of TNF-α. The percent of cells positive for TNF-α (A&B), the MFI (C) and the number of TNF-α+ cells (D) were determined. Data are representative of 2 independent experiments with $n \geq 4$. Two-tailed t-test.

Similar observations were made in the liver where a higher percentage of monocytes were TNF-α+ at 1 day post-infection with *Lm* (Figures 25A&B). Comparison of TNF-a production in the liver and spleen showed that a lower percentage of liver neutrophils and monocytes were positive for TNF-α in comparison to their splenic counterparts (Figures 24B & 25B). In line with observations made in the spleen, liver monocytes had a higher TNF-α MFI in comparison to neutrophils (Figure 25C). Unlike the spleen, however, a higher number of monocytes were making TNF-α in comparison to neutrophils at 1 day post *Lm* infection. These data indicate that in both the liver and spleen, monocytes are more effective at TNF-α production at one day post-infection.

TNF-α production by neutrophils and monocytes was also studied following 3 days of infection with *Lm*. In comparison to neutrophils, a higher percentage of monocytes were positive for TNF-α after *Lm* infection for 3 days (Figures 26A&B), similar to observations made at day 1 post-infection. Analysis of the MFI of TNF-α + cells also showed that monocytes have a higher TNF-α+ MFI in comparison to neutrophils in the spleen at 3 days post-infection with *Lm* (Figures 26A&C). However, there were no differences in the number of TNF-α producing neutrophils and monocytes in the spleen at 3 days of infection (Figure 26D) which are the same observations made at 1 day post-infection. Following 3 days of infection in the liver, a higher percentage of liver monocytes are positive for TNF-α in comparison to neutrophils (Figures 27A&B). In addition, the liver monocytes also had a higher TNF-α+ MFI in comparison to the neutrophils (Figures 27A&C), but there were also no differences in the number of TNF-α producing monocytes and neutrophils (Figure 27D). Similar to observations made at 1 day post infection, there are a higher percentage of a TNF-α+ monocytes in the spleen in comparison to the liver.

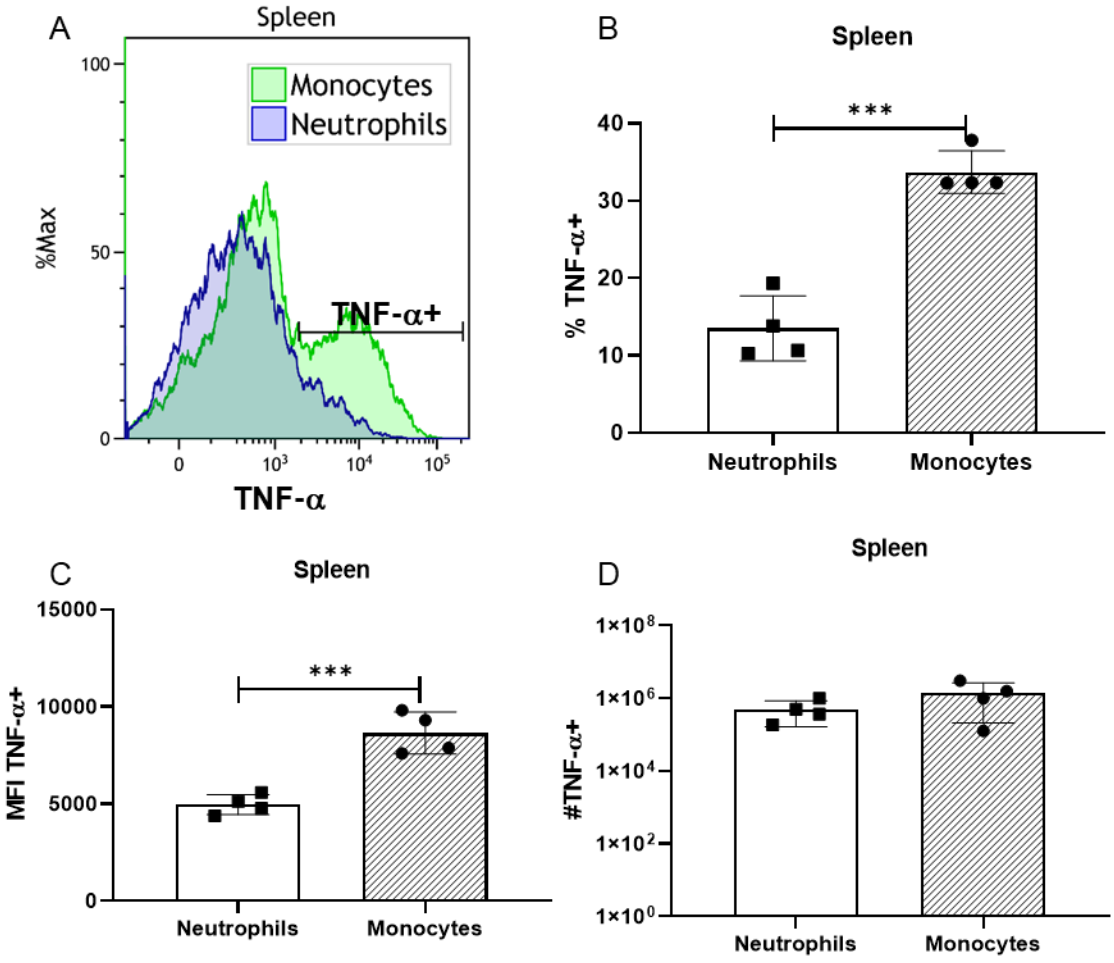

Figure 26: Splenic monocytes are more effective at TNF-α production at 3 days post *Lm* infection:

B6 mice were infected with $1x10^4$ *Lm* and at day 3 post-infection spleens were harvested. Splenocytes were stimulated with HKLM and then stained for neutrophil and monocyte markers as well as intracellular expression of TNF-α. The percent of cells positive for TNF-α (A&B), the MFI (C) and the number of TNF-α+ cells (D) were determined. Data are representative of 2 independent experiments with $n \geq 4$. Two-tailed t-test.

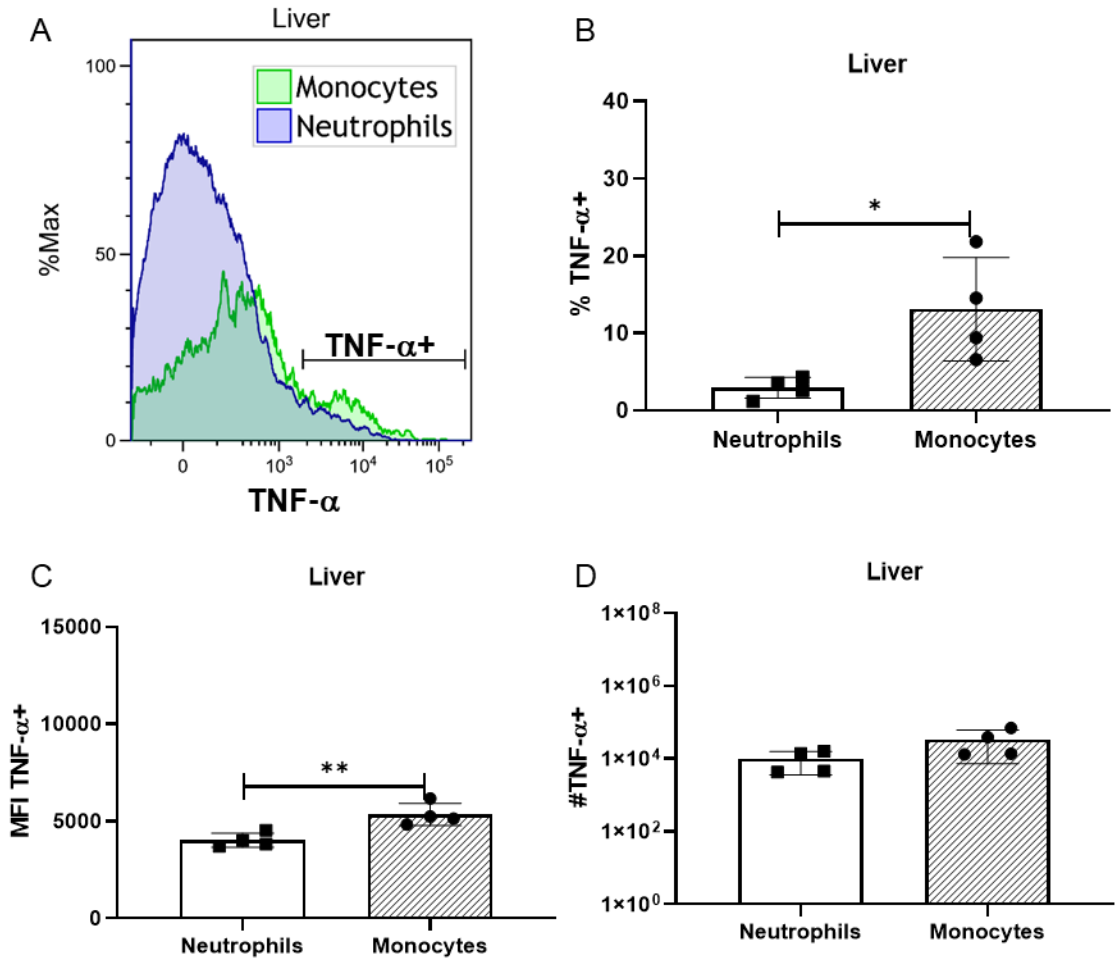

Figure 27: Liver monocytes are more effective at TNF-α production at 3 days post *Lm* infection:

B6 mice were infected with $1x10^4$ *Lm* and at day 3 post-infection livers were harvested. Liver leukocytes were stimulated with HKLM and then stained for neutrophil and monocyte markers as well as intracellular expression of TNF-α. The percent of cells positive for TNF-α (A&B), the MFI of TNF-α (C) and the number of TNF-α+ cells (D) were determined. Data are representative of 2 independent experiments with $n \geq 4$. Two-tailed t-test.

The above sets of data indicate that during the early innate immune response to *Lm* infection, monocytes, in comparison to neutrophils, are primarily responsible for TNF-α production in both the spleen and liver.

Monocytes are more effective at IL-1α production during Lm infection in the spleen

Another cytokine that could possibly be playing an important protective role during *Lm* infection is IL-1α. Neutralization of the IL-1 receptor (IL-1R) which binds to both IL-1α and IL-1β, leads to increased susceptibility to *Lm* infection (94). However, mice lacking IL-1β were just as resistant to infection as their wild-type counterparts (95). Therefore, it is possible that susceptibility to infection as a result of blocking the IL-1R is due to IL-1α, however, there are no studies reporting the production of IL-1α by neutrophils or monocytes during *Lm* infection. To gain a better understanding of the role neutrophils and monocytes play in IL-1α production, B6 mice were infected with *Lm* for 1 or 3 days. Splenocytes and liver leukocytes were harvested and following stimulation with HKLM, IL-1α production was measured using flow cytometry. A higher percentage of splenic monocytes were IL-1α+ in comparison to neutrophils (Figures 28A&B). The splenic monocytes also had a higher IL-1α+ MFI in comparison to neutrophils (Figures 28A&C). Although there were no differences in the number of IL-1α producing neutrophils and monocytes after a 1 day infection with *Lm* in the liver (Figure 29D), a higher percentage of liver neutrophils were positive for IL-1α (Figures 29A&B). However, in contrast to observations made in the spleen, there were no differences in the IL-1α+ MFI between liver neutrophils and monocytes at day 1 post *Lm* infection (Figures 29A&C). These data suggest that at day 1 post infection, monocytes contribute more than neutrophils to the IL-1α production in the spleen, whereas the opposite occurs in the liver.

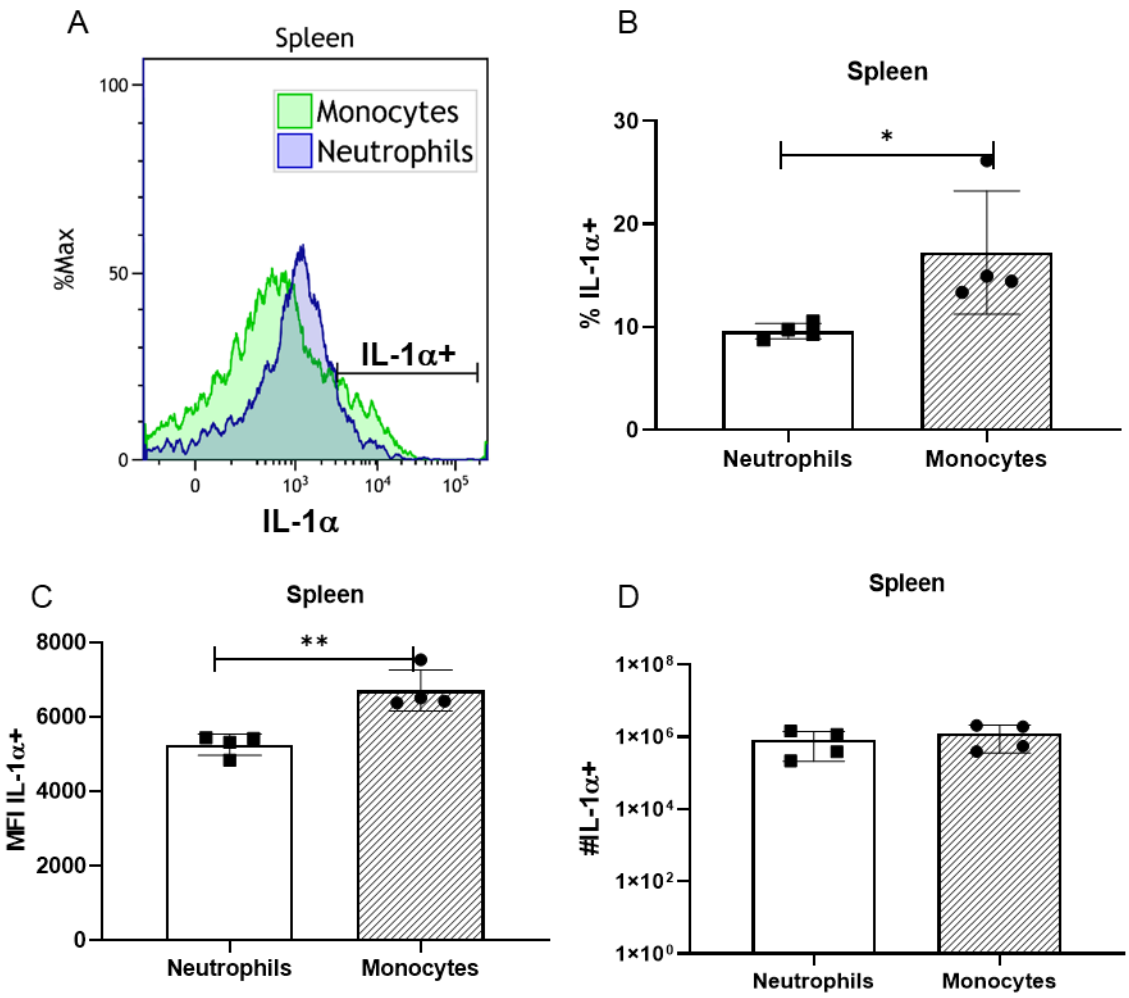

Figure 28: Splenic monocytes are more effective at IL-1α production at 1 day post *Lm* infection:

B6 mice were infected with $1x10^4$ *Lm* and at day 1 post-infection spleens were harvested. Splenocytes were stimulated with HKLM and then stained for neutrophil and monocyte markers as well as intracellular expression of IL-1α. The percent of cells positive for IL-1α (A&B), the MFI (C) and the number of IL-1α+ cells (D) were determined. Data are representative of 2 independent experiments with $n \geq 4$. Two-tailed t-test.

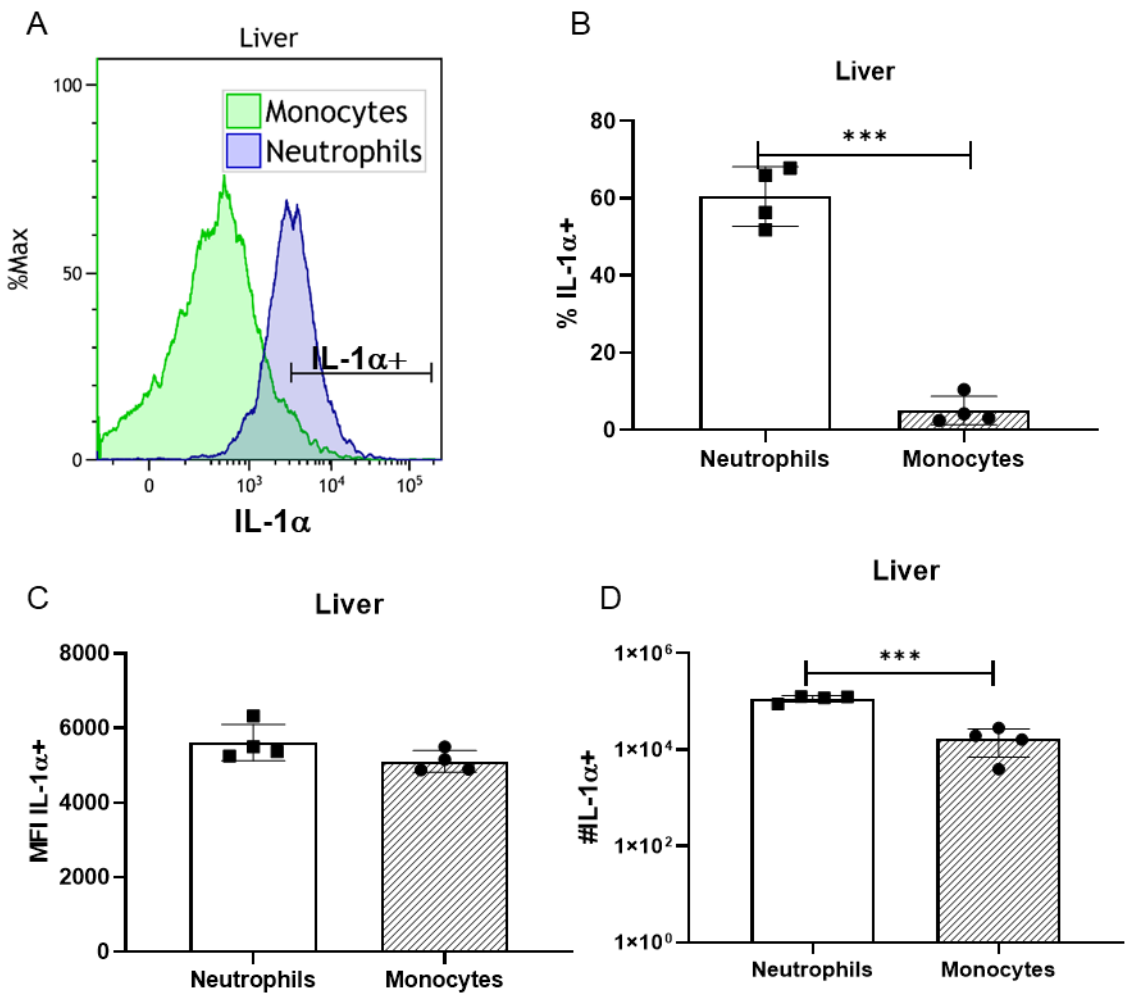

Figure 29: Liver neutrophils are more effective at IL-1α production at 1 day post *Lm* infection:

B6 mice were infected with $1x10^4$ *Lm* and at day 1 post-infection livers were harvested. Liver leukocytes were stimulated with HKLM and then stained for neutrophil and monocyte markers as well as intracellular expression of IL-1α. The percent of cells positive for IL-1α (A&B), the MFI (C) and the number of IL-1α+ cells (D) were determined. Data are representative of 2 independent experiments with $n \geq 4$. Two-tailed t-test.

IL-1α production by splenocytes and liver leukocytes was also investigated at 3 days post infection with *Lm*. B6 mice were infected with *Lm* and at 3 days post-infection, splenocytes and liver leukocytes were isolated and stimulated with HKLM. A higher percentage of splenic monocytes were positive for IL-1α in comparison to neutrophils (Figures 30A&B). In comparison to neutrophils, monocytes also express a higher IL-1α+ MFI (Figures 30A&C). There were, however, no differences in the number of neutrophils and monocytes expressing IL-1α in the spleen (Figure 30D). Analysis of liver leukocytes after 3 days of infection showed that there were no differences in the percentage of IL-1α+ neutrophils and monocytes producing IL-1α (Figures 31A&B) contrasting observations made in the spleen. However, in the liver, similarly to the spleen, the monocytes had a higher IL-1α+ MFI (Figures 31A&C). In addition, there were also no differences in the number of IL-1α producing neutrophils and monocytes (Figure 31D). These data suggest that in the liver and spleen, monocytes are more effective at IL-1α production in comparison to neutrophils at 3 days post-infection.

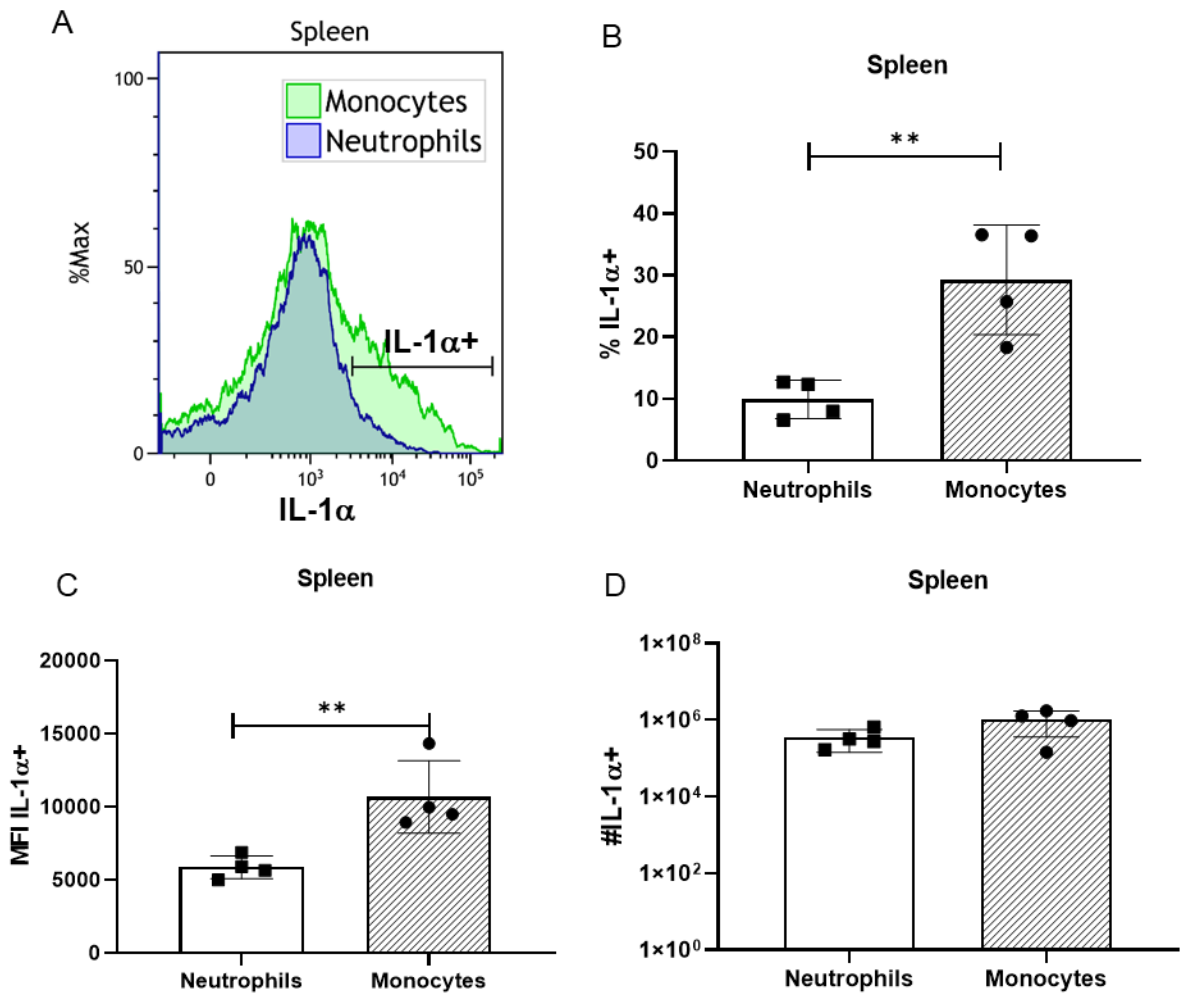

Figure 30: Splenic monocytes are more effective at IL-1α production at 3 days post *Lm* infection:

B6 mice were infected with $1x10^4$ *Lm* and at day 3 post-infection spleens were harvested. Splenocytes were stimulated with HKLM and then stained for neutrophil and monocyte markers as well as intracellular expression of IL-1α. The percent of cells positive for IL-1α (A&B), the MFI (C) and the number of IL-1α+ cells (D) were determined. Data are representative of 2 independent experiments with $n \geq 4$. Two-tailed t-test.

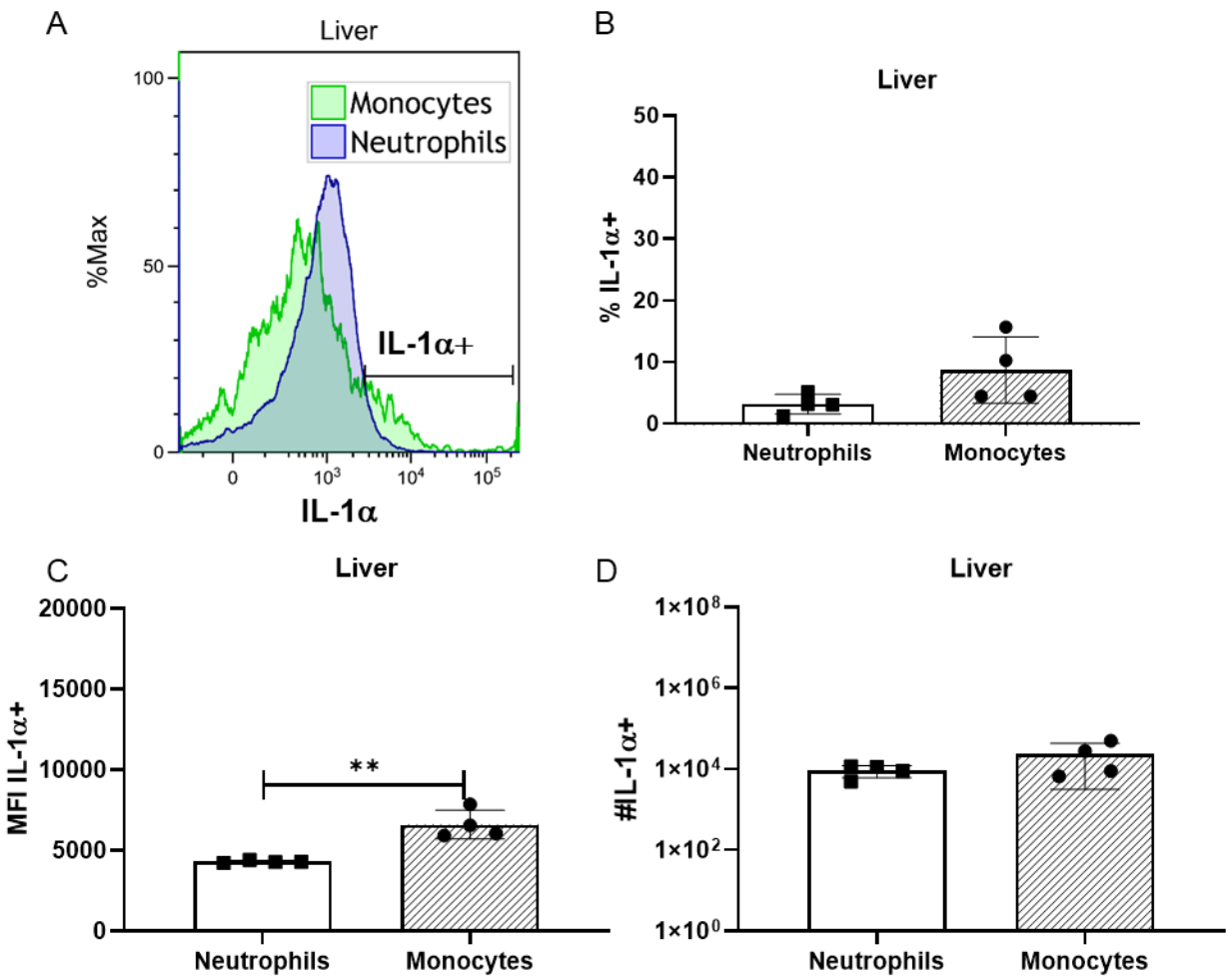

Figure 31: Liver monocytes are more effective at IL-1α production at 3 days post *Lm* infection:

B6 mice were infected with $1x10^4$ *Lm* and at day 3 post-infection livers were harvested. Liver leukocytes were stimulated with HKLM and then stained for neutrophil and monocyte markers as well as intracellular expression of IL-1α. The percent of cells positive for IL-1α (A&B), the MFI (C) and the number of IL-1α+ cells (D) were determined. Data are representative of 2 independent experiments with $n \geq 4$. Two-tailed t-test.

Monocytes are effective poly-functional cytokine producers

It has been demonstrated in a previous study conducted in a human cell line that IL-1α can induce the production of TNF-α (111). A previous study also demonstrated that inflammatory monocytes produce TNF-α and also express iNOS which leads to production of nitric oxide (2). This led to the question of whether the cells producing TNF-α were also producing IL-1α. To test this hypothesis, B6 mice were infected with *Lm* for 1 or 3 days and flow cytometry was utilized to detect cells that expressed both TNF-α and IL-1α. In the spleen, after 1 day of infection, a higher percentage of monocytes were double positive for IL-1α and TNF-α in comparison to neutrophils (Figures 32A&B). Unlike the spleen, a similar percentage of liver neutrophils and monocytes were double positive for TNF-α and IL-1α (Figure 32B).

Similar observations were made after 3 days of infection. A higher percentage of splenic monocytes were double positive for IL-1α and TNF-α (Figures 33A&B) whereas there were no differences in the percentage of double positive liver neutrophils and monocytes (Figures 33B&C). Another observation made during these studies was that following both 1 and 3 days of *Lm* infection, there was a higher percentage of double positive splenic monocytes in comparison to liver monocytes. To further support these findings, at 3 days post-infection, neutrophils and monocytes were imaged on an imaging cytometer to visualize expression of TNF-α and IL-1α in the cells. As shown in Figure 33D, both cytokines are expressed in puncta within the cells. In summation, these data indicate that monocytes are effective poly-functional cytokine producers.

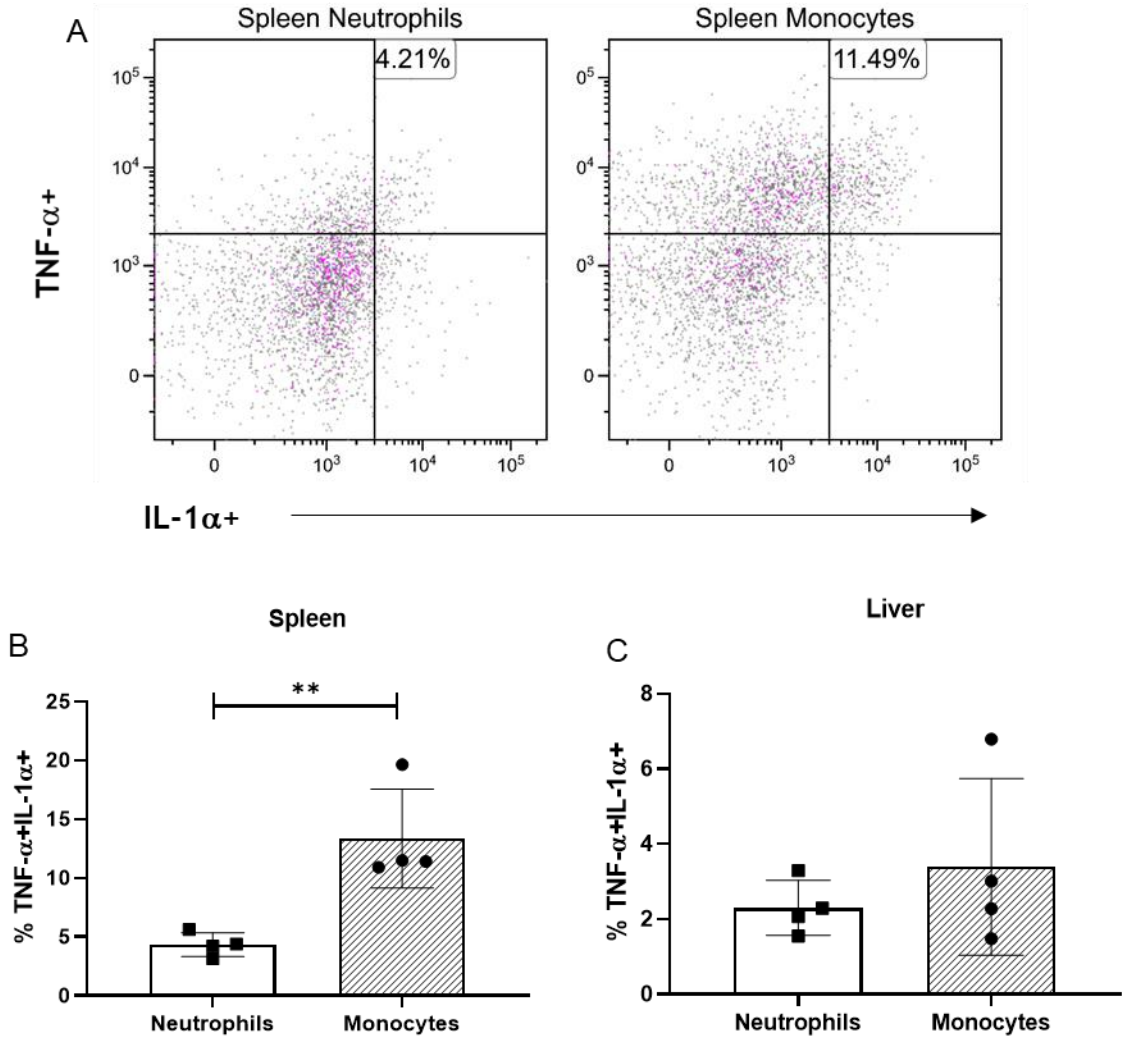

Figure 32: Monocytes are more effective poly-functional cytokine producers at 1 day post *Lm* infection:

B6 mice were infected with $1x10^4$ *Lm* and at day 1 post-infection spleens and livers were harvested. Splenocytes and liver leukocytes were stimulated with HKLM and then stained for neutrophil and monocyte markers as well as intracellular expression of TNF-α and IL-1α. Shown are representative dot plots displaying gating for neutrophils and monocytes that are double positive for TNF-α and IL-1α (A). The percentage of neutrophils and monocytes that are double positive for IL-1α and TNF-α in the spleen (B) and liver (C) was determined. Data are representative of 2 independent experiments with $n \geq 4$. Two-tailed t-test.

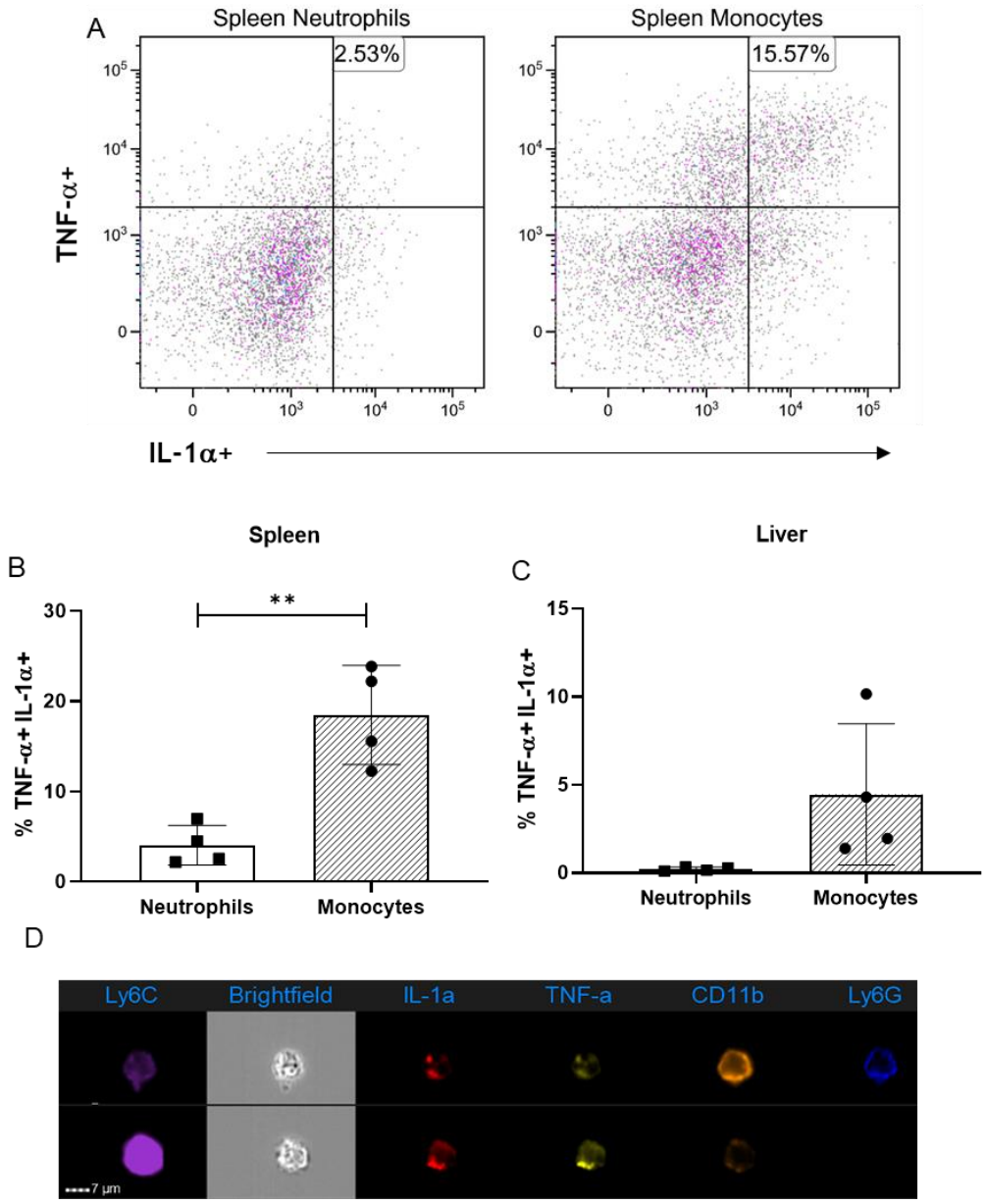

Figure 33: Monocytes are more effective poly-functional cytokine producers at 3 days post Lm infection:

B6 mice were infected with $1x10^4$ *Lm* and at day 3 post-infection spleens and livers were harvested. Splenocytes and liver leukocytes were stimulated with HKLM and then stained for neutrophil and monocyte markers as well as intracellular expression of TNF-α and IL-1α. Shown are representative dot plots displaying gating for neutrophils and monocytes that are double positive for TNF-α and IL-1α (A). The percentage of neutrophils and monocytes that are double positive for IL-1α and TNF-α in the spleen (B) and liver (C) was determined. Representative images of poly-functional IL-1α and TNF-α producing neutrophils (top panel) and monocytes (bottom panel) are shown (D). Data are representative of 2 independent experiments with $n \geq 4$. Two-tailed t-test.

Summary of Chapter IV

Neutrophils and monocytes were directly compared to delineate functional differences and similarities between the two cell types during *Lm* infection. The effects of bacteria opsonization was investigated first. Serum opsonization was found to increase neutrophil phagocytosis, reduce bacterial escape and increase containment of *Lm* in the phagosome. In monocytes, however, serum opsonization had no effect on splenic cells whereas, in bone marrow monocytes, it decreased uptake and escape but did not change containment. As a result of these data, it was concluded that *Lm* phagocytosis by neutrophils is most likely complement driven. Next, differences in the ability of neutrophils and monocytes to take up and contain *Lm* was directly compared. It was observed that neutrophils were more effective at bacterial uptake although both cells allowed for equal amounts of bacteria to escape out of the phagosome. Calculation of the containment index as a ratio of the total bacteria taken up versus escaped bacteria showed that neutrophils are more effective at containment of *Lm*. The ability of neutrophils and monocytes to kill *Lm* was also studied. Neutrophils generated more ROS, had higher expression of LAMP-1 and also subsequently killed the bacteria more effectively than monocytes.

Lastly, cytokine production by both cell types was also directly compared. Monocytes were more effective at TNF-α and IL-1α production after 3 days of infection with *Lm*, in comparison to neutrophils. At day 1 post infection, monocytes were also more effective at TNF-α production but neutrophils were found to be better at IL-1α production in the liver. However, in the spleen, monocytes more effectively produced IL-1α after 1 day of *Lm* infection. Furthermore, a subset of monocytes was also found to be capable of producing IL-1α and TNF-α simultaneously during *Lm* infection. From these data, it was surmised that over the course of infection with *Lm*, monocytes are more effective at cytokine production." (65)

CHAPTER V

DISCUSSION

Overview of Chapter III

In Chapter III, the role of ecSOD in the innate immune response to *Lm* infection was studied to gain a better understanding on how ROS regulates neutrophil functions. Our data demonstrated that although there are extracellular bacteria present in the spleen, this is not dependent on ecSOD activity until later on during infection. In addition, ecSOD activity was found to lead to a decrease in phagocytosis of *Lm* by neutrophils but had no effect on phagosomal containment of the bacteria. EcSOD KO neutrophils that associated with the bacteria also had a higher expression LAMP-1, likely indicative of more phagosomal lysosomal fusion, in comparison to ecSOD HI neutrophils. To gain a better understanding of mechanisms that drive phagocytosis and containment of *Lm*, neutrophils were treated with IFN-γ and recombinant SOD. Pre-activation with IFN-γ or treatment of neutrophils from ecSOD KO mice with recombinant SOD2 had no effect on uptake or phagosomal containment of *Lm*. However, inhibition of autophagy led to decreased phagocytosis and phagosomal containment of *Lm* by neutrophils.

Chapter III Discussion

These data build upon previous knowledge that demonstrated how modulations to certain neutrophil functions by ecSOD activity are likely detrimental to host resistance to *Lm* infection. Previous studies conducted with the ecSOD congenic mice highlighted dysfunctional modulation of neutrophil functions as a result of high ecSOD activity. The ecSOD HI mice, in comparison to the ecSOD KO mice, were found to have a higher percentage of neutrophils recruited to the liver and blood following infection with *Lm*. In addition, the ecSOD HI mice also had higher bacterial burden which was paradoxical to the presence of the high percentage of recruited neutrophils.

Furthermore, there was an observed lower production of TNF-α in the liver and spleen of the ecSOD HI mice and neutrophils from the ecSOD HI mice were less effective at TNF-α production, in comparison to neutrophils from the ecSOD KO mice(6). In the current study, the effects of ecSOD activity on the presence of extracellular bacteria was investigated. At 3 days post-infection, high activity of ecSOD activity led to the presence of extracellular bacteria in the spleen but not in the liver. However, at day 1 post infection, ecSOD had no effect on the observed presence of extracellular bacteria in the spleen. Additionally, a higher percentage of neutrophils from the ecSOD KO mice liver, spleen and bone marrow effectively phagocytose more *Lm* and also take up more bacteria per cell. Therefore, the presence of extracellular bacteria observed in the ecSOD HI spleen could be a result of the neutrophils not effectively phagocytosing the bacteria. In the ecSOD WT and KO mice, although there are extracellular bacteria present initially during the first day of infection, the effective phagocytosis of *Lm* by the neutrophils results in the lack of extracellular bacteria at day 3 post infection. These data highlight how efficient bacteria phagocytosis drives *Lm* clearance, and suggest that ecSOD activity may contribute in a detrimental manner to this process.

Containment of *Lm* in the phagosome is important as it prevents dissemination of the bacteria. Following phagocytosis of the bacteria, ecSOD activity does not appear to affect the ability of the neutrophils to keep the bacteria contained in the phagosome. This was an unexpected observation since there is a known higher bacterial burden in the ecSOD HI mice which is presumably due to the lack of effective phagocytosis and killing by the neutrophils (6). Phagosomal escape of the bacteria is directly dependent on the function of LLO since it is responsible for lysing the phagosomal membrane (75). However, a lack of differences in the LLO activity of *Lm* in the neutrophils from the three groups of mice could explain the observed

similarities in phagosomal escape and containment observed in these assays. Previous studies have demonstrated that LLO activity is pH dependent and redox sensitive (112). The gamma-interferon-inducible lysosomal thiol reductase (GILT), a thiol reductase present in lysosomes, has been reported to activate LLO in macrophages. Macrophages from GILT KO mice were observed to have reduced intracellular bacteria replication in comparison to WT mice (113). This led to the hypothesis that ecSOD may be directly interacting with *Lm* in the phagosome which would affect activation of LLO as it is redox sensitive. This is a likely possibility as a previous study reported the presence of ecSOD in the endosomes of endothelial cells when the enzyme was directly added to the cells in culture (114). The direct effect of ecSOD activity was tested by treating ecSOD KO mice splenocytes, liver and bone marrow leukocytes with recombinant SOD2 and determining if there were any differences in their ability to phagocytose and contain *Lm*. However, similarly to the previously stated observation, there were no differences in the ability of the ecSOD KO neutrophils to internalize and contain *Lm* when the cells are treated with exogenous SOD2. It can therefore be inferred that ecSOD activity has no effect on phagosomal containment of *Lm* by neutrophils. Therefore, an understanding of differences in LLO activity in the ecSOD congenic mice will provide more knowledge on the link between ecSOD and LLO activity.

Phagosomal maturation is a necessity for effective killing of the bacteria as the phagolysosome contains the hydrolytic enzymes as well as ROS components necessary for bacteria degradation (73). There were unexpectedly no observed differences in the ability of neutrophils from the three groups of mice to kill the bacteria. However, infection was shown to increase expression of LAMP-1 in ecSOD KO splenic neutrophils, *in vivo*, compared to ecSOD HI neutrophils. The increased expression of LAMP-1 is most likely indicative of the presence of more lysosomes, phagolysosome and/or autophagolysosomes which would subsequently lead to

more effective killing of *Lm*. The lack of differences observed in the killing assay could be a result of there being ecSOD present during the assay. To conduct the killing assay, the neutrophils had to be sorted, a time consuming process that could potentially have resulted in a complete loss of ecSOD which is normally localized extracellularly. Previously, our lab conducted a transfer study whereby neutrophils were isolated from ecSOD HI or ecSOD KO mice, fluorescently labeled, and transferred into ecSOD WT mice. Following infection with *Lm*, the percentage of recruited ecSOD HI and KO neutrophils in the WT mice was analyzed. It was observed that equal percentages of ecSOD HI and KO neutrophils transferred into ecSOD WT mice were recruited into the liver following infection with *Lm* (3). Essentially, both ecSOD HI and KO neutrophils are recruited similarly to sites of infection when they are in the same environment. Additionally, in another assay, neutrophils from either ecSOD HI or ecSOD KO mice were transferred into ecSOD WT mice followed by infection with *Lm*. CFUs were calculated and there were no differences in the bacterial burden between ecSOD WT mice reconstituted with either ecSOD KO or ecSOD HI neutrophils and infected with *Lm* (3). This led to the conclusion that the effects of ecSOD activity are not cell intrinsic but rather a result of the environment. This could therefore be another explanation for the lack of differences observed in both the killing and phagosomal containment assays.

In another previous study conducted in our lab, ecSOD congenic mice were infected with *Lm*, and neutrophils were sort purified from the 3 groups of mice and plated for CFUs. There were CFUs in the ecSOD HI and WT mice but no bacteria were recovered from neutrophils purified from the ecSOD KO mice (6). The combination of the observed increase in LAMP-1 expression *in vivo*, the more effective phagocytosis of *Lm*, and the previously reported lack of bacteria in

neutrophils from the ecSOD KO mice after *in vivo* infection with *Lm*, indicates that they are likely more effective at killing than the ecSOD HI neutrophils.

The effects of ecSOD activity on neutrophil autophagy was also investigated in these studies as phagosomal escape of *Lm* has been previously demonstrated to induce autophagy in macrophages (80, 81). As execution of autophagy is dependent on the cleavage of LC3 into LC3 I and LC3 II, this phenomenon can only be observed via western blotting due to the only available antibody against LC3 recognizing both forms. However, there was no observed cleavage of LC3 observed in neutrophils following infection with *Lm* (data not shown). Analysis of LC3 expression (LC3 I & II) with flow cytometry demonstrated that a higher percentage of neutrophils were expressing LC3 following infection with *Lm*, independent of ecSOD activity. Additionally, there were no differences in the amount of LC3 being expressed by the neutrophils from the three groups of mice. In support of these findings, previous study demonstrated cleavage of LC3 in neutrophils during the adaptive immune response to *Lm* but there was no observed cleavage during the innate immune response (77). Therefore, it is possible that autophagy is not a mechanism that the neutrophils engage in early on following uptake and escape of *Lm* into the cytosol.

Inhibition of autophagy was investigated to determine if there were effects independent of LC3 cleavage. Cells were treated with chloroquine, a pharmacological inhibitor of autophagy. Following infection with the bacteria, the observed decrease in uptake, phagosomal escape and containment of *Lm* following treatment with chloroquine could be due to the drug inhibiting effective phagocytosis of the bacteria as LC3 has also been previously reported to be involved in phagocytosis of *Lm* (115). Gluschko *et al.* observed that LC3 plays a role in the phagocytosis of *Lm* by macrophages during a process referred to as LC3 associated phagocytosis (LAP). During LAP, LC3 is recruited to the phagosomal membrane of the cells where it promotes phagosomal

lysosomal fusion (115). In regards to phagocytosis and containment, the observed decrease in the chloroquine treated cells could be a result of chloroquine inhibiting effective phagocytosis of the bacteria by possibly inhibiting effective recruitment of LC3 to the phagosome. However, the decrease in phagosomal escape observed in the chloroquine treated cells is most likely a direct result of the decrease in the amount of phagocytosed bacteria as the amount of escaped bacteria always correlates with how much *Lm* is taken up as shown in the dose response experiment in chapter III.

Overview of Chapter IV

In Chapter IV, although neutrophils and monocytes have been demonstrated to be essential for host protection against *Lm* infection, differences and/or similarities in their ability to perform specific functions have not been directly compared. The objective of this study was to delineate specific functions of neutrophils and inflammatory monocytes during *Lm* infection which can possibly be applied to other infection and disease model as well. Our data show that neutrophils are more effective at phagocytosis of *Lm* in comparison to monocytes, although monocytes from the spleen and liver also took up the bacteria. Neutrophils were also observed to be more effective at preventing *Lm* escape from the phagosome, probably as a result of more efficient phagosomal maturation and subsequent killing of the bacteria. These data also revealed differences in TNF-α production between the two cell types as well as the novel ability of monocytes and neutrophils to produce IL-1α during *Lm* infection. In addition, we were also able to demonstrate that a subset of inflammatory monocytes are capable of effectively producing both IL-1α and TNF-α.

Chapter IV Discussion

"This study lends credence to previous conclusions that neutrophils are the primary innate cells that associate with, and phagocytose, *Lm* early on during infection (1, 66, 116). Although

neutrophils and monocytes are rapidly mobilized to *Lm* foci during infection, the neutrophils are more important for bacterial uptake as they observably phagocytose more bacteria overall in comparison to monocytes. The receptors and ligands that facilitate neutrophil phagocytosis of *Lm* are currently up for debate (37). However, it has been reported that the complement system plays an important role in bacterial clearance during *Lm* infection. Macrophages more effectively phagocytosed bacteria that were C3b opsonized and lack of CR3, the complement receptor for iC3b, led to increased host susceptibility to *Lm* infection (68, 69). CR3, commonly known as Mac-1, is also expressed on both neutrophils and monocytes (67). Our experiments revealed that opsonization of the bacteria with serum increased neutrophil, but not monocyte, phagocytosis of *Lm*. When neutrophils and monocytes were directly compared following infection with opsonized *Lm*, neutrophils were more effective at phagocytosis in comparison to monocytes. Therefore, *Lm* phagocytosis by neutrophils is most likely complement driven although phagocytosis by monocytes still requires investigation.

A recent study by Jones *et. al.* reported that *Lm* primarily associate with monocytes in the gut, *in vivo*, during oral infection using the murinized *Lm* strain. However, when bone marrow derived monocytes were cultured with the bacteria, there was a lack of intracellular growth of *Lm* in the cells which led to the conclusion that *Lm* does not replicate effectively in monocytes (100). We postulate that the *in vivo* observation is most likely due to monocytes taking up some *Lm* but lacking the ability to contain and kill *Lm* as effectively as neutrophils. Using the unique actA:LMGFP reporter strain of *Lm*, we were able to demonstrate that there were no differences in the amount of bacteria escaping from the phagosome of neutrophils and monocytes when infected with *Lm*. The neutrophils, however, were more effective at containment of *Lm* as they took up significantly more bacterial, overall, but allowed for relatively equal amount of escape. LAMP-1,

a common lysosomal marker, necessary for phagosomal lysosomal fusion, was expressed at a higher level in neutrophils that had engulfed *Lm* than monocytes that had also phagocytosed the bacteria (73, 117). When the cells did not internalize *Lm*, there were no differences in LAMP-1 expression between neutrophils and monocytes. Additionally, phagocyosis of *Lm* by monocytes did not increase LAMP-1 expression in monocytes. This most likely indicates that following uptake of the bacteria, whilst phagolysosome formation is occurring in neutrophils it is not taking place in monocytes.

The combination of phagosomal containment and effective formation of phagolysosomes likely drives effective killing of *Lm* by neutrophils. Efficient assembly of phagolysosomes leads to effective killing of *Lm* as it would prevent the pore forming function of LLO from taking effect and allowing for escape of *Lm* before the bacteria is killed. In addition, neutrophils also generated more ROS than monocytes when stimulated following infection with *Lm*. However, this may not necessarily be the manner by which they kill as a recent finding by Pitts *et. al.* concluded that ROS is not required for killing of *Lm* by neutrophils (15). Mice lacking the gp91 subunit of the NADPH oxidase had previously been demonstrated to be more susceptible to *Lm* (118, 119). Interestingly, the recent study by Pitts *et. al*. showed that neutrophils from the gp91 mice were able to kill as effectively as neutrophils from WT mice (15). Therefore, though ROS may be contributing to the efficiency of neutrophil killing, it might not be the primary manner by which *Lm* is killed. The observed increase in bacteria burden in the gp91 KO mice may be due to ROS playing a much more significant role in macrophage rather than neutrophil killing.

The role of TNF-α during *Lm* infection has been well documented (14, 92, 93). TNF-α is a cytokine that induces phagocytosis as well as killing of pathogens by driving assembly and activation of the NADPH oxidase. It is also necessary necessary for the vasodilation that allows

for efficient recruitment of the phagocytes to sites of infection. Specific deletion of TNF-α in neutrophils, monocytes and macrophages has also been demonstrated to lead to an increase in host susceptibility to *Lm* infection (93). Carr *et. al.* demonstrated that simultaneous depletion of both neutrophils and monocytes with the anti-Gr-1 antibody led to a decrease in TNF-α production in both the liver and spleen. Therefore, lack of both neutrophils and monocytes during *Lm* infection decreases the amount of TNF-α produced in the spleen and liver. Additionally, specific depletion of neutrophils with the 1A8 antibody led to a decrease in TNF-α production in the liver but not the spleen. However, at a higher infectious dose, depletion of neutrophils resulted in a reduction in TNF-α production in both the spleen and liver (14). This highlights the contribution of neutrophils to overall TNF-α production in the liver and at higher doses of infection, the spleen as well. The data in the current study show that in both the liver and spleen, monocytes have a higher expression of TNF-α than neutrophils. In addition, a higher percentage and number of monocytes were producing the cytokine, in comparison to neutrophils. Furthermore, there was a higher TNF-α MFI in the splenic monocytes than liver monocytes. This is likely due to the differences in immune responses between the spleen and the liver. It can be inferred that in the liver, TNF-α production by neutrophils is more important whereas, production of TNF-α by monocytes is more important in the spleen. Our data support previous findings that inflammatory monocytes primarily function to produce TNF-α and NO during *Lm* infection, mainly in the spleen (2, 120). TNF-α production in neutrophils has previously been demonstrated to be driven by Sox2, a cytosolic sensor that is activated upon binding to *Lm* DNA. Sox2 is not expressed in macrophages but it is possible that monocytes also express Sox2 which could be what induces the observed higher production of TNF-α (121). This is also plausible as there is less phagosomal containment in the

monocytes than neutrophils and therefore, presumably more bacteria present in the monocyte cytosol over time to induce activation of Sox2.

Inflammatory monocytes are also capable of producing IL-1α as there was an observed significant population of TNF-α producing monocytes that also stained positive for IL-1α. Furthermore, similarly to TNF-α, splenic monocytes also have a higher IL-1α MFI in comparison to liver monocytes. Therefore, it is possible that during *Lm* infection, cytokines play a more important role in the spleen than in the liver. The role of IL-1α during *Lm* infection is largely unknown. IL-1β has been deemed dispensable during *Lm* infection, however, the IL-1R, to which both IL-1α and IL-1β bind, is necessary for host resistance to infection (94, 95). Treatment of *Lm* infected mice with recombinant IL-1α led to an increase in host resistance to *Lm* by reducing the bacterial burden (122). IL-1α also has a higher affinity for the IL-1R than IL-1β which could be the driving force for host susceptibility observed in the IL-1R neutralized mice. IL-1α, in contrast to IL-1β, does not need to be cleaved to become activated, suggesting its function is not entirely dependent on activation of the inflammasome (111). Therefore, the signaling for IL-1α is probably not dependent on Nod-like receptor (NLR) signaling and unlike IL-1β, it maybe be activated through other PRRs such as Toll-like receptors (TLRs). Production of IL-1α can also be driven by factors such as oxidative stress as well as other cytokines including IL-1α, itself. In turn, IL-1α can also induce the production of cytokines such as TNF-α (123). As a result, the role of IL-1α during *Lm* infection may be to drive production of TNF-α which would subsequently enhance phagocytosis and killing of *Lm* by neutrophils and macrophages.

The findings from Chapter IV demonstrate that there are similarities in function between neutrophils and monocytes during *Lm* infection. However, it also highlights that there are

differences in effectiveness of specific functions between the two innate cell types. The role of neutrophils during *Lm* infection appears to be more physical; they are more effective at phagocytosis, containment and killing of *Lm*. Inflammatory monocytes, however, are more effective at production of cytokines that can presumably enhance the functions of neutrophils, macrophages, and potentially T cells. In essence, this study adds further proof to the necessity of both neutrophils and monocytes for host resistance and effective clearance of *Lm*." (65)

Overall Conclusions & Future Direction

The ecSOD study highlighted a lack of differences in the ability of neutrophils with differing ecSOD activity to kill *Lm*. This finding suggests that ecSOD, and possibly ROS, is not a necessity for *Lm* killing. This is further emphasized by the observation in the neutrophil and monocyte studies whereby although neutrophils are making more ROS and kill a higher percentage of bacteria, monocytes were still able to kill a considerable percentage of *Lm*. In addition, a recently published study demonstrated that neutrophils with a defective gp91 component of the NADPH oxidase were able to kill just as effectively as neutrophils with the functional enzyme (15), although gp91 KO mice have previously been reported to be more susceptible to *Lm* infection (118). In contrast, mice lacking the p47 component of the NADPH oxidase were just as resistant as WT mice to infection with *Lm* (124). In totality, these findings indicate that phagocyte killing of *Lm*, although augmented by ROS, is not entirely dependent on ROS. Therefore, future studies on the regulation of *Lm* killing by neutrophils and monocytes are warranted. LAP has been reported to be a process by which macrophages are able to promote effective phagosomal lysosomal fusion, a process necessary for killing. It is therefore possible that as long as bacterial escape can be prevented and maturation of the phagosome achieved, the bacteria can still be killed in the absence

of ROS. Studies on the regulations of phagosomal lysosomal fusion during infection would therefore aid in better understanding of bacterial killing by phagocytic cells.

Data from chapter IV presented the novel finding of IL-1α production by neutrophils and monocytes during infection with *Lm*. Generally, monocytes were found to be more effective at IL-1α production, although, at day 1 post infection, liver neutrophils were more effective at IL-1α production. It is therefore imperative to gain a better understanding of the role of IL-1α as well as factors that regulate its production by neutrophils and monocytes. First, the role of IL-1α can be studied in the context of ecSOD activity as ROS has been previously reported to regulate production of IL-1α. Following infection with *Lm*, levels of IL-1α can be measured in the ecSOD congenic mice as well as expression of the cytokine in both neutrophils and monocytes. TNF-α has been also reported to induce production of IL-1α. Therefore, mice could be treated with TNF-α and in a separate experiment, anti-TNF-α, before and during infection with *Lm* and expression of IL-1α measured. The contributions of neutrophils and monocytes to IL-1α can be determined via measurement of the cytokine levels during depletion studies of either or both cell types during *Lm* infection. Neutrophil, monocyte and macrophage specific contributions to IL-1α production can be determined via the generation of lysosyme Cre mice bred to IL-1α floxed mice in order generate phagocytic cell specific IL-1α KO mice. Lastly, differences in the effect of pro- versus cleaved- IL-1α can also be investigated to determine how much of a role the inflammasome may have on IL-1α production during *Lm* infection. Data from the aforementioned studies will aid in a better understanding of the role of IL-1α in host protection during infection.

The *Lm* infection mouse model is an effective avenue for studying host pathogen interactions. Our studies made use of this model to gain a better understanding of the functional

roles and capabilities of neutrophils and monocytes. First, neutrophil function was studied in the context of ROS regulation and how ecSOD, an antioxidant, modulates neutrophil function during *Lm* infection. Second, comparisons of neutrophils and monocytes were performed in order to delineate functional similarities and differences between the two cell types. Neutrophils were found to be more effective at what can be referred to as more physical functions. They were more effective at bacterial phagocytosis, containment and killing, although, monocytes were still capable of performing these functions. Monocytes, on the other hand, were found to be more effective at production of cytokines which have been previously reported to potentially induce the primarily neutrophilic functions. In addition, high activity of ecSOD was detrimental to the ability of neutrophils to phagocytose *Lm* which also correlated with increased extracellular and intracellular bacterial burden in the spleen. Although this study was able to generate all aforementioned information, there is still much more knowledge on the functions and regulations of neutrophils and monocytes to be garnered. This is important to the development of effective treatments and vaccines for a variety of infectious and inflammatory diseases.

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