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Extracellular superoxide dismutase (ecSOD) regulates extracellular concentrations of reactive oxygen species (ROS) to protect tissues during infection and inflammation. Using ecSOD HI, ecSOD WT, and ecSOD KO mice, we have previously shown that ecSOD activity enhances neutrophil recruitment to the liver, yet inhibits the innate immune response against *Listeria monocytogenes* leading to increased host susceptibility. Using adoptive transfer experiments, we observed that ecSOD activity does not affect neutrophil recruitment or function in a cell-intrinsic manner. Additionally, we noted that ecSOD activity results in decreased retention of immature neutrophils in the bone marrow without altering granulopoiesis. Furthermore, we determined that ecSOD activity protects the extracellular matrix (ECM) and increases concentrations of neutrophil-attracting chemokines leading to an increase in immature neutrophils in the liver.

Since ecSOD can be produced by cells from the hematopoietic lineage as well as non-hematopoietic cells, we used bone marrow chimeric mice to investigate the relative contribution of ecSOD produced by cells from each lineage. Ultimately, it was determined that ecSOD from both hematopoietic and non-hematopoietic cells contributes to the overall phenotype observed in ecSOD congenic mice. Collectively, our data suggest that ecSOD activity inhibits degradation of the ECM and promotes egress of

immature neutrophils out of the bone marrow and into the liver where they provide inadequate protection against *L. monocytogenes*. These studies highlight the potential therapeutic value of ecSOD inhibitors to enhance immune responses during bacterial infections.

Extracellular Superoxide Dismutase Indirectly Enhances the Release of Immature Neutrophils from the Murine Bone Marrow

Dissertation

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By

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LIST OF ABBREVIATIONS

Name	Abbreviation
Analysis of Variance	ANOVA
Bone marrow chimeric	BMC
Brain-heart infusion	BHI
Colony-forming unit	CFU
Dulbecco's modified eagle medium	DMEM
Enzyme-linked immunosorbent assay	ELISA
Extracellular matrix	ECM
Extracellular superoxide dismutase	ecSOD
Fetal calf serum/fetal bovine serum	FCS/FBS
Granulocyte-colony stimulating factor	G-CSF
Hank's balanced salt solution	HBSS
Heat-killed *Listeria monocytogenes*	HKLM
Hyaluronic acid	HA
Interleukin	IL
Intravenous	i.v.
Mean fluorescence intensity	MFI
Myeloperoxidase	MPO
Neutrophil extracellular trap	NET
Phosphate-buffered saline	PBS

Post-infection	p.i.
Reactive nitrogen species	RNS
Reactive oxygen species	ROS
Red blood cell	RBC
Tumor necrosis factor	TNF

CHAPTER I

INTRODUCTION

Specific Aims

Antioxidants have become a buzzword in both medicine and in the popular media, however the implications of antioxidants, particularly their effects on the immune system, are incompletely understood. The role of extracellular superoxide dismutase (ecSOD), an antioxidant enzyme important in the regulation of reactive oxygen species (ROS), has not been thoroughly investigated in the context of immune responses during bacterial infection. In particular, the potential effects of this antioxidant enzyme on immune cell development and recruitment out of the bone marrow require further investigation. While it has been previously shown that ecSOD activity leads to increased neutrophil recruitment and decreased neutrophil function, the mechanisms behind these differences are unclear and the effect of ecSOD activity on neutrophil maturation in the bone marrow has not been studied. We hypothesize that parenchymal cells produce ecSOD which reduces neutrophil retention in the bone marrow and protects the extracellular matrix from degradation leading to increased egress of immature neutrophils from the bone marrow. Furthermore, this enhances recruitment of immature neutrophils to the liver where they are unable to protect against *Listeria monocytogenes*.

Specific Aim I: How does ecSOD activity reduce neutrophil activity in the liver?

It has been shown that neutrophils are essential for protection against *L. monocytogenes* infection in the liver (1). It has also been published that ecSOD activity leads to increased recruitment of neutrophils to the liver, however these neutrophils are unable to provide sufficient protection during *L. monocytogenes* infection (2). Furthermore, depletion of neutrophils in ecSOD congenic mice leads to increased susceptibility to *L. monocytogenes* infection in ecSOD WT and ecSOD KO mice but decreased susceptibility in ecSOD HI mice (2). We hypothesize that ecSOD activity leads to increased presence of phenotypically and functionally immature neutrophils in the liver. To determine if ecSOD activity alters neutrophil maturity, expression levels of L-selectin (CD62L) on neutrophils from ecSOD congenic mice will be analyzed in the absence of infection and at day one post-infection (d1p.i.). Since ecSOD is an antioxidant enzyme and neutrophils are important for production of ROS, including superoxide, during bacterial infections, ecSOD activity may be directly inhibiting neutrophil maturity and function. Neutrophil transfer protection studies will be implemented to determine if ecSOD activity is altering neutrophil function in a cell-intrinsic manner.

Specific Aim II: Does ecSOD activity alter neutrophil development in the bone marrow?

Neutrophils are generated by granulopoiesis in the bone marrow and develop into mature neutrophils that are essential for protection against bacterial infections (3). The increased presence of phenotypically and functionally immature neutrophils in the liver of ecSOD HI mice suggests potential defects in neutrophil development or generation. To evaluate neutrophil development, bone marrow neutrophils will be analyzed for maturity by expression of c-kit, a marker for granulocyte precursor cells. Since G-CSF is important for neutrophil development and generation, this could lead to differences in neutrophil granulopoiesis (4). In order to

determine if there are differences in granulopoiesis in the congenic ecSOD mice, granulopoiesis colony assays will be performed. Neutrophils from ecSOD HI mice are unable to protect against *L. monocytogenes* infection in the liver (2). In order to determine if ecSOD also mediates neutrophil function in the bone marrow, experiments to evaluate ROS production, via H_2DCFDA, and phagocytosis, via *in vitro* infections with *L. monocytogenes*-GFP, will be performed. Differences in G-CSF concentrations, as observed in the serum of ecSOD congenic mice (manuscript submitted), may also suggest potential differences in the CXCR4/CXCL12 bone marrow retention axis (5, 6). In order to investigate these differences, flow cytometry analysis of CXCR2 and CXCR4 on bone marrow neutrophils will be determined. In addition, CXCL12 ELISAs will be performed on bone marrow extracellular fluid (ECF) to determine if there are differences between the congenic mice.

Specific Aim III: Does ecSOD produced by hematopoietic cells or non-hematopoietic cells play a role in mediating differences in neutrophil responses?

Since ecSOD is expressed in multiple tissues throughout the body (7) and can be produced by both immune and non-immune cells (8), it is important to determine relative contributions of ecSOD activity from either hematopoietic or non-hematopoietic cells on both neutrophil recruitment and function. In order to investigate the effects of lack of ecSOD from hematopoietic or non-hematopoietic cells, as well as the effects of enhanced ecSOD activity from hematopoietic or non-hematopoietic cells, bone marrow chimeric (BMC) mice will be generated. Once fully reconstituted, these BMC mice will be infected with *L. monocytogenes* and evaluated for differences in bacterial burden and neutrophil recruitment.

Extracellular Superoxide Dismutase

Extracellular superoxide dismutase (ecSOD, SOD3) is an antioxidant enzyme responsible for converting superoxide into hydrogen peroxide in the extracellular milieu and is, therefore, an important antioxidant enzyme for protecting host tissues from damage caused by reactive oxygen and reactive nitrogen species (ROS, RNS) (9). ROS are common byproducts and important components of cellular processes. In addition, during infection, the upregulation of ROS production is essential for efficient clearance of pathogens (10-12). However, since ROS are not targeted against pathogens, they can cause oxidative damage to the host and therefore ROS concentrations must be tightly regulated (11). Enzymes such as ecSOD play an important role in regulating ROS concentrations to find the appropriate balance between normal cellular functions, pathogen killing, and tissue damage.

There are three members of the superoxide family, however, SOD1 and SOD2 are present within cells, with SOD1 found in cytosplasm, nuclear compartments, and lysosomes, and SOD2 found in mitochondria, making ecSOD (SOD3) the only extracellular member of the family (8). ecSOD has a heparin sulfate binding domain which enables it to bind to the ECM and maintain its localization within tissues (7, 9). ecSOD is expressed in multiple tissues throughout the body including the heart, lungs, kidneys and liver (7) and ecSOD can be produced by both immune and non-immune cells (8), which is important since this enzyme plays important roles in homeostatic regulation of ROS as well as during inflammatory diseases (7). Importantly, one of the ways ecSOD reduces inflammation is by protecting the extracellular matrix (ECM) from degradation (13-15).

ecSOD has been shown to be protective in multiple disease models, particularly during non-infectious inflammatory lung injury models, and its activity leads to decreased neutrophil recruitment to the lungs (16-21). The protective effects of ecSOD during disease models, and the

correlation between increased pathology and decreased ecSOD levels (22), has led to suggestions of using ecSOD as a therapy for oxidative stress-induced disease (7, 23).

Neutrophils

Neutrophils are one of the first responding cells during inflammation or infection. They are classic phagocytic cells, able to take up and kill pathogens by releasing degrading enzymes from their granules and producing both ROS and RNS. Neutrophils are also well known for their ability to enhance the immune response by production of pro-inflammatory cytokines, particularly TNF-α. Recently, they have been shown to expunge DNA and other intracellular molecules to form neutrophil extracellular traps (NETs) that are effective in consolidating the spread of pathogens (24).

"Neutrophils are hematopoietic-derived immune cells that are generated, and continue to develop, in the bone marrow until recruited into circulation and then to sites of infection or inflammation. Steady-state neutrophil granulopoiesis is modulated by common stem cell cytokines, such as IL-3 and IL-6, as well as granulocyte colony stimulating factor (G-CSF) and granulocyte-macrophage colony stimulating factor (GM-CSF). Under infectious or inflammatory conditions, neutrophil granulopoiesis can be increased, typically termed "emergency granulopoiesis", in order to restore homeostasis in the bone marrow after recruitment of neutrophils to peripheral sites (25). While IL-3, IL-6, G-CSF and GM-CSF have all been shown to contribute to emergency granulopoiesis, it has also been demonstrated that the production of reactive oxygen species (ROS) by bone marrow myeloid cells is critical for this process during infection (26)." (27).

Once a granulocyte precursor has differentiated into a neutrophil, it exists in a bone marrow niche with non-hematopoietic bone marrow cells that express membrane-bound stem

cell factor and CXCL12 (SDF-1), the ligands for c-kit and CXCR4, respectively. Neutrophils that express high c-kit, CXCR4, or both, are considered immature and are retained in the bone marrow by binding their respective ligands (28). CXCR4 is gradually reduced as neutrophils mature, correlating with the gradual increase of CXCR2, the receptor for neutrophil-attracting chemokines such as CXCL1 and CXCL2, which leads to the release of mature neutrophils from the bone marrow. Neutrophil progenitors upregulate expression of Ly6G as they differentiate, and neutrophils increase expression of CD11b as they mature and prepare to exit the bone marrow (3).

Neutrophil recruitment from the bone marrow may also be induced by damage-associated molecular patterns (DAMPS) that can be host-derived as well as pathogen-derived (29). Under basal conditions, G-CSF may also play an essential role in mobilization of neutrophils out of the bone marrow, likely by decreasing concentrations of CXCL12 (5). It has also been shown that in a sepsis model, inhibition of CXCL12 actually prevented the release of neutrophils from the bone marrow, though this contrasts with much of the established literature for other models (30).

L-selectin (CD62L, Mel14) is present on immature neutrophils but is shed from neutrophils as they mature and traffic to peripheral sites (31). In addition, expression of both CD11b and CD18 adhesion molecules is increased on mature neutrophils as these molecules are essential for efficient neutrophil rolling and diapedesis. Neutrophils also express CD44 which is often used as a marker for neutrophil activation but, more importantly, CD44 binds to the ECM, specifically hyaluronic acid (HA), in order for neutrophils to traffic and extravasate into the liver (32-35).

“Neutrophils that traffic into tissues in the absence of infection or inflammation commonly become apoptotic rather than returning to circulation. It has been shown that these

neutrophils are phagocytosed by resident macrophages and dendritic cells in the liver, which could potentially induce a feedback loop that decreases further granulopoiesis (36). Alternately, the chemokine receptor CXCR4 is upregulated as circulating neutrophils age, leading to trafficking back to the bone marrow where they are ingested by macrophages (3, 37, 38). Although neutrophil production is constitutive during homeostasis, an enhanced neutrophil response is often essential for host survival." (27).

Listeria monocytogenes

"*Listeria monocytogenes* is a gram-positive bacterium with a primarily intracellular life cycle after infection of a host organism. *L. monocytogenes* infection occurs following ingestion of contaminated foods and is the third leading cause of death among foodborne pathogens (39). Immunocompromised individuals, pregnant women and newborns are particularly susceptible to infection which can result in septicemia, meningitis, and loss of fetus. While foci of infection are generally established in the spleen and liver, *L. monocytogenes* can travel through the circulation to the heart, the brain, and to the bone marrow (40-42).

L. monocytogenes is able to cross the intestinal wall by binding to E-cadherin with one of its virulence factors, Internalin A. From the intestine, *L. monocytogenes* disseminates through the lymphatics and the bloodstream to the spleen and liver, where it can enter target cells via phagocytosis or induced endocytosis. In the liver, one of the primary target cells for infection are hepatocytes, which are initially directly infected through utilization of the virulence factor Internalin B binding to hepatocyte growth factor receptor (HGFR, Met, c-Met). Once internalized, *L. monocytogenes* is able to escape into the cytosol of host cells by secretion of listeriolysin O (LLO). Cytoplasmic *L. monocytogenes* then replicates and spreads to neighboring cells by polymerizing host actin with the aid of actin-assembly-inducing protein A (ActA) (43,

44). This indirect infection via cell-to-cell spread is effective, particularly in the liver, because it allows *L. monocytogenes* to infect neighboring cells without being exposed to opsonization or recognition and killing by innate immune cells." (27).

Neutrophils and Listeria monocytogenes infection

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"**Requirement of neutrophils during *L. monocytogenes* infection**

Initial immune responses against *L. monocytogenes* are managed by innate immune cells, with macrophages, monocytes and neutrophils playing a central role. Early depletion studies using the anti-GR-1 monoclonal antibody (RB6-8C5) concluded that neutrophils are critically important for host defense during *L. monocytogenes* infection (45-51). More recently, it was shown that the anti-GR-1 antibody binds to Ly6G, which is expressed exclusively by neutrophils, and Ly6C, which is expressed by neutrophils, monocytes, subsets of dendritic cells, and subsets of memory CD8+ T cells (51, 52). However, use of the anti-Ly6G monoclonal antibody (1A8) for neutrophil-specific depletion studies has shown that neutrophils are essential for clearance of *L. monocytogenes*, particularly from the liver (1, 53). Our studies have also established that neutrophils are particularly important during high *L. monocytogenes* inoculum, consistent with previous reports indicating that following the administration of a high dose infection, neutrophils ingest *L. monocytogenes* in the liver (1, 50). Another recent report presented data that suggest neutrophils are not required for protection against *L. monocytogenes*. However, a relatively low dose of infection was used and importantly, neutrophils were depleted

via intraperitoneal injection of the 1A8 antibody at the time of infection (54). It is possible that after intravenous infection and subsequent rapid arrival of *L. monocytogenes* at the target organs, neutrophils were still present and contributed to early bacterial uptake and killing. Further highlighting the importance of neutrophils, mice lacking G-CSF, or its receptor, display severe neutropenia and are more susceptible to *L. monocytogenes* infection than wild-type mice (55, 56). Additionally, an increased presence of neutrophils in peripheral organs, resulting in increased resistance against *L. monocytogenes*, was observed in mice deficient in B7-H4, a molecule which inhibits growth of neutrophil progenitors (57). Another study showed that over-activation of the innate immune response, by high-dose *L. monocytogenes* infection or pre-activation with either heat killed *L. monocytogenes* or synthetic TLR2-ligand followed by low-dose *L. monocytogenes* infection, led to significant neutrophil apoptosis in the bone marrow and a subsequent increase in host susceptibility (58).

Besides neutrophils, other phagocytic cells have well-established roles during *L. monocytogenes* infection. Depletion of splenic and liver macrophages during *L. monocytogenes* infection results in increased mortality and bacterial burden (59, 60). Tissue macrophages are also involved in the production of TNF-α and IL-12 in response to recognition of *L. monocytogenes* (61, 62). Inflammatory monocytes have been shown to respond to *L. monocytogenes* by producing IL-12 and IL-15 (63). Additional studies identified a differentiated population of CCR2+ inflammatory monocytes, also termed Tip-DCs, which produce both TNF-α and iNOS (inducible nitric oxide synthase) and are essential for clearance of *L. monocytogenes* infection (64). Ultimately, the production of innate cytokines from macrophages and inflammatory monocytes induces the production of IFN-γ from multiple cell types, leading to increased macrophage phagocytosis and killing of the bacteria (62, 65). The complex interplay

between tissue resident and recruited phagocytic cells is critical for protection of the host against *L. monocytogenes*.

Neutrophil recruitment during *L. monocytogenes* infection

Neutrophil release from bone marrow

During homeostasis, neutrophils are retained in the bone marrow by the interaction of CXCR4 with its ligand CXCL12 (SDF-1), which is expressed by non-hematopoietic bone marrow cells. Following *L. monocytogenes* infection, neutrophils are rapidly recruited out of the bone marrow primarily due to the downregulation of CXCR4 followed by the upregulation of CXCR2, the receptor for neutrophil-attracting chemokines such as CXCL1 (KC) and CXCL2 (MIP-2α) (66, 67). Myeloid-lineage specific loss of CXCR4 was shown to lead to premature release of neutrophils from the bone marrow into the blood during basal conditions but also contributed to an impaired release of neutrophils in response to G-CSF, CXCL2 or *L. monocytogenes* infection (66). This suggests that during *L. monocytogenes* infection CXCR4 is essential for regulating neutrophil release from the bone marrow. Under basal conditions, G-CSF may also play an essential role in mobilization of neutrophils out of the bone marrow, likely by decreasing concentrations of CXCL12 (5).

Neutrophil recruitment from the bone marrow may also be induced during infection or inflammation by host-derived damage-associated molecular patterns (DAMPs) as well as pathogen-associated molecular patterns (PAMPs) (29, 68). There are no current studies illustrating *L. monocytogenes*-specific PAMPs that can induce neutrophil release or the role of CXCL1, CXCL2 or CXCL12 in the bone marrow during infection. However, one could speculate that increased concentrations of G-CSF induced by *L. monocytogenes* infection would

lead to decreased concentrations of CXCL12 followed by a downregulation in neutrophil CXCR4 expression and enhanced release of neutrophils from the bone marrow into circulation.

Neutrophil extravasation into tissue

During *L. monocytogenes* infection, neutrophils that are released from the bone marrow are subsequently recruited to infected organs, primarily the liver. Efficient neutrophil chemotaxis is dependent on chemoattractant molecules that induce signaling pathways leading to the rearrangement of intracellular structural molecules and upregulation of surface adhesion molecules. Formylated peptide receptors (FPRs) are highly expressed on neutrophils and can bind *L. monocytogenes*-derived formylated peptides (such as fMIVIL) resulting in a signaling cascade that induces neutrophil migration (69). Accordingly, mice deficient in FPR1, FPR2 or both, are more susceptible to *L. monocytogenes* infection and have delayed recruitment of neutrophils to the liver (69-71). This occurs in the absence of differences in concentrations of common neutrophil-attracting chemokines, suggesting that FPRs are responsible for initial chemotactic signals to recruit neutrophils into the liver during *L. monocytogenes* infection (70, 71).

The neutrophil-attracting chemokines CXCL1 and CXCL2 are produced in the liver following *L. monocytogenes* infection (67). Treatment with anti-CXCL2 (anti-MIP-2) antibody decreases neutrophil recruitment to the liver after *L. monocytogenes* infection in wild-type mice and antibody blockade of CXCR2 completely ablated efficient neutrophil recruitment (67). Conversely, mice deficient in the murine IL-8 receptor homolog (CXCR2) have previously been shown to be more resistant to acute *L. monocytogenes* infection (72). However, this increased resistance to infection is likely attributed to the extreme neutrophilia observed in these mice (73, 74). Mice lacking the type I interferon receptor (IFNAR) show increased neutrophil recruitment

to sites of infection and increased resistance to *L. monocytogenes* infection compared to wild-type mice. Pharmacological inhibition of CXCR2 in IFNAR deficient mice reversed both the enhanced neutrophil recruitment and the increased resistance to infection (75). Collectively, these data suggest that FPRs are required for initial extravasation into the liver with subsequent chemokine receptor signaling implicated in neutrophil recruitment to the site of *L. monocytogenes* infection within the tissue.

Mature neutrophils upregulate expression of adhesion molecules resulting in efficient extravasation into tissues. Specific adhesion molecules expressed on neutrophils include LFA-1 (CD11a/CD18) and Mac-1 (CD11b/CD18, CR3) (76). Interestingly, CD18 KO mice (deficient for LFA-1 and Mac-1) display increased resistance to *L. monocytogenes* infection, probably due to increased presence of neutrophils in the periphery caused by increased concentrations of G-CSF (77). Similarly, mice deficient in CD11a (LFA-1) are more resistant to *L. monocytogenes* infection, have increased infiltration of neutrophils into the liver, and increased concentrations of both G-CSF and IL-17. Furthermore, neutrophil depletion (using the GR-1 antibody) in LFA-1 deficient mice abrogated the increased resistance to *L. monocytogenes* (78). Conversely, antibody blockade of CD11b (Mac-1) results in a reduction of neutrophil recruitment to the site of *L. monocytogenes* infection in the liver and decreased resistance to *L. monocytogenes* infection (59, 79). Collectively, these data suggest that LFA-1 expression restricts neutrophil recruitment to sites of infection leading to increased bacterial burden, while Mac-1, particularly the CD11b component, is essential for neutrophil recruitment and control of *L. monocytogenes* infection.

<u>Indirect regulation of neutrophil chemotaxis</u>

During *L. monocytogenes* infection, IL-23 regulates the production of IL-17A and IL-17F from γδ T cells, resulting in optimal liver neutrophil recruitment and enhanced bacterial clearance presumably due to increased chemokine production. Mice lacking IL-23p19, IL-17A, or IL-17RA have increased bacterial burdens in the liver, which corresponds with decreased neutrophil recruitment (80, 81). One study showed that increased concentrations of CCL8 (MCP-2) led to an influx of IL-17-producing γδ T cells in mice conditionally knocked out for B lymphocyte-induced maturation protein-1 (BLIMP1) in macrophages. This resulted in a subsequent enhancement of neutrophil recruitment to sites of infection and increased clearance of *L. monocytogenes* (82). These studies demonstrate the importance of chemokine regulation in effective neutrophil recruitment and ultimately, clearance of bacteria.

Other cytokines that have been suggested to be important during *L. monocytogenes* infection, likely through indirect effects on neutrophils, include IL-1 and IL-6. IL-1α and IL-1β are produced in the liver and spleen after *L. monocytogenes* infection and exogenous IL-1α has been shown to increase neutrophil recruitment to sites of infection and decrease bacterial burden (83-86). Blocking the type 1 IL-1 receptor (IL-1R), which binds both IL-1α and IL-1β, leads to increased susceptibility to *L. monocytogenes* (87). Interestingly, IL-1β deficient mice show no difference in susceptibility to *L. monocytogenes*, suggesting a more central role for IL-1α (88). Studies performing IL-1 depletion in SCID mice show increased susceptibility to *L. monocytogenes* compared to untreated SCID mice, suggesting the mechanism of IL-1 protection is not mediated by T cells (89). However, further studies are required to determine how IL-1α mediates its protective effects, particularly in relation to neutrophils, during *L. monocytogenes* infection.

Mice deficient for IL-6 are more susceptible to *L. monocytogenes* infection with increased bacterial burden in both the spleen and liver and deficient neutrophil recruitment into the blood compared to wild-type mice (90, 91). Addition of rIL-6 to wild-type mice was able to provide enhanced protection against *L. monocytogenes*. Use of the anti-GR-1 antibody for depletion, though not specific for neutrophils, eliminated the IL-6 induced protective effect, suggesting IL-6 can directly or indirectly enhance recruitment, and possibly function, of neutrophils (90). Interestingly, in IL-6 KO mice no differences were observed in NK cell or macrophage activation and there was no difference in IFN-γ production during *L. monocytogenes* infection, further highlighting the potential link between IL-6 and neutrophils (90). Although it has been shown that classical IL-6 signaling, rather than IL-6 trans-signaling, is required for protection against *L. monocytogenes*, the cells responsible for producing and responding to IL-6 during infection are not known (92).

Extracellular superoxide dismutase (ecSOD) is the enzyme responsible for regulating extracellular concentrations of ROS and protecting host tissues during inflammation. Our lab has shown that ecSOD activity results in increased neutrophil recruitment to the liver during *L. monocytogenes* infection, possibly facilitated by the enzyme's ability to protect the extracellular matrix from degradation leading to enhanced neutrophil trafficking. However, the increased number of neutrophils did not correlate with protection during infection, as mice with high ecSOD activity are more susceptible to *L. monocytogenes* than mice with wild-type ecSOD activity or mice deficient in ecSOD. Furthermore, in mice with high ecSOD activity, neutrophils did not effectively co-localize with bacterial lesions in the liver, suggesting not only chemotactic, but potentially functional defects (2). Ultimately, rapid recruitment out of the bone marrow and

efficient chemotaxis to sites of infection are essential preludes to neutrophil function and clearance of *L. monocytogenes* infection.

Neutrophil function during *L. monocytogenes* infection

Neutrophil phagocytosis and containment of bacteria

Upon recruitment to foci of infection, particularly within the liver, neutrophils recognize and phagocytose *L. monocytogenes*. Specific receptors, including the complement receptor of the immunoglobulin superfamily (CRIg), have been shown to be required for macrophage phagocytosis of *L. monocytogenes* (93); however, the receptors and ligands that induce phagocytosis by neutrophils are currently unknown. The majority of bacteria recovered from the liver immediately following high-dose intravenous infection with *L. monocytogenes* are presumed to be extracellular and many are associated with hepatocytes. However, the rapid influx of neutrophils to the liver during the first 6 hours post-infection leads to a significant reduction in the bacterial burden. In addition, depletion using the anti-GR-1 antibody prior to infection, though not specific for neutrophils, led to markedly increased hepatocyte damage and increased bacterial burden (50). Collectively, these data suggest that early phagocytosis of *L. monocytogenes* by incoming neutrophils is essential for protection of the liver against infection.

Neutrophil phagocytosis of bacteria may not necessarily result in bacterial killing but may instead limit the spread of *L. monocytogenes*. The bacteria-filled neutrophils could then be phagocytosed by macrophages, ultimately killing the bacteria. One study demonstrated the presence of *L. monocytogenes* inside liver neutrophils that were located inside Kupffer cells (59). Furthermore, the Mac-1 receptor on neutrophils can bind to ICAM-1 (CD54) on the surface of macrophages and potentially facilitate phagocytosis of infected neutrophils by Kupffer cells. It

has been shown that inhibition of either CD11b (Mac-1) or ICAM-1 resulted in a reduced clearance of *L. monocytogenes* in the liver, although this could be related to altered recruitment in addition to blockade of neutrophil-macrophage interactions (59). Therefore, one could speculate that the contribution of neutrophils to protection against *L. monocytogenes* infection is purely related to bacterial containment.

Neutrophils produce matrix metalloproteinase-8 (MMP8) and store it in granules until bacterial sensing induces degranulation at sites of phagocytosis allowing MMP8 to be taken into the phagosome with *L. monocytogenes* where it was shown to degrade LLO. It was further proposed that this leads to bacterial containment by preventing *L. monocytogenes* escape from the phagosome. These *in vitro* studies showed that inhibition of neutrophil degranulation led to increased cell damage and inhibition of proteases led to decreased LLO degradation and increased intracellular neutrophil bacterial burden, supporting a potential role for MMP8 and other granule contents in protecting the host against *L. monocytogenes* infection (94).

Neutrophil production of ROS

Direct sensing of *L. monocytogenes* by murine neutrophils is thought to be mediated primarily by formylated peptides binding to the receptor FPR1 on neutrophils, which induces a signaling cascade leading to calcium efflux and subsequent superoxide ($O_2^{\cdot -}$) and hydrogen peroxide (H_2O_2) production (69). Lack of FPR1 results in increased bacterial burden and decreased production of $O_2^{\cdot -}$ and H_2O_2 by neutrophils (70). Regulation of calcium entry is an important factor in neutrophil function, including production of ROS, and is mediated by molecules such as stromal-interacting molecule 1 (STIM1). Mice deficient in STIM1 have decreased production of ROS and increased susceptibility to *L. monocytogenes* infection (95). Mice deficient for 4-1BB (CD137), a member of the TNF receptor superfamily constitutively

expressed by neutrophils, are more susceptible to *L. monocytogenes* infection than wild-type mice, which correlates with defective calcium mobilization and decreased ROS production from neutrophils (96). Furthermore, pre-treatment of wild-type mice with a 4-1BB agonist antibody led to decreased bacteria burden and increased neutrophil ROS production suggesting a role for TNF receptors in activation of signaling pathways leading to production of ROS during *L. monocytogenes* infection (97).

Neutrophil activation during *L. monocytogenes* infection induces production of $O_2^{\cdot -}$ and H_2O_2, both of which are anti-microbial and thought to be important for bacterial killing. The NADPH oxidase complex assembles on the phagosome in neutrophils and converts molecular oxygen (O_2) into $O_2^{\cdot -}$ (98). The NADPH oxidase complex is comprised of 6 subunits: $gp91^{phox}$ and $p22^{phox}$ are membrane-bound components while $p47^{phox}$, $p67^{phox}$, and $p40^{phox}$ are all cytosolic components that assemble with the membrane-bound portion, and either Rac1 or Rac2 GTPases, upon activation of the cell (99). Mice that lack the essential $gp91^{phox}$ component of NADPH oxidase are more susceptible to *L. monocytogenes* during the early stages of infection (100-102). Interestingly, mice deficient in the $p47^{phox}$ component have equivalent bacterial burden to wild-type mice during *L. monocytogenes* infection (103). Lack of the $p47^{phox}$ subunit may be compensated for by high concentrations of the $p67^{phox}$ subunit, leading to efficient $O_2^{\cdot -}$ production; however, this has not been thoroughly investigated *in vivo* (104). In addition to ROS production by the NADPH oxidase complex, it has been shown that mitochondria-generated ROS is important for phagocyte-mediated bacterial killing, although *L. monocytogenes* was not used in this study (105). Furthermore, efficient phagosome localization with the mitochondria, mediated by the Mst1 and Mst2 kinases, is required for optimal induction of ROS downstream of

TLR signaling, and mice lacking both Mst1 and Mst2 show increased susceptibility to *L. monocytogenes* compared to wild-type mice (106).

While ROS are potent bactericidal molecules, they can also cause host tissue damage and must therefore be properly regulated. The negative regulator of ROS (NRROS) is a recently described protein important for preventing tissue damage to host organs by limiting phagocytic production of ROS. Increased ROS production, increased resistance to *L. monocytogenes* infection, and increased tissue damage, were all observed in mice deficient in NRROS (107). The $O_2^{\cdot -}$ generated by NADPH oxidase is converted into H_2O_2 by superoxide dismutases (SODs). In the extracellular milieu, ecSOD catalyzes the conversion of $O_2^{\cdot -}$ to H_2O_2 to protect the host from excessive tissue damage. Our lab has previously shown that during *L. monocytogenes* infection, mice with high ecSOD activity have increased bacterial burden and neutrophil apoptosis as well as impaired neutrophil-specific production of TNF-α, compared to ecSOD wild-type or ecSOD KO mice. Depletion of neutrophils in mice with high ecSOD activity slightly decreases bacterial burden while neutrophil depletion in ecSOD wild-type or ecSOD KO mice results in increased bacterial burden, suggesting ecSOD activity leads to impaired neutrophil function (2). Though $O_2^{\cdot -}$ and H_2O_2 are produced by activated neutrophils, hypochlorous acid (HOCl) is believed to be a more potent bactericidal ROS molecule (108, 109). Production of HOCl is catalyzed by myeloperoxidase (MPO) in the presence of H_2O_2 and chloride ions. Neutrophil-specific MPO activity against *L. monocytogenes* has not been determined, though it has been implicated as being important for neutrophil anti-microbial activity against other bacterial pathogens (110).

In addition to ROS, neutrophils can generate reactive nitrogen species (RNS) through the expression of inducible nitric oxide synthase (iNOS or NOS2), an enzyme that converts O_2 to

nitric oxide ($NO^{\cdot}$). NOS2 deficient mice were found to be more susceptible to *L. monocytogenes* infection than wild-type mice (101, 111). Conversely, a more recent study showed that pharmacologic inhibition of NOS2 resulted in decreased bacteria burden in the liver after infection with *L. monocytogenes* (112). Why different approaches to eliminating NOS2 function resulted in different outcomes, and importantly, whether or not NOS2 is required for neutrophil killing *in vivo* during *L. monocytogenes* infection still needs to be resolved. Peroxynitrite ($NO_3^{\cdot-}$), a ROS molecule produced by a reaction between $O_2^{\cdot-}$ and $NO^{\cdot}$, is also thought to have very potent bactericidal activities, including the ability to kill *L. monocytogenes in vitro* (113). However, these activities have not yet been identified as neutrophil specific, nor have they been shown to be required for killing *in vivo*.

Neutrophil production of cytokines

Neutrophil activation induced by *L. monocytogenes* infection also results in the production of several cytokines that have been deemed important for resistance to bacterial infection. Generally, recognition of microbial products by pattern recognition receptors (PRRs) initiates signaling pathways through adaptor molecules leading to activation of the NF-κB transcription factor and ultimately, production of cytokines.

It has been shown that mice lacking MyD88 (a TLR adaptor protein) are very susceptible to *L. monocytogenes* infection, have reduced production of IL-6, IL-12, IL-18, IFN-γ and TNF-α and decreased neutrophil recruitment to the spleen (61, 114). Mice with MyD88 expression exclusive to dendritic cells responded comparably to wild-type mice during infection with *L. monocytogenes* (115). These data suggest that MyD88 is required in dendritic cells for optimal

responses to *L. monocytogenes* but neutrophil recruitment and function are independent of MyD88 signaling.

While TLRs are responsible for recognizing a wide array of extracellular or vesicular pathogens, Nod-like receptors (NLRs) are positioned in the cytosol in order to recognize PAMPs expressed by pathogens that escape the phagosome. Mice deficient in NOD1 have increased bacterial burden during *L. monocytogenes* infection which correlates with a decrease in neutrophil recruitment. However, it was determined that NOD1 signaling was essential in non-hematopoietic, but not hematopoietic cells, during infection (116). Furthermore, neutrophils from RIP2 KO mice, which cannot signal through NOD1 or NOD2, did not display altered production of IL-6, TNF-α, CXCL1 or CXCL2 in response to *L. monocytogenes* infection, as compared to neutrophils from wild-type mice (117). This suggests that NLR signaling may be a redundant, rather than essential, pathway to induce neutrophil cytokine production during infection with *L. monocytogenes*. One member of the NLR family, NLRP6, has been suggested to be a negative regulator of inflammatory responses during infection with *L. monocytogenes*. Mice deficient in NLRP6 show increased survival and decreased bacterial burden following *L. monocytogenes* infection. In addition, NLRP6-deficient mice exhibit increased IL-6 and CXCL1 concentrations in circulation and in the peritoneum correlating with increased recruitment of GR-1+ cells to sites of infection (118).

A recent study identified the Sox2 transcription factor acting in the cytosol of neutrophils as a sensor of bacterial DNA. Upon recognition of bacterial DNA, such as that from *L. monocytogenes*, Sox2 initiates a signaling cascade ultimately resulting in production of pro-inflammatory cytokines, including TNF-α and IL-6. In addition, mice with phagocyte-specific Sox2 deficiency exhibited increased susceptibility to *L. monocytogenes* infection and since Sox2

is not expressed in macrophages, this indicates that Sox2 is a novel and essential sensor of *L. monocytogenes* in neutrophils (119). Mice deficient in Toso, an Fc receptor for IgM with previously unknown function, predominantly displayed decreased production of TNF-α, IL-6, and IL-12, as well as decreased phagocytic ability by granulocytes and a concurrent increase in bacterial burden following *L. monocytogenes* infection (120). These recent studies highlight novel bacterial sensors and signaling pathways in neutrophil activation during *L. monocytogenes* infection.

Deficiency in IFN-γ or the IFN-γ receptor renders mice highly susceptible to infection with *L. monocytogenes* (61, 65, 121, 122). Multiple subsets of lymphocytes can produce IFN-γ during *L. monocytogenes* infection (123), and our studies have shown that antigen-independent responses of memory CD8+ T cells are superior to NK cells at providing protection when transferred into IFN-γ deficient hosts (124). Interestingly, one study observed neutrophil-specific production of IFN-γ during *L. monocytogenes* infection and showed that transferring neutrophils from wild-type mice into IFN-γ KO mice increased bacterial clearance (125), suggesting that multiple cells have the capacity to provide IFN-γ mediated protection against *L. monocytogenes*. Mice deficient in TNF-α, a pro-inflammatory cytokine produced by immune cells, or its receptor TNFR1, are highly susceptible to *L. monocytogenes* infection (126-129). Additionally, mice conditionally knocked out for phagocyte-specific TNF-α display extreme susceptibility to *L. monocytogenes* infection characterized by increased bacterial burden in the spleen and liver and decreased host survival (130). Furthermore, depletion of neutrophils during *L. monocytogenes* infection decreases the amount of TNF-α produced, correlating with increased bacterial burden (1). Decreased neutrophil activation due to deficiency of FPR1 or 4-1BB, also resulted in a decrease in TNF-α production (69, 97). Although the precise role of TNF-α produced by

neutrophils during *L. monocytogenes* infection is not known, it is possible that hepatocytes are lysed by the actions of neutrophil specific TNF-α production. A previous study has shown that neutrophil depletion with the GR-1 antibody led to increased liver damage assessed by increased AST concentrations in serum (59). Additional studies using microscopy have suggested that depletion of neutrophils with the GR-1 antibody results in decreased hepatocyte death at 24 hours post-infection (46). Furthermore, TNF-α has been shown to directly induce hepatocyte lysis (131, 132). Initially, neutrophil phagocytosis of *L. monocytogenes* could limit hepatocyte infection and at later time points neutrophil TNF-α production could induce hepatocyte death, thus reducing the cell-to-cell spread of *L. monocytogenes*."

Factors that influence neutrophil recruitment and function during *L. monocytogenes* infection are summarized in Figure 1.

ecSOD, Neutrophils and L. monocytogenes

Our laboratory has published data showing that ecSOD activity leads to an increase in the recruitment of neutrophils to the murine liver in both the absence and the presence of *L. monocytogenes* infection. Surprisingly, increased percentages of neutrophils in ecSOD HI mice did not translate to increased protection against *L. monocytogenes* infection. Conversely, ecSOD activity actually results in increased host susceptibility to infection and increased bacterial burden in the spleen and liver at day 3 post-infection (d3p.i.). In addition, ecSOD activity was determined to result in increased apoptosis of liver neutrophils, decreased co-localization with bacteria, decreased TNF-α and an overall decrease in peroxynitrite in the liver. Ultimately, it was shown that depletion of neutrophils in the ecSOD HI mice led to a decrease in host susceptibility

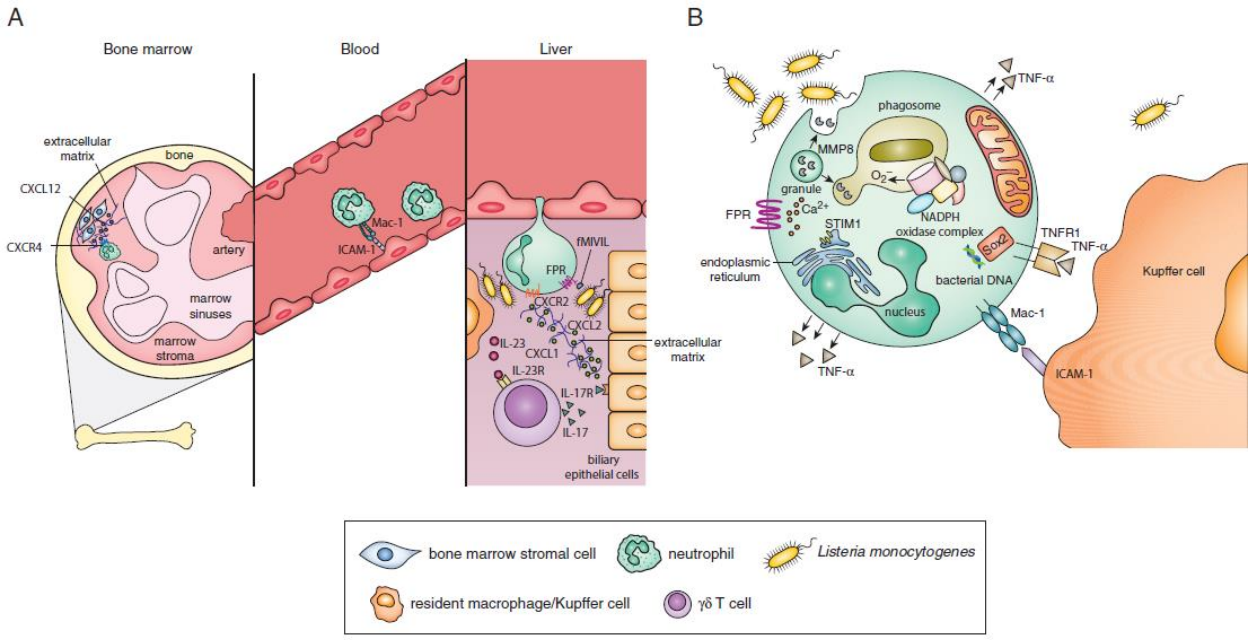
to *L. monocytogenes* infection indicating ecSOD activity inhibits the ability of neutrophils to protect against infection (2).

Significance

Although there have been great strides in understanding neutrophil biology in the last decade, there is still much we do not understand about factors that influence neutrophil development and function. This research proposes to investigate a novel relationship between a common host antioxidant enzyme, ecSOD, and a prominent immune cell type, neutrophils. Importantly, this research suggests that ecSOD activity leads to the recruitment of phenotypically and functionally immature neutrophils out of the bone marrow. In addition to the contribution to basic immunology and neutrophil biology that this research will contribute, it also suggests that inhibition of ecSOD, and potentially other antioxidants, could lead to short-term benefits against severe acute infections where neutrophils play essential roles. This research could also provide some cautions to researchers and clinicians who propose to use antioxidant treatments in diseases mediated by reactive oxygen species (7, 133, 134), which might simultaneously be severely inhibiting host immune responses.

Figure 1. Neutrophil recruitment and function during *L. monocytogenes* infection.

(A) Upon *L. monocytogenes* infection, neutrophils are rapidly released out of the bone marrow, where they travel through the circulation and into sites of infection, especially the liver. Direct interactions via chemokine/chemokine receptors, adhesion molecules, and FPRs, as well as indirect effects of cytokines/cytokine receptors are involved. (B) Neutrophil function upon recognition of bacteria or bacterial products includes phagocytosis, killing via ROS/RNS, and production of pro-inflammatory cytokines.



CHAPTER II

MATERIALS AND METHODS

Mice

ecSOD KO mice were originally provided by Dr. Cheryl L. Fattman and were backcrossed to C57Bl/6 mice at least eight times (University of Pittsburg). ecSOD HI and ecSOD WT mice were generated as previously described (135) with ecSOD HI mice expressing the ecSOD allele found in 129P3/J backcrossed to C57Bl/6 mice at least eight times, and ecSOD WT mice expressing the "wild-type" ecSOD allele (found in multiple mouse strains, including C57Bl/6 mice) backcrossed to C57Bl/6 mice for at least eight generations. B6-Ly5.1/Cr mice (expressing the CD45.1 allele), used for bone marrow chimera studies, were purchased from Charles River Frederick Animal Facility. Gender and age-matched (7-12 week) mice were used for all experiments. All mice were provided food and water *ad libitum*, except under conditions for bone marrow chimeras described below, and were housed in sterile microisolator cages with sterile bedding at the University of North Texas Health Science Center AAALAC accredited animal facility. All animal studies were performed in compliance with the U.S. Department of Health and Human Services Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee at the University of North Texas Health Science Center.

L. monocytogenes infections

L. monocytogenes 10403s serotype 1 was grown on brain-heart infusion (BHI) agar plates (BD Bacto, Sparks, MD) and virulence was maintained by routine passage through C57Bl/6 mice. *L. monocytogenes*-GFP is on the 10403S background and expresses GFP off the NF8 plasmid (a generous gift from Dr. Laurel Lenz at University of Colorado Denver) and it is selectively maintained on BHI plates with 15µg/mL erythromycin. For infections, *L. monocytogenes* was cultured overnight in BHI broth to reach log phase, washed twice with 1x phosphate buffered saline (PBS), and diluted to the appropriate concentration in sterile 1x PBS. The route of infection was intravenous for all *in vivo* experiments and a dose of ~10^4 was used for all experiments. Bacterial burden was determined by homogenizing organs, resuspending in desired volume of sterile double-distilled water (ddH_2O), serially diluting, and plating 50µL on BHI agar plates. Plates were incubated for 24 hours at 37˚C and then colony-forming units (CFUs) were counted. For *in vitro* infections, *L. monocytogenes*-GFP was added at a multiplicity of infection (MOI) of 20:1 and cultured for 1 hour.

Organ processing and tissue culture

To isolate peripheral blood leukocytes, blood was collected from the lateral tail vein into Hanks' Balanced Salt Solution (HBSS) containing 2% FCS (Atlanta Biologicals, Norcross, GA) and heparin. Samples were then centrifuged at 1500 x g for 6 minutes and treated with Tris-Ammonium Chloride (TAC; pH 7.2) to lyse red blood cells (RBCs). Splenocytes were isolated by grinding whole spleens between two sterile glass slides. Livers were homogenized with Dounce homogenizers, then a discontinuous Percoll gradient was used to isolate liver leukocytes. Liver homogenates were resuspended in a 35% Percoll solution, then layered over 67.5% Percoll solution. Following centrifugation at 600 x g for 20 minutes, cells were harvested from the interface. RBC lysis of splenoctyes and liver leukocytes was performed with TAC. Bone marrow

cells were isolated by flushing femurs and tibiae with HBSS + 2% FBS + 2mM EDTA through a 70µm nylon cell strainer (BD Falcon). Red blood cells were lysed using a 0.2% solution of NaCl in ddH_2O followed by quenching with 1.6% NaCl in ddH_2O after 20 seconds (adapted from (136)). Bone marrow cells were incubated in Dulbecco's Modified Eagle Medium (DMEM) + 10% FCS, L-glutamine, vitamins, and penicillin/streptomycin (Invitrogen-Gibco, Carlsbad, CA) overnight at 37˚C with 5% CO_2 in the presence or absence of HKLM (MOI 50:1). Supernatants were collected and stored at -20˚C until needed.

In vitro* infection with *L. monocytogenes

Bone marrow cells were incubated 24 well tissue culture plates in DMEM + 10% FCS, L-glutamine, vitamins, and penicillin/streptomycin (Invitrogen-Gibco, Carlsbad, CA) for one hour at 37˚C with 5% CO_2. Next, *L. monocytogenes*-GFP was added at a MOI of 20:1, plates were centrifuged for 2 minutes at 1200 rpm on a Beckman Coulter Allegra X-12 centrifuge, and incubated for 2 hours followed by addition of gentamicin at 50µg/mL and incubation for another 30 minutes. Finally, cells were washed and transferred to 96 well plates for cell surface staining, as described below.

Flow cytometry based assays

For cell staining, the following antibodies were used from BD Biosciences (San Diego, CA): anti-Ly6G FITC (1A8), anti-CD11b PE-Cy7 (M1/70), anti-CD62L biotin (MEL-14); BioLegend (San Diego, CA): anti-CD16/CD32 (93), anti-Ly6G PE and PE-Cy7 (1A8), anti-CD117 PE (c-kit, 2B8), anti-CXCR2 AF647 (TG11), anti-CXCR4 PE (L276F12), anti-CD45.1 APC (A20), anti-CD45.2 FITC (104); Invitrogen (Grand Island, NY): anti-CD11b PE-TR (M1/70.15)

For cell surface staining, liver leukocytes, splenocytes, peripheral blood leukocytes, or bone marrow cells were incubated with saturating amounts of antibodies, in addition to anti-CD16/32 to block Fc receptor binding, in FACS buffer (1x PBS + 2% FCS + 0.1% sodium azide) for 15 minutes at 4˚C. Cells were fixed with either 1% paraformaldehyde or BD Stabilizing fixative.

To determine ROS production using 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) (Life Technologies), bone marrow cells were incubated with 10μM H_2DCFDA, with or without HKLM stimulation, in DMEM + 10% FCS, L-glutamine, vitamins, and penicillin/streptomycin (Invitrogen-Gibco, Carlsbad, CA) for one hour at 37˚C with 5% CO_2. Following this, cells were washed and stained for cell surface markers.

Neutrophil transfer

Bone marrow cells were harvest from ecSOD HI and ecSOD KO mice, then neutrophils were isolated via Histopaque gradient (Sigma)(136). Host ecSOD WT mice were infected for 1 day with 10^4 *L. monocytogenes* then $3.5x10^6$ neutrophils from either ecSOD HI or ecSOD KO mice were transferred into the host mice. Control mice were infected ecSOD WT mice that received no transferred neutrophils. At d3p.i., spleens and livers were isolated and analyzed for bacterial burden (CFUs).

Granulopoiesis assay

Bone marrow cells were isolated from uninfected ecSOD congenic mice and cultured in duplicate in 6-well tissue culture plates with two wells per plate full of ddH_2O to maintain humidity. Cells were cultured in Methocult M3231 methylcellulose media (StemCell Technologies, Vancouver, BC, Canada), with recombinant IL-3 (10 ng/mL), IL-6 (10 ng/mL),

and G-CSF (50 ng/mL) (Biolegend, San Diego, CA), and incubated at 37˚C with 5% CO_2 for 7 days. Colonies were identified via microscopy on day 7 (137, 138).

Quantification of G-CSF and CXCL12

To obtain bone marrow extracellular fluid (ECF), one femur and one tibia from each mouse was flushed with 2mL of 1x PBS (6). These samples were centrifuged at 1500 x g for 6 minutes and supernatant was harvested and stored at -20˚C until needed.

CXCL12 and G-CSF were measured by DuoSet ELISA kits (R&D Systems, Minneapolis, MN).

Bone Marrow Chimera generation

Approximately 18 hours prior to irradiation, food was removed from all host mice. 12 ecSOD HI or ecSOD KO mice and 12 B6-Ly5.1/Cr mice were irradiated at ~1100 Rads from a ^{137}Cs source. These mice were then transferred to recently autoclaved, sterile microisolator cages where they remained without food, and where water was also removed. Bone marrow cells were isolated from 6-8 ecSOD HI or ecSOD KO mice and 6-8 B6-Ly5.1/Cr mice. These cells were processed as described above, resuspended in 1x PBS, then pooled with cells of the same genotype. To reconstitute the host mice, 10^7 donor bone marrow cells were injected i.v. into the lateral tail vein. Food was then returned to these mice as well as antibiotic-containing water (2g/L neomycin sulfate in ddH_2O). Newly generated bone marrow chimeric (BMC) mice were monitored daily, and kept on antibiotic water for 2 weeks then returned to usual housing conditions described above. At 8 weeks post-reconstitution, approximately 100 µL of tail blood was taken from the lateral tail vein of BMC mice and stained for flow cytometry as described

above, using CD45.1 and CD45.2 to determine successful chimerism. BMC mice were then infected with *L. monocytogenes*, as described previously, and used for experiments.

Statistical Analyses

Each experiment was repeated at least one time with 3-6 mice per group unless stated otherwise in the figure legend. One-way and two-way ANOVAs were conducted on the data where appropriate followed by Bonferroni *post hoc* analysis to determine significant differences between groups. An *, **, or ***, indicates that the groups differ at $p < 0.05$, $p < 0.01$, or $p < 0.001$, respectively. Bacterial burden (CFU) data was log transformed prior to analysis, as represented in the figures.

Chapter III

ECSOD ACTIVITY INHIBITS NEUTROPHIL FUNCTION IN THE MURINE LIVER

Previous studies from our laboratory determined that ecSOD activity leads to increased bacterial burden and decreased host survival while also resulting in increased recruitment of neutrophils to the liver. Preliminary characterization of the neutrophils recruited to the liver of ecSOD HI mice determined that these neutrophils did not co-localize with *L. monocytogenes* lesions and were impaired in their ability to produce TNF-α. Furthermore, depletion of neutrophils in ecSOD congenic mice resulted in decreased susceptibility to infection and determined that ecSOD HI liver neutrophils are not protective (2).

Further studies were performed to follow up on the ecSOD-mediated effects on both neutrophil recruitment and function. In order to determine if differences in neutrophil recruitment were due to cell-intrinsic alterations in the neutrophils, transfer studies were performed. Bone marrow cells were isolated from either ecSOD HI or ecSOD KO mice and labeled with a fluorescent dye, either CFSE or eFluor670, to mark their origins. Then an equivalent number of bone marrow cells (with equivalent percentage of neutrophils) were transferred into host ecSOD WT mice that were either left uninfected or had been infected for one day with 10^4 *L. monocytogenes*. Livers were removed at 18 hours post-transfer and stained for neutrophils ($Ly6G^+CD11b^+$) then flow cytometry was performed to determine the percentage of neutrophils in the liver of host mice that were transferred from either ecSOD HI or ecSOD KO mice, based on the fluorescent dye labeling. Percent recovery of transferred neutrophils was determined from the livers of host mice and no differences in recovery were observed in either uninfected or d1p.i. mice. These data suggested that the ecSOD-mediated differences in

neutrophil recruitment were not due to cell-intrinsic differences. This led to further evaluation of potential differences in the environment that could lead to altered recruitment. Since ecSOD is known to protect the ECM from degradation in models of inflammation, concentrations of the ECM component hyaluronic acid (HA) were measured by ELISA. There were higher concentrations of HA in both the liver and bone of ecSOD HI mice compared to ecSOD WT and ecSOD KO mice at both uninfected and d1p.i. time points. This indicates that ecSOD is protecting the ECM from degradation which likely contributes to the increased recruitment of neutrophils in the ecSOD HI mice. Since neutrophil trafficking into tissues is partially mediated by CD44 binding to HA, both neutrophil expression of CD44 and their ability to bind to HA was measured. There were no differences between neutrophils from ecSOD congenic mice in expression of CD44 or their ability to bind to HA either prior to or during infection, further supporting the theory that ecSOD activity mediates neutrophil recruitment by modulating the environment, not by affecting cell-intrinsic changes in the neutrophils.

Since the ECM is also important for chemokine binding to create a chemotactic gradient, concentrations of the common neutrophil-attracting chemokines CXCL1 and CXCL2, as well as the neutrophil attractant and growth factor G-CSF, were measured in the serum and liver of ecSOD congenic mice at d1p.i.. Concentrations of CXCL1, CXCL2 and G-CSF were all higher in the serum of ecSOD HI mice and CXCL1 and CXCL2 concentrations were highest in the ecSOD HI livers. These data suggest that ecSOD activity leads to higher concentrations of neutrophil-attracting chemokines which would contribute to increased neutrophil recruitment to the livers of ecSOD HI mice.

Finally, studies were also performed on the neutrophils from ecSOD congenic to determine their functional ability. Flow cytometry based assays were used to measure the

intracellular and extracellular levels of MPO, oxidative burst generation, phagocytic ability, and neutrophil-specific intracellular bacterial burden. Neutrophils from the livers of ecSOD KO mice displayed the highest expression of both intracellular and extracellular MPO compared to ecSOD HI and ecSOD WT mice, indicating they are more functional. In addition, neutrophils from ecSOD HI mice were significantly impaired in their ability to generate oxidative burst compared to ecSOD KO mice. Furthermore, while liver neutrophils from ecSOD HI were slightly better at phagocytosing pH-sensitive bioparticles, neutrophil-associated bacterial burden was highest in these neutrophils. Overall, these data suggest that while ecSOD HI liver neutrophils have a slight advantage in phagocytosis, there functional ability to eliminate *L. monocytogenes* is impaired, likely due to decreased production of oxidative burst and MPO (submitted manuscript).

ecSOD activity leads to decreased shedding of L-selectin by liver neutrophils

To investigate the phenotypic maturity status of neutrophils in the livers of ecSOD congenic mice, cell surface expression of L-selectin (CD62L) was determined by flow cytometry. Mice were left uninfected or infected with 10^4 *L. monocytogenes* for one day. Livers were harvested, processed, and stained for neutrophils ($Ly6G^+CD11b^+$) and L-selectin followed by flow cytometry analysis. In uninfected mice, nearly all neutrophils (~98%) from the liver of ecSOD congenic mice expressed L-selectin on the cell surface (Figure 2A for representative histograms and 2B). However, in uninfected mice, neutrophils from ecSOD KO mice expressed less L-selectin on a per cell basis (as measured by MFI) compared to the ecSOD HI and ecSOD WT mice (Figure 2D). After infection with *L. monocytogenes* for 1 day, there was no difference in either the percentage of neutrophils expressing L-selectin (Fig 2C) or the relative neutrophil expression of L-selectin (Fig 2E). These data suggest that neutrophils in the liver of ecSOD KO mice are initially more phenotypically mature than those in ecSOD HI or WT mice, but after

infection these neutrophils are activated and shed L-selectin until there are no differences between the congenic mice. Importantly, L-selectin shedding only indicates phenotypic maturity and activation, it does not indicate functional maturity.

ecSOD activity does not permanently alter the ability of neutrophils to protect against L. monocytogenes infection

Despite the phenotypic and functional maturity differences observed in liver neutrophils from the ecSOD congenic mice, it remained unclear whether ecSOD activity resulted in cell-intrinsic differences in the neutrophils' ability to protect against *L. monocytogenes* infection. In addition, the results of the neutrophil transfer that determined ecSOD does not lead to cell-intrinsic differences in neutrophil recruitment may not correlate with ecSOD mediated differences in neutrophil function. Therefore, neutrophil transfer studies, similar to those used to evaluate neutrophil recruitment, were performed. As diagramed in Figure 3A, neutrophils were isolated from the bone marrow of ecSOD HI and ecSOD KO mice via Histopaque gradient and $3.5x10^6$ neutrophils were transferred into ecSOD WT mice that had been infected with 10^4 *L. monocytogenes* for 1 day. At d3p.i., spleens and livers were harvested from control mice – infected ecSOD WT mice that did not receive any transferred neutrophils – and from ecSOD WT mice that received either ecSOD HI or ecSOD KO neutrophils. No differences in bacterial burden were observed in the spleen, however, this result is not surprising since previous observations have determined neutrophils are more important for protecting against *L. monocytogenes* infection in the liver than the spleen (1). Bacterial burden was decreased in the livers of ecSOD WT mice that had received transferred neutrophils, from either ecSOD HI or ecSOD KO mice, compared to the control mice which demonstrated that these transferred neutrophils are able to protect against infection in the liver. Importantly, there was no difference

in the decreased bacterial burden between ecSOD WT mice that received either ecSOD HI or ecSOD KO neutrophils, indicating that these transferred neutrophils are equivalently protective when placed into the same environment (Figure 3B). These data strongly suggest that while ecSOD activity inhibits neutrophil function, this is not a permanent cell-intrinsic defect.

Summary

The results presented in this chapter, in combination with the previously described data, suggest that in the liver, ecSOD activity modulates the environment leading to differences in recruitment and function of neutrophils. ecSOD activity does not cause cell-intrinsic differences in the ability of neutrophils to be recruited, rather ecSOD protects the ECM from degradation and leads to increased production of neutrophil-attracting chemokines. This likely increases the interactions between CD44 on neutrophils and the ECM, as well as binding of chemokines to the ECM and to their receptors on neutrophils, ultimately leading to increased recruitment of neutrophils to the liver in ecSOD HI mice.

Neutrophils recruited to the liver in ecSOD KO mice were determined to be more phenotypically and functionally mature. Neutrophils from the liver of ecSOD KO mice expressed less L-selectin, more MPO, were better able to generate oxidative burst, and had lower neutrophil-associated bacterial burden compared to liver neutrophils from ecSOD WT or ecSOD HI mice. In addition, neutrophil transfer studies showed that ecSOD activity does not lead to cell-intrinsic differences in neutrophil function since ecSOD HI and ecSOD KO neutrophils are equally protective when placed into the same environment. This further suggests that the functional differences in neutrophils between ecSOD congenic mice, like the recruitment differences, are due to environmental effects on the neutrophils.

Figure 2. ecSOD activity leads to decreased shedding of L-selectin by liver neutrophils.

ecSOD congenic mice were left uninfected or infected for 1 day with 10^4 *L. monocytogenes* then livers were harvested and liver leukocytes isolated. Flow cytometry was performed to determine the percentage of neutrophils ($Ly6G^+CD11b^+$) that express L-selectin (CD62L) during uninfected (A for representative histograms, B) and d1p.i. (C) time points. In addition, the expression of L-selectin on a per cell basis was measure by mean fluorescence intensity (MFI) at both uninfected (D) and d1p.i. (E) time points. One-way ANOVAs were performed to determine statistical differences between groups. An * indicates that the groups differ at $p < 0.05$. All data are presented as mean ± SEM (n=3-5/group). These data are representative of at least three independent experiments for each time point.

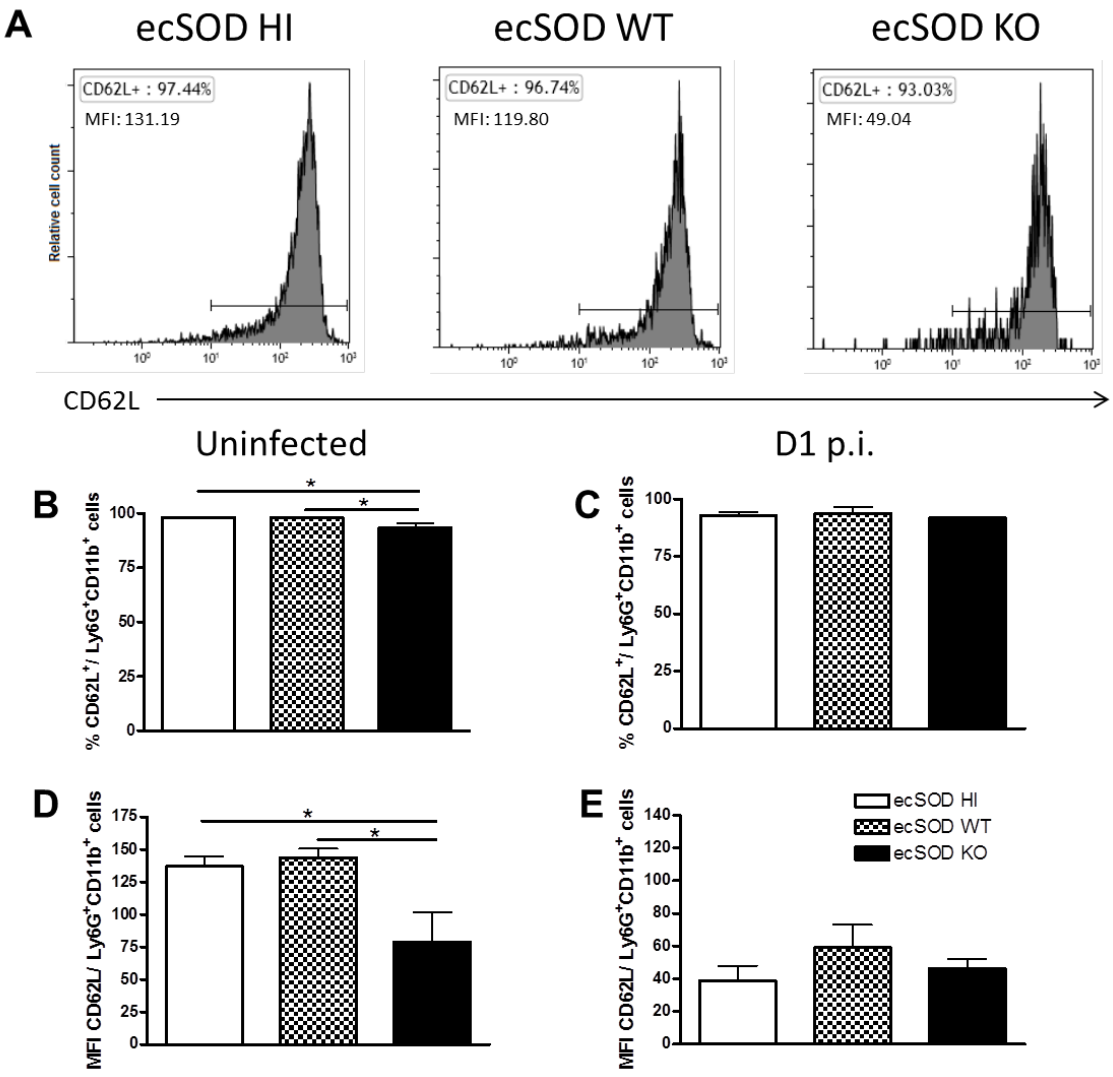
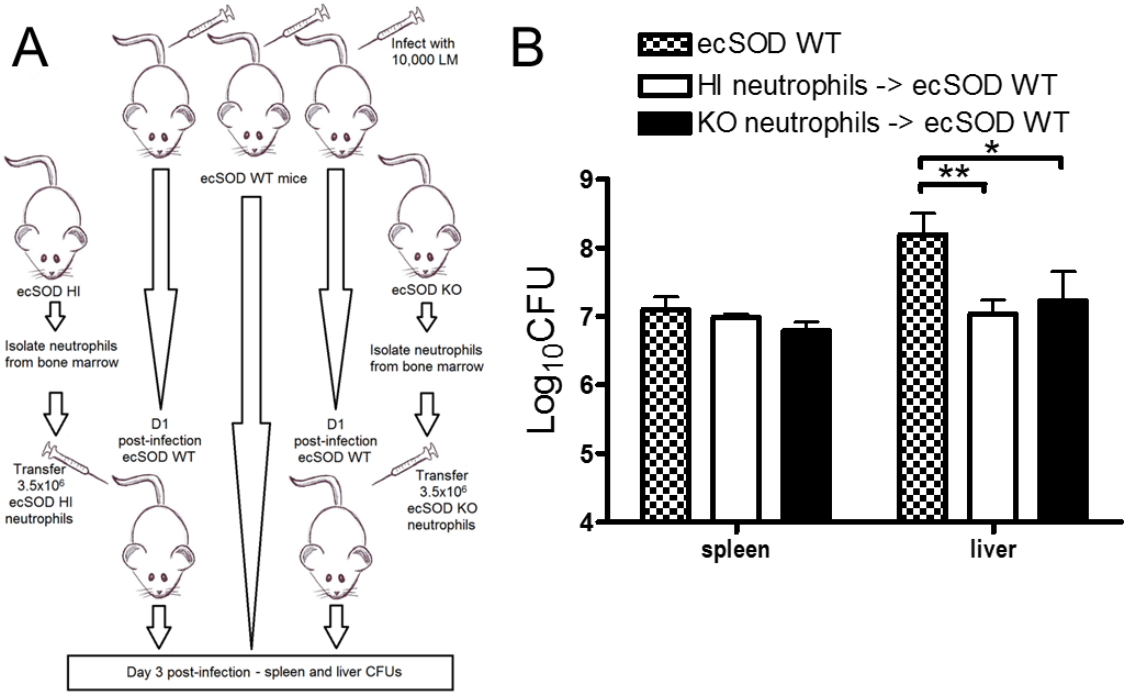


Figure 3. ecSOD activity does not permanently alter the ability of neutrophils to protect against *L. monocytogenes* infection.

(A) Neutrophils were isolated from ecSOD HI or ecSOD KO bone marrow by Histopaque gradient then $3.5x10^6$ neutrophils were transferred into host ecSOD WT mice that were infected for 1 day with 10^4 *L. monocytogenes*. Control mice were infected ecSOD WT mice that received no transferred neutrophils. (B) At day 3 post-infection spleens and livers were isolated and analyzed for bacterial burden (CFUs). Two-way ANOVA was performed to determine statistical differences between groups. An *, or **, indicates that the groups differ at $p < 0.05$, or $p < 0.01$, respectively. All data are presented as mean ± SEM (n=3-5/group). These data are representative of at least three independent experiments.



Chapter IV

ECSOD PROMOTES EGRESS OF IMMATURE MURINE BONE MARROW NEUTROPHILS

It has been established that functional differences in neutrophils are not permanent, since neutrophils from either ecSOD HI or ecSOD KO mice, placed into the same environment, are equally protective against *L. monocytogenes*. Neutrophils are generated and develop in the bone marrow, and functional defects mediated by ecSOD activity's effects on the tissue environment may also indicate differences in neutrophil function or development in the bone marrow.

ecSOD activity leads to increased immature neutrophils in the bone marrow and blood

Granulocyte precursors express high levels of c-kit, and downregulate it as they mature into neutrophils. Evaluation of c-kit expression on bone marrow neutrophils from ecSOD congenic mice by flow cytometry determined that ecSOD HI mice have higher percentage of c-kit$^+$ neutrophils than ecSOD KO mice at both uninfected (Figure 4A for representative histograms and 4B) and d1p.i. (Figure 4D) time points. In addition, neutrophils from ecSOD HI mice express more c-kit both in the absence of infection (Figure 4C) and at d1p.i. (Figure 4E) as compared to ecSOD KO mice, while neutrophils from ecSOD HI and ecSOD WT mice have comparable expression of c-kit. This indicates that there are more immature neutrophils in the bone marrow of ecSOD HI and ecSOD WT mice both prior to, and during, infection as compared to ecSOD KO mice. This finding was corroborated by utilizing a flow cytometry gating strategy for evaluating mature and immature neutrophils by Ly6G and CD11b expression (137). Developing neutrophils upregulate their expression of Ly6G and mature neutrophils

express higher levels of CD11b as they prepare to exit the bone marrow and traffic through the blood (3). Therefore, mature neutrophils in the bone marrow can be gated as $Ly6G^{hi}CD11b^{hi}$ while immature neutrophils can be gated as $Ly6G^{hi}CD11b^{int}$ (Figure 5A)(137). This gating strategy further indicates that there are more immature neutrophils, and hence fewer mature neutrophils, in uninfected ecSOD HI mice as compared to ecSOD KO mice (Figure 5B). In addition, while there were no differences in immature neutrophils at d1p.i., there were more mature neutrophils in the ecSOD KO mice (Figure 5C). Furthermore, while this gating strategy cannot be used to identify immature versus mature neutrophils outside the bone marrow, analysis of CD11b expression on neutrophils from the blood indicated that ecSOD HI neutrophils express less CD11b than ecSOD KO neutrophils both at uninfected (Figure 5D) and d1p.i. (Figure 5E) time points. This finding suggests that there are more immature neutrophils in the blood and bone marrow of ecSOD HI mice. This supports the previously described data indicating there are more immature neutrophils in the livers of ecSOD HI mice (as shown by L-selectin expression in Figure 2).

ecSOD activity leads to decreased production of G-CSF in the bone marrow

Differences in neutrophil maturity could indicate ecSOD mediated defects in neutrophil development in the bone marrow. In addition, the neutrophil growth factor G-CSF, which was decreased in the serum of ecSOD HI mice (submitted manuscript), is important for proper neutrophil maturation. To evaluate concentrations of G-CSF, bone marrow cells were isolated from uninfected or d1p.i. ecSOD congenic mice and cultured overnight with or without HKLM. Supernatants were collected and an ELISA was performed to measure G-CSF concentrations. There were no differences in G-CSF concentrations from unstimulated cells either from uninfected or d1p.i. mice. Cells from ecSOD HI mice cultured with HKLM produced less G-CSF

than ecSOD WT or ecSOD KO mouse bone marrow cells (Figure 6). These data suggest that decreased G-CSF concentrations in the bone marrow of ecSOD HI mice may result in inhibited neutrophil development leading to decreased neutrophil maturity in the blood, bone marrow and liver.

ecSOD activity does not alter potential for granulopoiesis

Since G-CSF is an essential growth factor for neutrophils in the bone marrow, differences in G-CSF concentrations could also indicate differences in neutrophil generation, or granulopoiesis. Flow cytometry analysis of the neutrophil progenitor populations in the bone marrow ($Ly6G^{lo}CD11b^{+}$)(Figure 7A) showed no difference in percentages between the ecSOD congenic mice at either uninfected (Figure 7B) or d1p.i. (Figure 7C) time points. In addition, a granulopoiesis assay was performed by isolating bone marrow cells from ecSOD congenic mice and culturing them for 7 days with appropriate cytokines and growth factors at equivalent concentrations, then evaluating the number of granulocytic colonies based on morphology. There were no differences between the ecSOD congenic mice in number of colonies recorded from the granulopoiesis assay (Figure 7D). These data suggest that there are no defects in neutrophil granulopoiesis in the ecSOD congenic mice.

ecSOD activity does not alter chemokine receptor expression on bone marrow neutrophils

The high percentage of immature neutrophils and low concentrations of G-CSF observed in the ecSOD HI mice may indicate that there is decreased neutrophil retention in these mice. In addition, the previously described high concentrations of CXCL1 and CXCL2 may contribute to increased recruitment out of the bone marrow in the ecSOD HI mice. The expression of chemokine receptors CXCR4 and CXCR2 on bone marrow neutrophils from the ecSOD

congenic mice was determined by flow cytometry. There were no differences in the percentages of neutrophils in the bone marrow expressing either CXCR4 or CXCR2 at either uninfected or d1p.i. time points (Figure 8). Furthermore, there were no differences in relative expression of CXCR4 or CXCR2 on neutrophils in the bone marrow from either uninfected or d1p.i. mice (MFI data not shown). These data suggest that differences in release or recruitment out of the bone marrow are mediated by differences in chemokine concentrations not receptor expression.

ecSOD activity leads to decreased CXCL12 in the bone marrow

While there were no differences in expression of CXCR4 or CXCR2 on neutrophils, differences in neutrophil maturity in the bone marrow and the blood suggest that concentrations of the chemokine ligand for CXCR4 may be altered. Therefore, concentrations of CXCL12 were measured from supernatants of flushed bone marrow from uninfected ecSOD congenic mice. There were higher concentrations of CXCL12 in the bone marrow of ecSOD KO mice compared to ecSOD HI and ecSOD WT mice (Figure 9). This suggests that chemokine signaling responsible for retaining neutrophils in the bone marrow until they are mature is decreased in the ecSOD HI mice, which supports the data indicating more immature neutrophils in bone marrow and blood of these mice.

ecSOD activity decreases oxidative burst and phagocytosis by bone marrow neutrophils

To determine if the decreased neutrophil maturity in the bone marrow of ecSOD HI mice correlated with decreased function, flow cytometry based assays were used to measure oxidative burst and phagocytosis. H_2DCFDA, an indicator of oxidative burst, was used to measure the ability of bone marrow neutrophils from the ecSOD congenic mice to generate ROS. Fewer bone marrow neutrophils from uninfected ecSOD HI mice were able to generate ROS after stimulation

with HKLM than neutrophils from ecSOD WT or ecSOD KO mice (Figure 10A). In addition, ecSOD HI neutrophils were less able to generate oxidative burst in response to HKLM (Figure 10B). In order to evaluate the phagocytic ability of bone marrow neutrophils from the ecSOD congenic mice, neutrophils were isolated and infected *in vitro* with *L. monocytogenes*-GFP then flow cytometry was used to determine the percentage of GFP^+ neutropihls. There were fewer GFP^+ neutrophils from the ecSOD HI mice compared to the ecSOD WT and ecSOD KO mice, suggesting these neutrophils may be less able to efficiently phagocytose bacteria (Figure 9). Taken together, these findings indicate that ecSOD activity leads to functionally impaired neutrophils in the bone marrow.

Summary

The effects of ecSOD on neutrophil recruitment and function were first observed in the liver, however, a role for ecSOD activity in the bone marrow, where neutrophils are generated and develop, was necessary to expand our understanding of the relationship between ecSOD and neutrophils. The data presented here show that ecSOD activity leads to decreased concentrations of the neutrophil retention factor CXCL12, although it does not affect neutrophil expression of chemokine receptors CXCR4 or CXCR2. This decreased retention correlates with high percentages of immature neutrophils in the bone marrow and blood of ecSOD HI mice and these neutrophils are less able to generate oxidative burst or phagocytose *L. monocytogenes*. In addition, observed differences in G-CSF concentration indicate a potential role for this growth factor in mediating effects on neutrophil recruitment and function in response to ecSOD activity, however, these effects do not include decreased potential for granulopoiesis. Ultimately, it appears that ecSOD activity in the bone marrow leads to decreased retention and early release of phenotypically and functionally immature neutrophils.

Figure 4. ecSOD activity leads to increased immature neutrophils in the bone marrow.

ecSOD congenic mice were left uninfected (A for representative histograms, B and C) or infected for 1 day with 10^4 *L. monocytogenes* (D and E) and flow cytometry was performed to determine the percentage of bone marrow neutrophils (Ly6G+CD11b+) that expressed c-kit (B and D) as well the level of c-kit expression on neutrophils (C and E). One-way ANOVAs were performed to determine statistical differences between groups. An *** indicates that the groups differ at $p < 0.001$. All data are presented as mean ± SEM (n=3-5/group). These data are representative of at least two independent experiments.

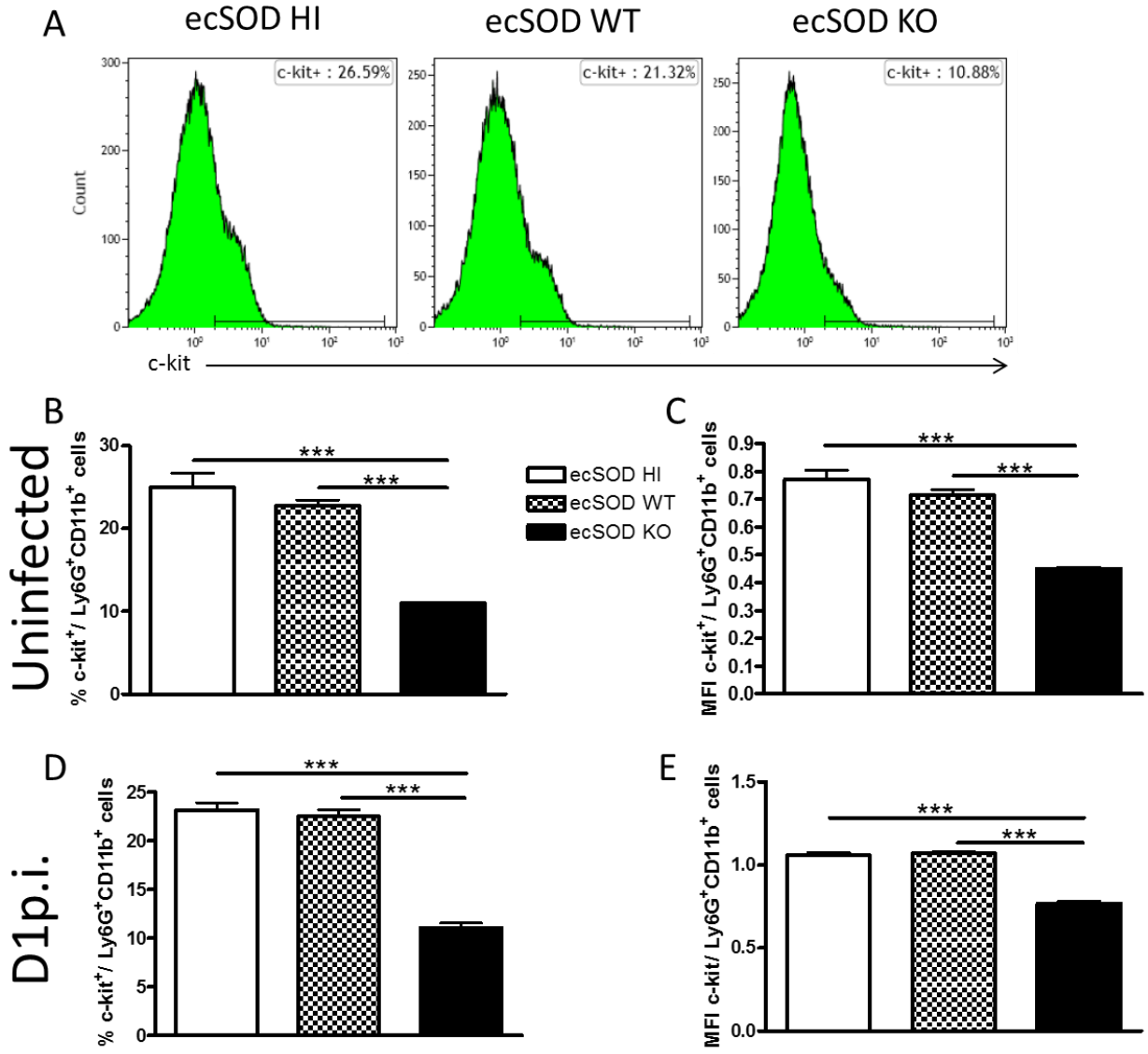


Figure 5. ecSOD activity leads to increased immature neutrophils in the bone marrow and blood.

ecSOD congenic mice were left uninfected or infected for 1 day with 10^4 *L. monocytogenes*. Flow cytometry analysis was performed to determine the percentage of mature neutrophils ($Ly6G^{hi}CD11b^{hi}$) and immature neutrophils ($Ly6G^{hi}CD11b^{int}$) in the bone marrow at uninfected (A for representative histograms, B) and d1p.i. (C) time points. Relative expression of CD11b was measured on peripheral blood neutrophils at uninfected (D) and d1p.i. (E) time points. One-way ANOVAs were performed to determine statistical differences between groups. An *, **, or ***, indicates that the groups differ at $p < 0.05$, $p < 0.01$, or $p < 0.001$, respectively. All data are presented as mean ± SEM (n=3-5/group). These data are representative of at least three independent experiments.

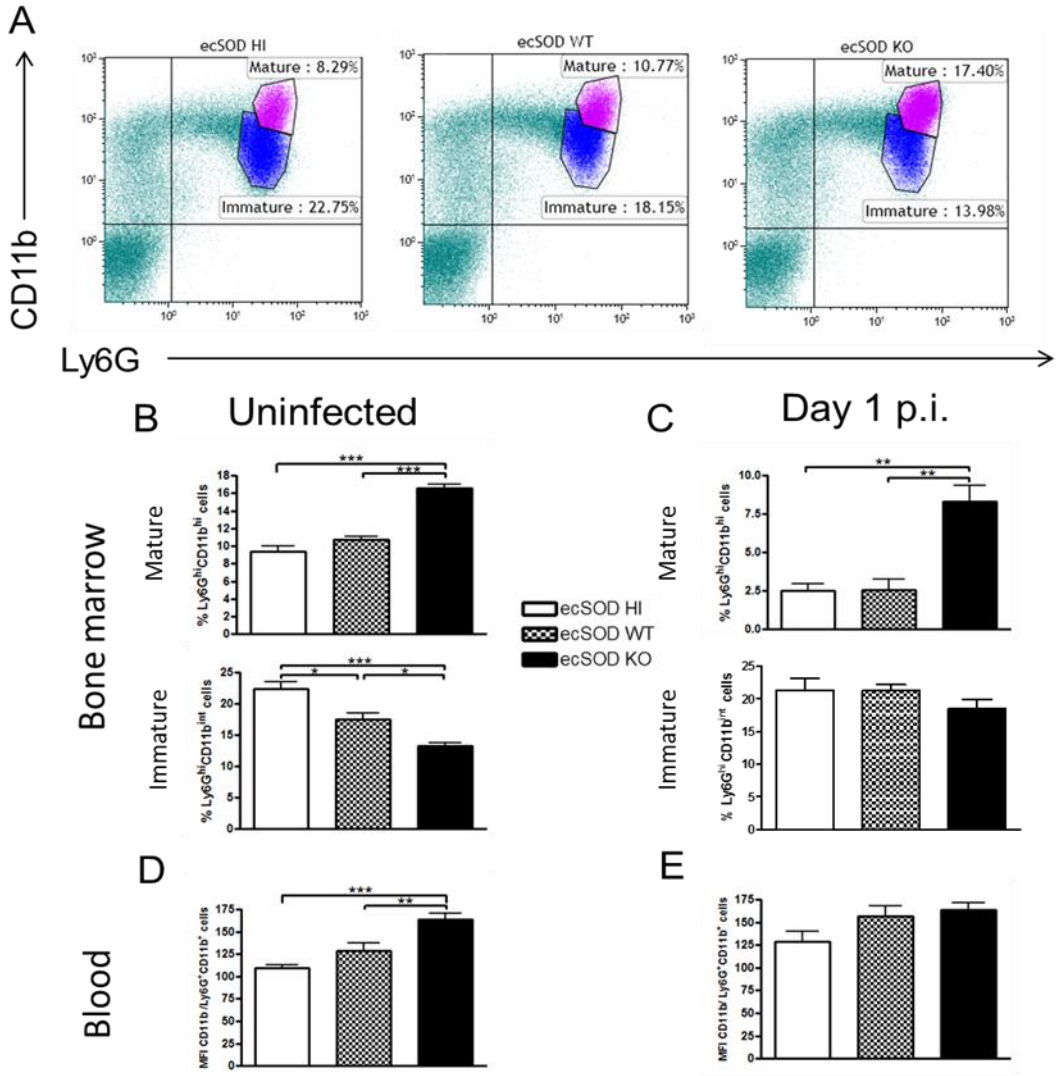


Figure 6. ecSOD activity leads to decreased production of G-CSF in the bone marrow.

ecSOD congenic mice were left uninfected (A) or infected for 1 day (B) with 10^4 *L. monocytogenes*. Bone marrow cells were isolated and cultured overnight in the presence and absence of HKLM stimulation. Supernatants were harvested and G-CSF concentrations were measured by ELISA. Two-way ANOVAs were performed to determine statistical differences between groups. An *, **, or ***, indicates that the groups differ at $p < 0.05$, $p < 0.01$, or $p < 0.001$, respectively. All data are presented as mean ± SEM (n=3-5/group). These data are representative of one independent experiment for each time point.

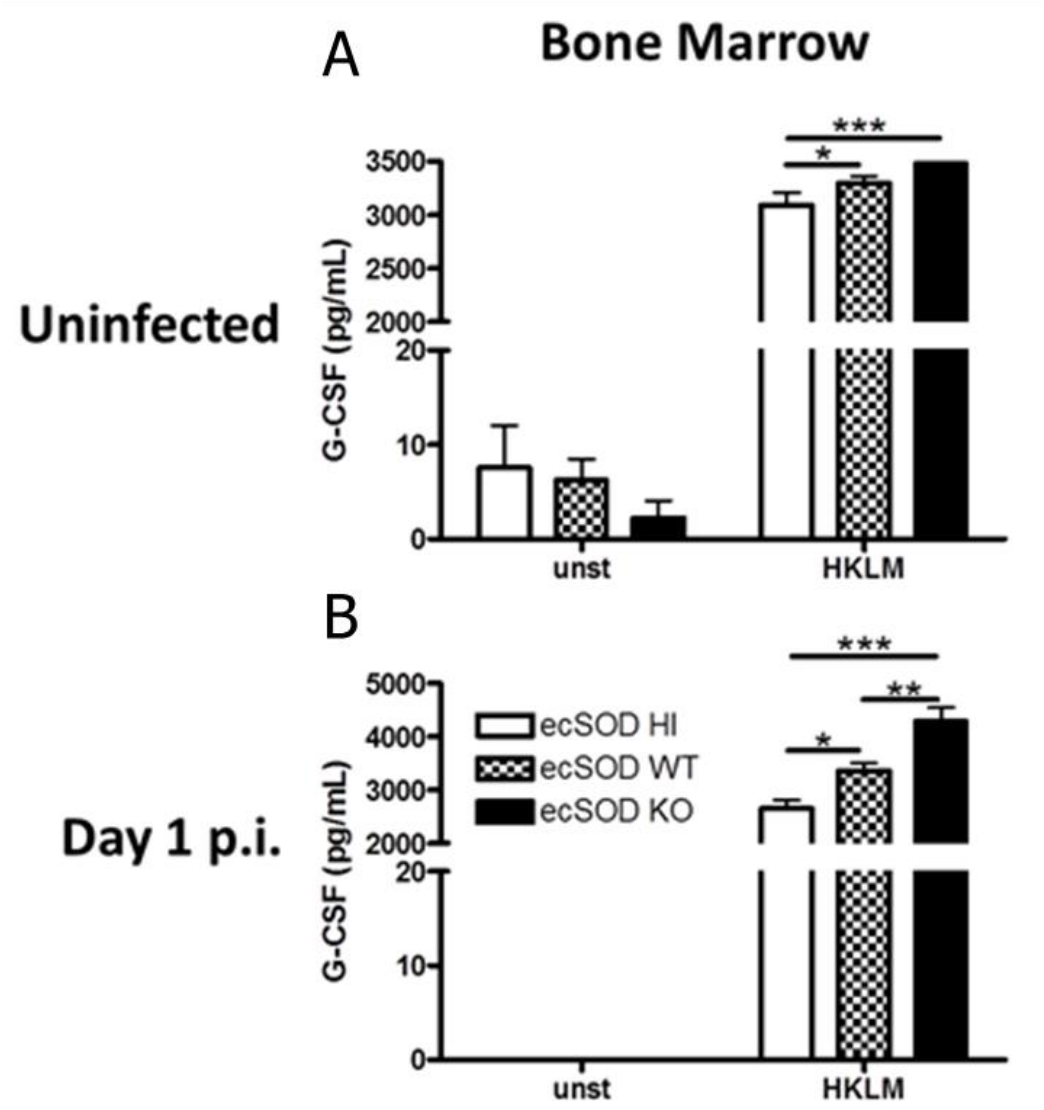


Figure 7. ecSOD activity does not alter potential for granulopoiesis.

ecSOD congenic mice were left uninfected or infected for 1 day with 10^4 *L. monocytogenes*. Flow cytometry analysis was performed to determine the percentage of neutrophil progenitors ($Ly6G^{lo}CD11b^{+}$) in the bone marrow at uninfected (A for representative histograms, B) and d1p.i. (C) time points. (D) Bone marrow cells were isolated from uninfected ecSOD congenic mice and cultured in duplicate in Methocult media, with IL-3, IL-6 and G-CSF, for 7 days. Colonies were identified via microscopy on day 7. One-way ANOVAs were unable to detect any differences between groups. All data are presented as mean ± SEM (n=2-5/group). These data are representative of three independent experiments.

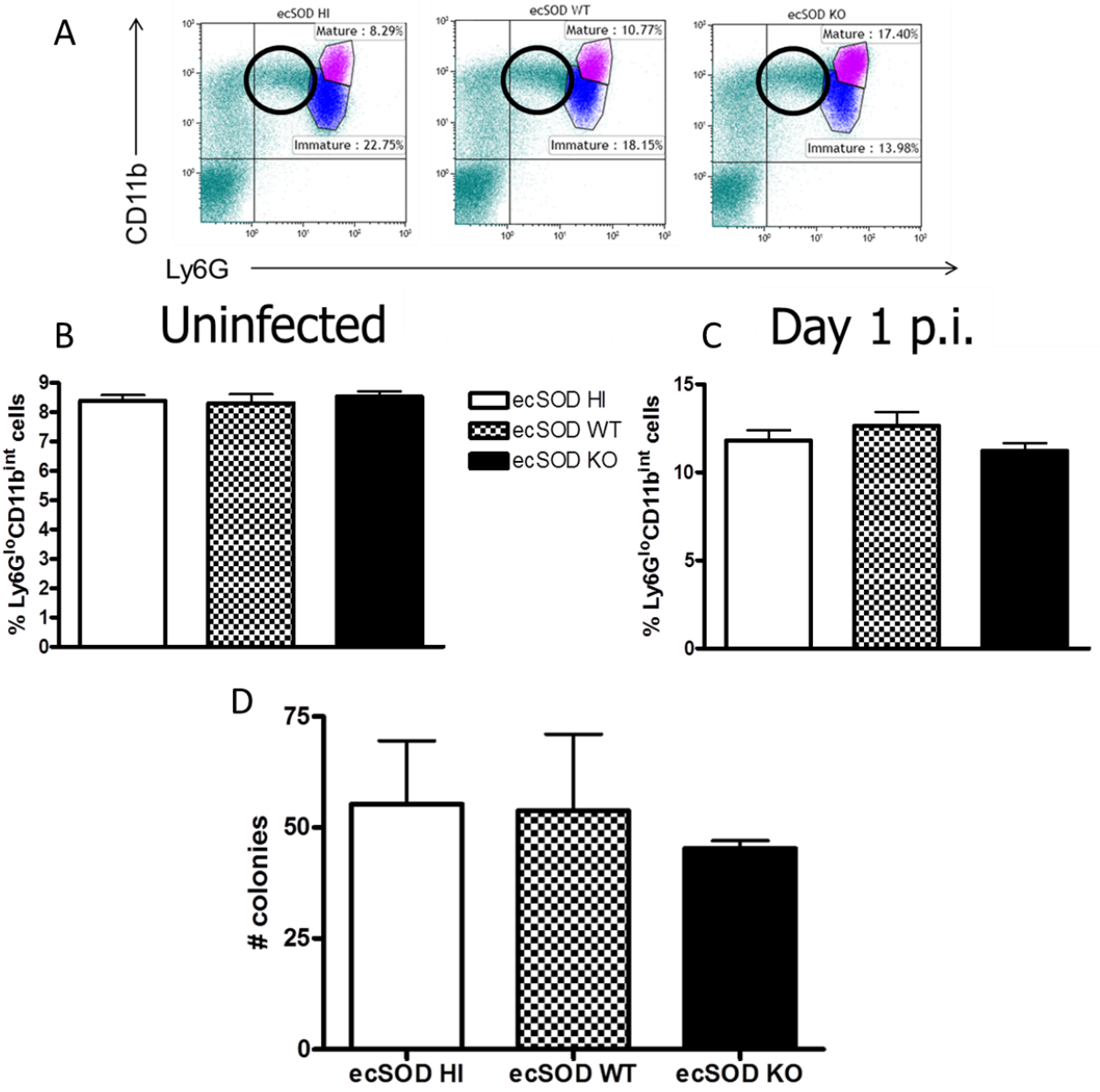


Figure 8. ecSOD activity does not alter chemokine receptor expression on bone marrow neutrophils.

ecSOD congenic mice were left uninfected (A and B) or infected for 1 day (C and D) with 10^4 *L. monocytogenes*. Flow cytometry was performed to determine the percentage of neutrophils ($Ly6G^+CD11b^+$) that expressed CXCR4 (A and C) or CXCR2 (B and D). One-way ANOVAs were unable to determine statistical differences between groups. All data are presented as mean ± SEM (n=3-5/group). These data are representative of at least three independent experiments for each time point.

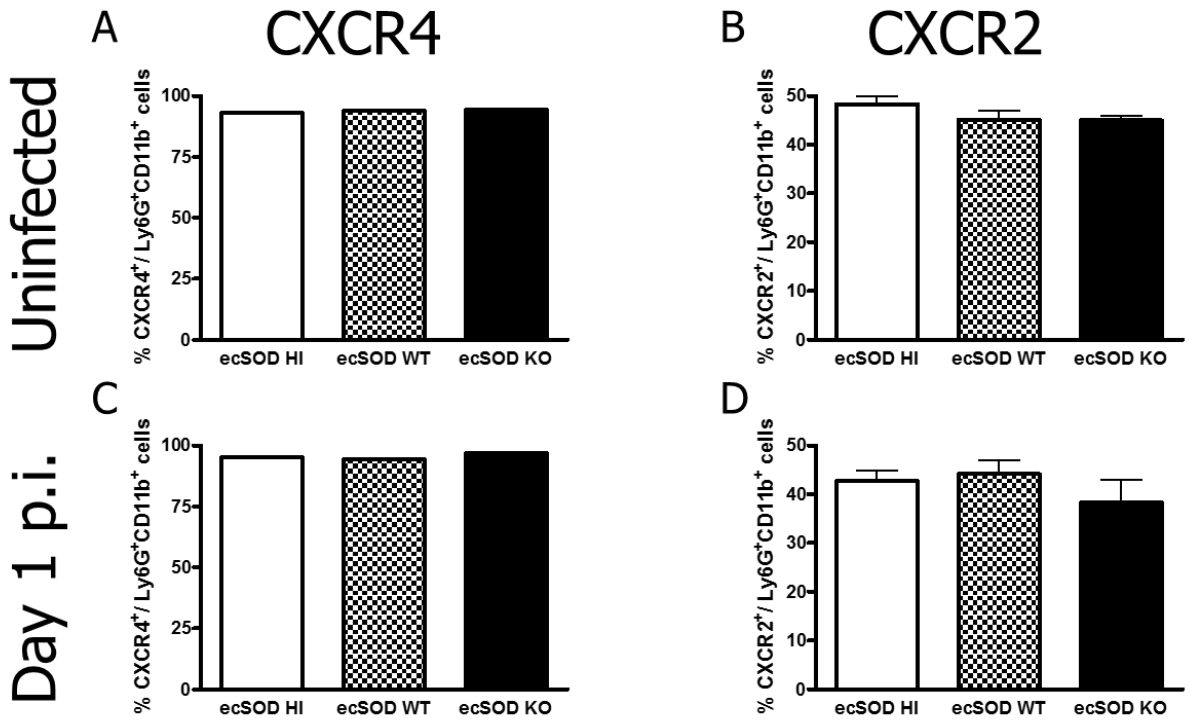


Figure 9. ecSOD activity leads to decreased CXCL12 in the bone marrow.

Bone marrow extracellular fluid (ECF) was flushed from uninfected ecSOD congenic mice. Concentrations of CXCL12 were measured from the supernatants of ECF by ELISA. One-way ANOVAs were unable to determine statistical differences between groups. All data are presented as mean ± SEM (n=3-5/group). These data are representative of one independent experiment.

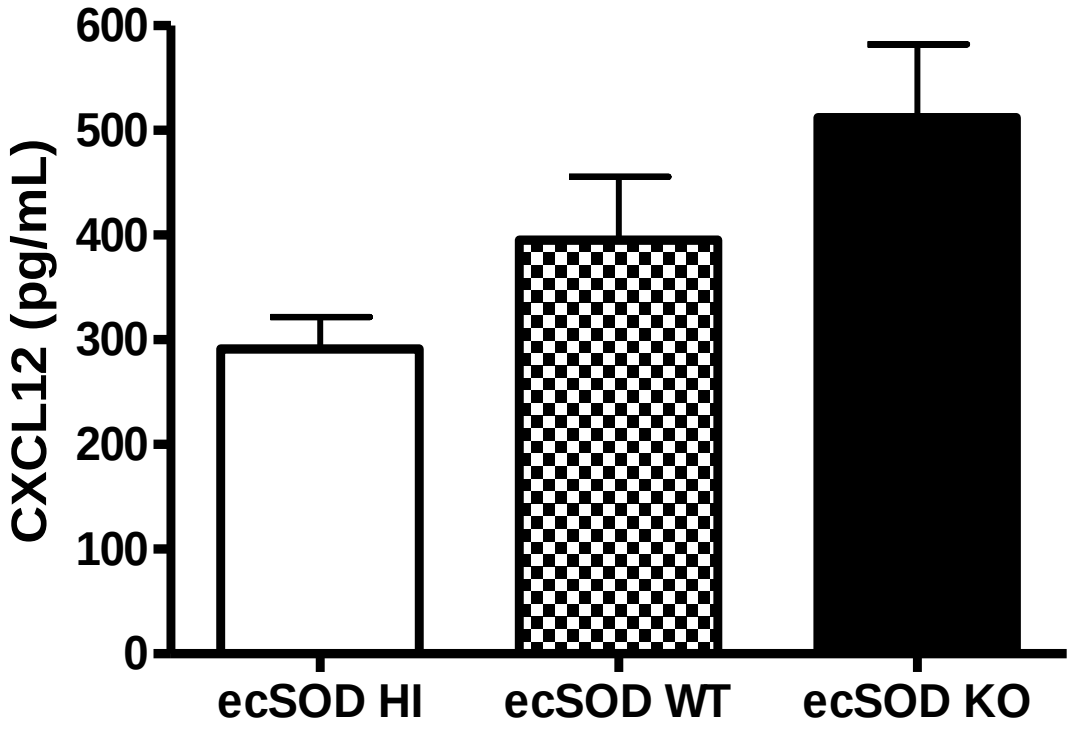


Figure 10. ecSOD activity decreases oxidative burst by bone marrow neutrophils.

Bone marrow cells were isolated from uninfected ecSOD congenic mice and cultured with 10μM H_2DCFDA in the presence or absence of HKLM stimulation. Flow cytometry analysis determined the percentage of neutrophils ($Ly6G^+CD11b^+$) that generated ROS (A) and their relative ROS generation (B). Two-way ANOVAs were performed to determine statistical differences between groups. An *** indicates that the groups differ at $p < 0.001$. All data are presented as mean ± SEM (n=3-5/group). These data are representative of two independent experiments.

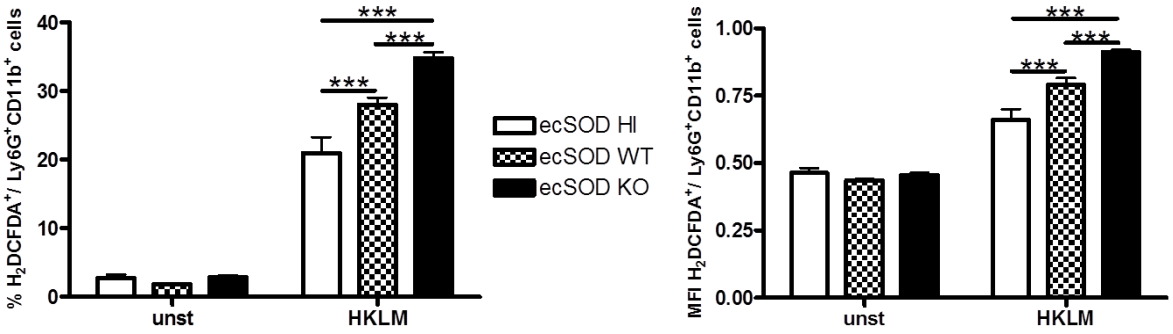
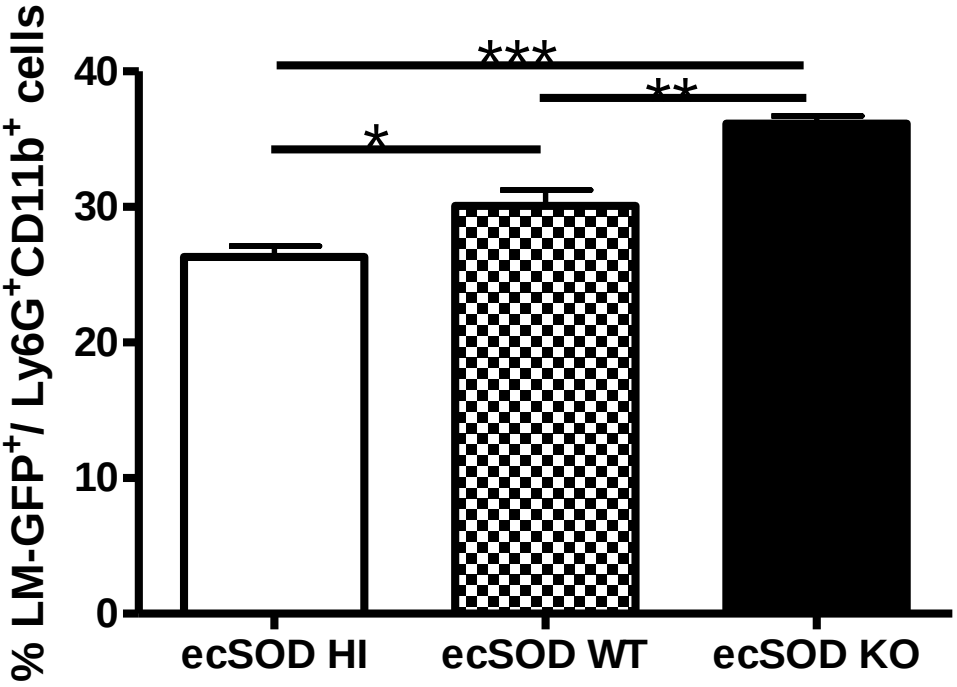


Figure 11. ecSOD activity decreases phagocytosis by bone marrow neutrophils.

Bone marrow cells were isolated from uninfected ecSOD congenic mice and incubated with *L. monocytogenes*-GFP (MOI 20:1). Flow cytometry was performed to determine what percentage of neutrophils ($Ly6G^+CD11b^+$) were associated with *L. monocytogenes*-GFP. One-way ANOVA was performed to determine statistical differences between groups. An *, **, or ***, indicates that the groups differ at $p < 0.05$, $p < 0.01$, or $p < 0.001$, respectively. All data are presented as mean ± SEM (n=3-5/group). These data are representative of two independent experiments.



Chapter V

ECSOD PRODUCED BY BOTH HEMATOPOIETIC AND NON-HEMATOPOIETIC CELLS CONTRIBUTE TO DIFFERENCES IN NEUTROPHIL RECRUITMENT AND FUNCTION

Since ecSOD can be produced by multiple cell types throughout the body, it was important to determine if there were differing contributions for ecSOD produced by hematopoietic versus non-hematopoietic cells in the altered neutrophil recruitment and function observed in ecSOD congenic mice. Previous data demonstrating that ecSOD activity impacts the tissue environment but does not permanently affect neutrophil recruitment and function, suggest that ecSOD production from different cell lineages may vary in their contribution to generating the neutrophil phenotype in ecSOD congenic mice. In order to investigate the effects of enhanced ecSOD activity (ecSOD HI) or lack of ecSOD activity (ecSOD KO) from hematopoietic or non-hematopoietic cells, bone marrow chimeric (BMC) mice were generated.

Absence of ecSOD in either hematopoietic or non-hematopoietic cells leads to increased L. monocytogenes clearance and decreased neutrophil recruitment

BMC mice were generated by lethally irradiating a host mouse and reconstituting with bone marrow cells from a donor mouse. After eight weeks, successful bone marrow engraftment was determined by harvesting blood, staining and performing flow cytometry to analyze the congenic marker CD45. Once chimerism was determined, mice were infected with *L. monocytogenes*. BMC mice were generated using ecSOD KO and C57/Bl6 mice (wild-type ecSOD activity) in groups as shown in Figure 12A, and infected for 3 days prior to harvest. While there were no significant differences in bacterial burden between the four groups of BMC mice, the control groups did follow the trend established with the ecSOD congenic mice with

WT -> WT mice having higher bacterial burden than ecSOD KO -> ecSOD KO mice in both the spleen and liver. In addition, the trend indicates that when ecSOD activity is knocked out from either hematopoietic or non-hematopoietic cell lineages (ecSOD KO -> WT, WT -> ecSOD KO), bacterial burden in the liver is decreased as compared to WT -> WT control mice (Fig 12B). Neutrophil percentages also followed the expected trend seen in the ecSOD congenic mice with WT -> WT mice showing a higher percentage of neutrophils in the liver than ecSOD KO -> ecSOD KO mice. Furthermore, the trend indicates that when ecSOD activity is knocked out from either hematopoietic or non-hematopoietic cell lineages, neutrophil recruitment to the liver is decreased (Fig 12C). This suggests that ecSOD produced from both cell lineages is important for maintaining both increased neutrophil recruitment and impaired neutrophil function observed in ecSOD WT and ecSOD KO mice.

Enhanced ecSOD activity reveals divergent roles for ecSOD produced by either hematopoietic or non-hematopoietic cells

Bone marrow chimera mice were generated using ecSOD HI and C57/Bl6 mice (wild-type ecSOD activity) in groups as shown in Figure 13A, and infected for 3 days prior to harvest. Although there were no differences in bacterial burden between the four groups of mice, the control mice did follow the trend established in the ecSOD congenic mice. The HI -> HI mice had higher bacterial burden than the WT -> WT mice. Mice with high activity ecSOD produced by the hematopoietic cells (HI -> HI and HI -> WT) displayed higher bacterial burden than mice with wild-type activity ecSOD (WT -> WT and WT -> HI) (Fig 13B). This suggests that enhanced ecSOD activity in immune cells leads to decreased clearance of *L. monocytogenes*.

The trend from ecSOD congenic mice regarding neutrophil recruitment was also observed in the control bone marrow chimeras with HI -> HI mice having higher percentages of

neutrophils in the liver compared to WT -> WT mice. However, mice with high activity ecSOD produced by the non-hematopoietic cells (HI -> HI and WT -> HI) displayed higher neutrophil recruitment than mice with wild-type activity ecSOD from non-hematopoietic cells (WT -> WT and HI -> WT) (Fig 13B). This suggests that enhanced ecSOD activity from somatic cells leads to increased neutrophil recruitment to the liver.

Summary

The data presented here suggest that absence of ecSOD activity from either hematopoietic cells or non-hematopoietic cells is sufficient to decrease bacterial burden and decrease neutrophil recruitment to the liver. However, enhanced ecSOD activity, such as that observed in ecSOD HI mice, indicates there may be duality in the role of ecSOD produced by each cell lineage. When only hematopoietic derived cells produce ecSOD with high activity, the mice display increased susceptibility to *L. monocytogenes* infection. However, when only non-hematopoietic cells produce ecSOD with high activity, the increase in neutrophil recruitment is enhanced. Ultimately these data suggest that at d3p.i., ecSOD activity is required from both hematopoietic and non-hematopoietic cells in order to maintain the increased neutrophil recruitment, but this also leads to increased bacterial burden.

Figure 12. Absence of ecSOD in either hematopoietic or non-hematopoietic cells leads to increased *L. monocytogenes* clearance and decreased neutrophil recruitment.

(A) Bone marrow chimeras were generated by irradiation of host mice and reconstitution with bone marrow of donor mice in order to generate the groups shown. Then bone marrow chimeric mice were infected for 3 days with 10^4 *L. monocytogenes*. (B) Bacterial burden (CFUs) was determined from the spleen and liver of bone marrow chimeras at d3p.i. (C) Flow cytometry was used to determine percentages of neutrophils in the spleen, liver and blood of bone marrow chimeras at d3p.i. Two-way ANOVAs were performed to determine statistical differences between groups. An *, or *** indicates that the groups differ at $p < 0.05$, or $p < 0.001$, respectively. All data are presented as mean ± SEM (n=4-6/group). These data are representative of two independent experiments.

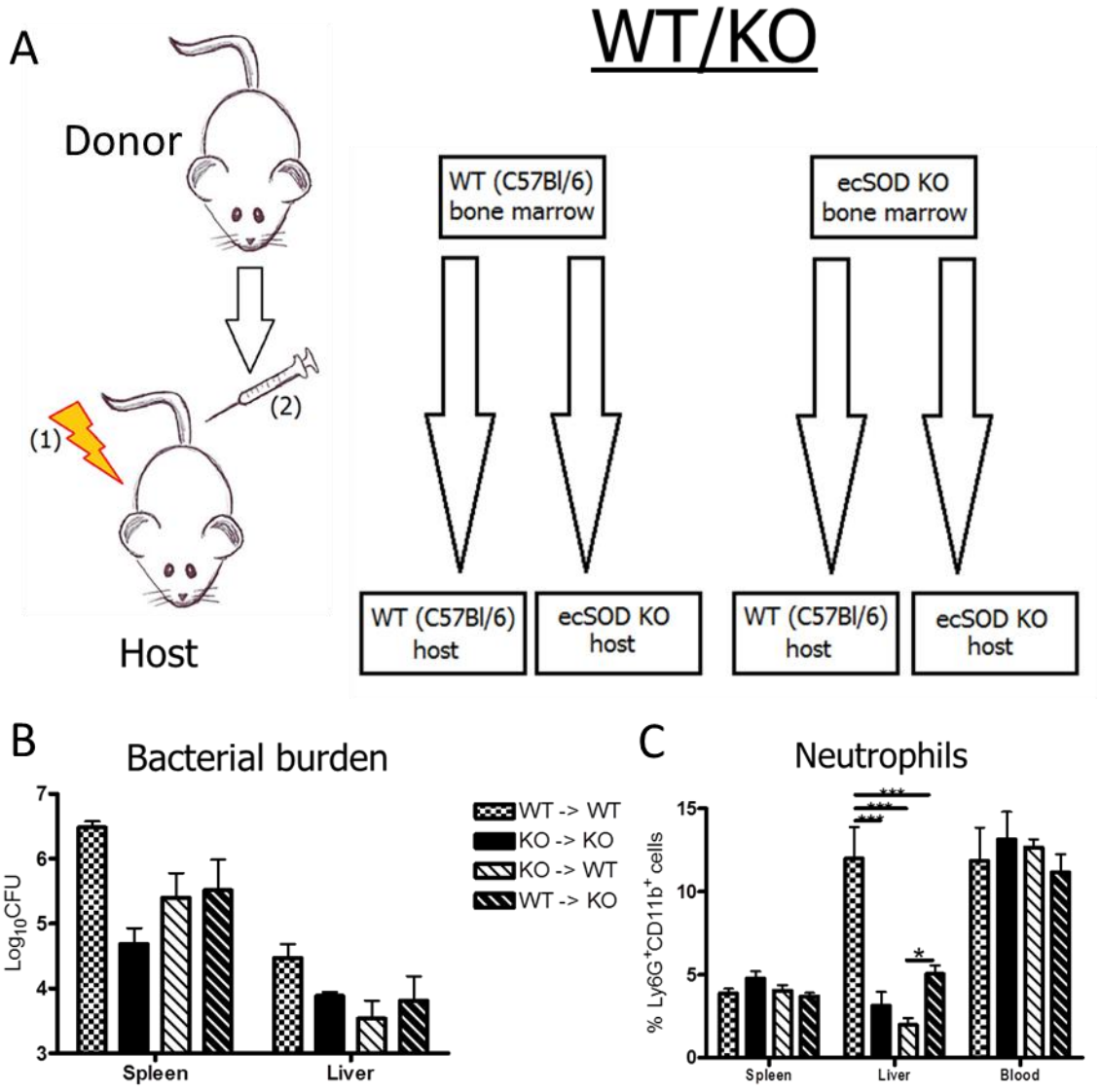
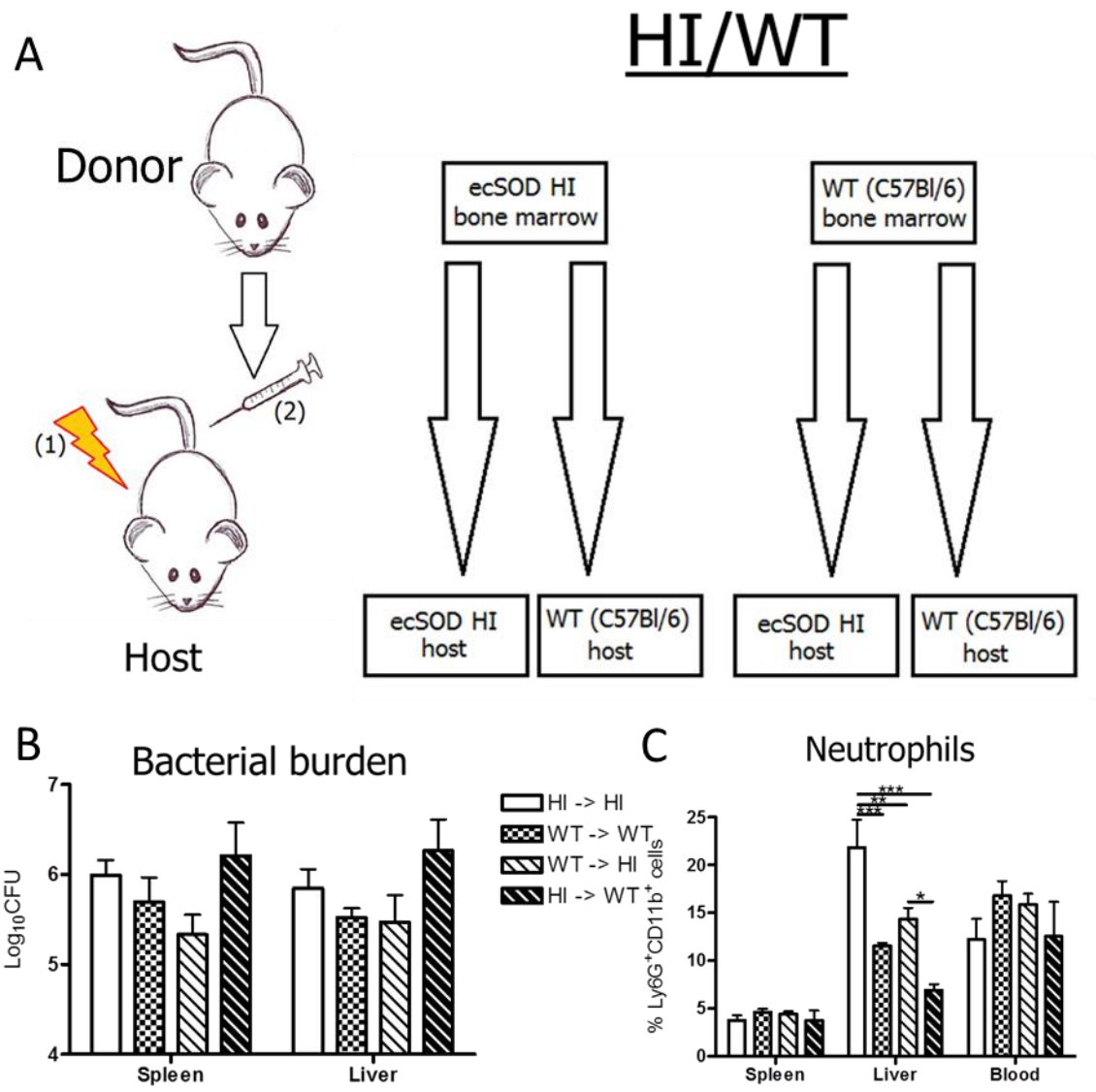


Figure 13. Enhanced ecSOD activity reveals divergent roles for ecSOD produced by either hematopoietic or non-hematopoietic cells.

(A) Bone marrow chimeras were generated by irradiation of host mice and reconstitution with bone marrow of donor mice in order to generate the groups shown. Then bone marrow chimeric mice were infected for 3 days with 10^4 *L. monocytogenes*. (B) Bacterial burden (CFUs) were determined from the spleen and liver of bone marrow chimeras at d3p.i. (C) Flow cytometry was used to determine percentages of neutrophils in the spleen, liver and blood of bone marrow chimeras at d3p.i. Two-way ANOVAs were performed to determine statistical differences between groups. An *, or *** indicates that the groups differ at $p < 0.05$, or $p < 0.001$, respectively. All data are presented as mean ± SEM (n=4-6/group). These data are representative of at least three independent experiments.



Chapter VI

DISCUSSION

Chapter III

Previous studies from our laboratory have established a role for ecSOD activity in the regulation of neutrophil responses. ecSOD activity leads to increased recruitment of neutrophils in both the absence and presence of infection with the intracellular bacterial pathogen *L. monocytogenes*. However, this enhanced neutrophil recruitment does not correlate with protection during infection, rather, mice with high ecSOD activity are more susceptible to *L. monocytogenes* (2). A range of studies were performed to evaluate the phenotypic and functional maturity of neutrophils in mice with different ecSOD activity. Bone marrow transfer studies using fluorescent dye labeled neutrophils determined that ecSOD activity does not lead to cell-intrinsic differences in the ability of neutrophils to be recruited to the liver under basal or infectious conditions (manuscript submitted).

Since differences in neutrophil recruitment in ecSOD congenic mice were not cell-intrinsic, further studies focused on the effects of ecSOD activity on the tissue environment and the subsequent impact on recruitment. ecSOD is responsible for converting superoxide to hydrogen peroxide, which can be rapidly cleared, in order to reduce damage to host tissues (9). In models of lung inflammation, ecSOD has been shown to protect the ECM from degradation (13-15, 32). Importantly, interactions between CD44 present on neutrophils and the HA

component of the ECM mediate efficient neutrophil trafficking into tissues, including the liver (33-35). In addition, chemokines bind to components of the ECM in order to establish concentration gradients important for neutrophil chemotaxis and recruitment (139). Concentrations of HA were observed to be higher in the liver and bone of ecSOD HI mice at both uninfected and d1p.i. time points, indicating that ecSOD is protecting the ECM from degradation in our model. There were no differences in the expression of CD44 on neutrophils or their ability to bind HA either prior to or during infection (submitted manuscript), further suggesting that neutrophils are not altered in their ability to be recruited, rather they are dependent on the quality of the environment. Supporting this theory, concentrations of neutrophil-attracting chemokines CXCL1 and CXLC2 were found to be higher in the serum and livers of ecSOD HI mice (submitted manuscript), which likely augments the effects of increased intact ECM leading to increased neutrophil recruitment.

While the increased ECM and chemokine concentrations provides a reasonable explanation behind the increased recruitment observed in ecSOD HI mice, it does not provide insight into the lack of protection displayed by neutrophils from ecSOD HI mice. Studies of neutrophil function indicated that neutrophils from the livers of ecSOD KO mice have the highest expression of intracellular and extracellular MPO, and while ecSOD HI liver neutrophils are slightly better at phagocytosis, they have impaired oxidative burst generation, and the highest neutrophil-specific intracellular bacterial burden. Overall, these data suggest that liver neutrophils from ecSOD HI mice are functionally impaired resulting in decreased host resistance to *L. monocytogenes*. It is known that ecSOD expression can be downregulated by TNF-α signaling (7, 140), and interestingly, in the ecSOD HI mice, we see decreased production of TNF-α (2), therefore it might be beneficial to explore a feedback loop involving both ecSOD

activity and TNF-α signaling pathways. Interestingly, it has also been shown that TNF-α can induce the down-regulation of CXCR2 on the surface of neutrophils (141), and while there was no difference in CXCR2 expression on bone marrow neutrophils, this could still suggest a complex mechanism involving ecSOD activity leading to reduced TNF-α and increased CXCR2-induced neutrophils chemotaxis.

Degraded HA has been shown to stimulate immune cells through TLR4 (142-144). In our model, it would be predicted that more degraded HA would be generated in the ecSOD KO mice since ecSOD is absent and there is no protection of the tissues against oxidative damage. This could lead to increased activation of neutrophils in the ecSOD KO mice, suggesting a mechanism for their increased function. However, *ex vivo* splenocytes and liver leukocytes from ecSOD WT mice cultured with different molecular weight HA, including HA degraded with hyaluronidase, showed no differences in production of TNF-α or IFN-γ (data not shown). This does not exclude the possibility that degraded HA in combination with other damage-associated molecular patterns (DAMPs) could induce or enhance neutrophil responses. One DAMP that would be interesting to investigate in our model is high-mobility group box 1 (HMGB1) protein, which was previously known to be an important regulator of transcription in the nucleus but has more recently been shown to induce immune responses when released from activated or damaged cells (145). Importantly, the oxidative state of HMGB1 regulates its function which could be particularly relevant in our model since differences in ecSOD activity affect the oxidative environment. HMGB1 has three cysteine residues and when completely oxidized, the protein has no immune function. However, partially reduced HMGB1 can bind to toll-like receptor 4 (TLR4) and lead to production of pro-inflammatory cytokines like TNF-α. Finally,

fully reduced HMGB1 has been shown to have chemotactic functions to recruit immune cells to sites of inflammation (146, 147).

L-selectin is minimally expressed on bone marrow neutrophils but highly upregulated on circulating neutrophils since it is an important adhesion molecule for trafficking into tissues. Once neutrophils have successfully extravasated into a tissue, they shed L-selectin, and upon activation further shedding occurs, ultimately allowing mature and activated neutrophils to be identified by their low L-selectin expression (31). Evaluation of neutrophil L-selectin expression in ecSOD congenic mice indicated that neutrophils in the liver of ecSOD HI mice are less mature prior to infection that ecSOD WT or ecSOD KO neutrophils. Interestingly, expression of L-selectin on neutrophils appears to decrease from the uninfected state to the infected state and differences in MFI between the ecSOD KO mice and the ecSOD HI and WT mice are abrogated at d1p.i. Both of these findings support the idea that L-selectin should be decreased on the surface of mature neutrophils and further shed upon activation, in this case, during *L. monocytogenes* infection. Importantly, while L-selectin shedding indicates maturity or activation, it does not necessarily indicate the functional ability of neutrophils. These data suggest that neutrophils in the liver of ecSOD HI mice are phenotypically and functionally immature prior to infection and that during infection with *L. monocytogenes*, these cells are activated but still functionally impaired.

Differences in liver neutrophil maturity could indicate that, in contrast to the neutrophil recruitment findings, there are cell-intrinsic defects in ecSOD HI neutrophils that lead to decreased function and decreased protection during infection. Neutrophil transfer studies to measure protective ability indicated that there are no permanent or cell-instrinsic differences in neutrophils from ecSOD congenic mice. This correlates with the similar finding regarding

neutrophil recruitment and indicates that ecSOD mediates its effects on both neutrophil recruitment and function by modulating the environment, not by permanently altering the neutrophils. Neutrophil transfers performed in mice that lack neutrophils, termed Genista mice (148), would be even more compelling to show that in the absence of host neutrophils, transferred neutrophils are able to provide protection, and that this protection is equivalent regardless of whether neutrophils originated from ecSOD HI or ecSOD KO mice. In addition, the neutrophil protection transfer experiments utilize neutrophils isolated from the bone marrow of donor mice in order to isolate sufficient numbers of neutrophils to transfer and observe protection. However, bone marrow neutrophils might still retain enough plasticity to alter their phenotype and function based on a new environment. However, functional defects in neutrophils may be permanent once those neutrophils reach the liver. Ideally, in future studies, neutrophils isolated from the liver would be transferred into host Genista mice to measure their protective ability in an environment where even a small number of neutrophils could provide resistance against *L. monocytogenes* infection.

Based on all these data, it is clear that ecSOD activity modulates the liver environment by protecting the ECM from degradation and also enhances concentrations of neutrophil-attracting chemokines leading to increased neutrophil recruitment into the liver both prior to, and during, infection with *L. monocytogenes*. Importantly, it has also been shown that these neutrophils are not protective during infection due to functional defects including reduced MPO production and reduced oxidative burst generation. While these functional defects do not result from permanent alterations in neutrophils, but rather from ecSOD mediated effects on the environment which in turn inhibit neutrophil function, the mechanism behind these defects is still unclear. Since

neutrophils develop and mature in the bone marrow, it is possible that ecSOD activity affects the bone marrow environment, leading to decreased neutrophil maturity and function.

Chapter IV

Downregulation of c-kit expression and increased Ly6G expression are characteristic of the transition from granulocyte precursors to mature neutrophils. Furthermore, neutrophils preparing to leave the bone marrow upregulate the CD11b adhesion molecule, allowing them to be identified as a mature population of bone marrow neutrophils (3). Evaluation of c-kit expression and Ly6G/CD11b expression on bone marrow neutrophils in ecSOD congenic mice confirmed that these neutrophils are predominantly immature in ecSOD HI mice prior to infection. In addition, CD11b expression on blood neutrophils mirrored this finding with ecSOD HI neutrophils expressing less CD11b than ecSOD WT or ecSOD KO neutrophils. This suggests that there are more immature neutrophils in the blood, as well as the bone marrow, of ecSOD HI mice. During infection, it is expected that more neutrophils are released from the bone marrow to traffic to sites of infection and that granulopoiesis would be increased in response (3). While the differences in percentages of mature bone marrow neutrophils also appear at d1p.i., the differences in immature neutrophil percentages are abrogated, most likely due to increased granulopoiesis in response to infection. In addition, while percentages of immature neutrophils in the bone marrow remain the same, or increase, from uninfected to infected state, overall percentages of mature neutrophils decrease upon infection, indicating increased release into circulation in response to infection.

Concentrations of G-CSF produced by bone marrow cells after stimulation with HKLM indicated that cells from both uninfected and d1p.i. ecSOD HI mice are less able to produce G-CSF than cells from ecSOD WT or ecSOD KO mice. This could contribute to the differences in

neutrophil maturity observed in the congenic mice as G-CSF is important for efficient neutrophil generation and development. This method of measuring G-CSF may only provide insight into the ability of immune cells in the bone marrow to generate G-CSF rather than providing data about concentrations of G-CSF in the bone marrow under basal or infectious conditions. To address this limitation, G-CSF concentrations in the bone marrow also need to be measured in extracellular fluid supernatants from bone marrow to generate more accurate results of what is occurring *in vivo*. In addition, differences in G-CSF receptor expression, particularly on neutrophils, should be investigated since this would also lead to differences in neutrophil maturity.

In the granulopoiesis assay, the data showing no difference in colonies across the ecSOD congenic mice indicates that ecSOD does not affect the potential for granulopoiesis in these mice. However, since we have firmly established that neutrophils isolated from the different mice exhibit plasticity and are recruited and function equivalently when placed in the same environment, we cannot completely rule out that ecSOD activity may lead to differences in granulopoiesis *in vivo*. This is further supported by the fact that there are differences in G-CSF production from the bone marrow of ecSOD congenic mice, however, in the granulopoiesis assay the concentrations of all growth factors and cytokines is equivalent across the three groups, potentially eliminating the ecSOD-mediated environmental impact.

Early release of immature neutrophils from the bone marrow of ecSOD HI mice could be due to increased release factors, decreased retention factors, or both. As discussed, there are increased concentrations of CXCL1 and CXCL2 in the serum of ecSOD HI mice which would bind to CXCR2 on neutrophils and enhance their release from the bone marrow. No differences were observed in CXCR2 expression on neutrophils suggesting that differences in neutrophil

release signals depend on the chemokine concentrations. Measuring concentrations of CXCL1 and CXCL2 in the bone marrow may provide additional insight into involvement of these chemokines in the release of neutrophils from the bone marrow. There were no differences in expression of CXCR4 on bone marrow neutrophils, which, combined with the CXCR2 data, further supports the idea that ecSOD does not mediate neutrophil recruitment by directly acting on neutrophils but rather by altering the tissue environment. Despite the lack of differences in CXCR4, there was decreased concentrations of CXCL12 in the bone marrow of ecSOD HI mice. Since CXCL12 binding to CXCR4 is the major signal regulating neutrophil retention in the bone marrow, this suggests a decrease in neutrophil retention in ecSOD HI mice. The combination of increased release signaling and decreased retention signaling likely contributes to the increased recruitment of neutrophils into the blood and the liver in ecSOD HI mice.

A potential mechanism behind ecSOD-mediated differences in chemokine concentrations and neutrophil retention and release factors involves the influence of G-CSF on neutrophil mobilization. Previous studies have shown that administration of G-CSF leads to decreased CXCL12 expression in the bone marrow (5, 149, 150), and it has been suggested that G-CSF can lead to cleavage of the CXCL12/CXCR4 interactions that retain neutrophils in the bone marrow (66). This cleavage has been attributed to various proteases, including matrix metalloproteinases (MMPs)(139). Furthermore, studies have shown an additional mechanism for G-CSF mediated upregulation of CXCR2 on neutrophils, which would not be relevant to our model, as well as increased responsiveness of neutrophils to CXCL2 via STAT3 signaling and thrombopoietin release (151, 152). Since G-CSF is elevated in the serum of ecSOD HI mice, and CXCL12 is decreased in the bone marrow, the effects of G-CSF through the previously mentioned mechanisms needs to be further investigated in ecSOD congenic mice. The decreased G-CSF

produced in response to HKLM from cultured bone marrow cells contrasts with this theory (Figure 6), however, the previously mentioned caveats about using this method of measuring G-CSF concentrations from the bone marrow mean we cannot rule out a relationship between G-CSF and CXCL12.

It was determined that neutrophils from the bone marrow of ecSOD HI mice are less functional than those of ecSOD WT or ecSOD KO mice. These neutrophils were less able to generate oxidative burst upon stimulation and were less able to phagocytose *L. monocytogenes in vitro*. Further studies to evaluate the function of these neutrophils should include degranulation assays and analysis of pro-inflammatory cytokine production. In addition, assays to clarify phagocytic ability of bone marrow neutrophils should be performed since the *in vitro* assay used to measure phagocytosis may not reflect *in vivo* neutrophil activity. The *in vitro* assay also cannot differentiate between cells associated with *L. monocytogenes*-GFP as a result of infection, versus resulting from phagocytosis.

Chapter V

It has been shown that ecSOD is produced in multiple tissues by multiple cell types, including both immune and non-immune cells (7, 8). Therefore, it is important to attempt to determine if hematopoietic or non-hematopoietic production of ecSOD play a more significant role in the differences in neutrophil recruitment and function observed in ecSOD congenic mice. Bone marrow chimeras allow for the generation of mice that express ecSOD from either hematopoietic-derived cells or somatic cells to differentiate the relative contributions of ecSOD activity from either cell lineage. In addition, bone marrow chimeras can be generated that have enhanced ecSOD activity from either hematopoietic or non-hematopoietic cells to determine if the enhanced activity significantly alters the phenotype.

Bone marrow chimeras between ecSOD KO and C57/Bl6 (wild-type ecSOD) mice determined that only when ecSOD is produced by both hematopoietic and non-hematopoietic cells are the increased bacterial burden and increased neutrophil recruitment observed. Absence of ecSOD from either lineage (ecSOD KO -> WT or WT -> ecSOD KO), or from both lineages (ecSOD KO -> ecSOD KO), leads to decreased bacterial burden and decreased neutrophil recruitment into the liver.

Potential divergent roles for ecSOD produced by each cell lineage was apparent in BMC mouse studies using the ecSOD HI and C57/Bl6 mice. Enhanced ecSOD activity (ecSOD HI) produced only from hematopoietic cells resulted in increased bacterial burden while enhanced ecSOD activity produced by non-hematopoietic cells resulted in increased neutrophil recruitment. However, these results were observed as trends not as statistically significant differences so further studies are needed to explore potential differences in ecSOD activity from each cell lineage.

Generation of bone marrow chimeras between ecSOD HI and ecSOD KO mice would be the most revealing in determining the differences between ecSOD produced from hematopoietic cells and non-hematopoietic cells in regulating neutrophil recruitment or function since these mice have the largest differences in congenic studies. Unfortunately, both ecSOD HI and ecSOD KO mice express the CD45.2 allele so there would be no internal control to check for successful chimerism in these studies. To overcome this limitation, ecSOD HI and ecSOD KO mice could be crossed onto the B6-Ly5.1/Cr background while still retaining their respective ecSOD alleles. While differences in bacterial burden may not be significantly different at d3p.i., functional studies of neutrophils from the liver of BMC mice at this time point should be performed. All the data presented are from d3p.i., therefore these experiments need to be completed at d1p.i. as well

to further explore potential differences in recruitment and function of neutrophils. Future studies should also be performed on uninfected BMC mice to determine if the differences in neutrophil recruitment and function observed in uninfected congenic mice are also present in BMC mice in the absence of infection. Finally, since differences in chemokine concentrations appears to be an important mechanism behind ecSOD-mediated differences in both neutrophil recruitment and function, CXCL1 and CXCL2 should be measured in liver homogenates, serum, and bone marrow of BMC mice and CXCL12 should be measured in the bone marrow extracellular fluid.

Conclusions

While these studies provide further insight into the role of ecSOD activity in regulating neutrophil recruitment and function in both the absence and presence of infection, there are still potential mechanisms of action that need to be further explored. One area to investigate further is how ecSOD activity directly or indirectly alters chemokine concentrations. The ability of ecSOD activity to protect the ECM from degradation may contribute to longer half-life or reduced clearance of chemokines that bind to the ECM. Furthermore, chemokines possess cysteine residues that may make them susceptible to oxidative stress which could result in increased degradation of chemokines in ecSOD KO mice.

Future studies should also attempt to elucidate the role of ecSOD produced by different cell types by expanding on the bone marrow chimera experiments performed here, as well as additional studies to determine if ecSOD produced by neutrophils can act in an autocrine manner to regulate recruitment or function, or if the phenomena observed in ecSOD congenic mice is purely due to ecSOD affecting the environment and subsequently altering neutrophil responses.

Finally, an avenue of ecSOD activity that has not been discussed is the potential effects of ecSOD activity directly on *L. monocytogenes* and the ability of neutrophils to kill the bacteria. It has been proposed that ecSOD may be taken up into the phagosome, along with bacteria, where it might contribute to the conversion of superoxide into hydrogen peroxide and lead to decreased killing of *L. monocytogenes*. Studies to investigate this possibility would include extensive microscopy and use of novel *L. monocytogenes* bacterial mutants, particularly the *L. monocytogenes*-GFP used in *in vitro* studies (Figure 11) and the mutant that transcribes GFP from the *actA* promoter so that it only expresses GFP when the bacteria has escaped into the cytosol of a host cell (DH-L1245; Darren Higgins, Harvard Medical School) (153).

Other studies investigating the role of ecSOD during non-infectious inflammatory lung models have shown that ecSOD protects the tissue from inflammation, a finding that we also observe in our model. However, the mechanism of protection by ecSOD in the lung appears to involve an overall decrease in the inflammatory response, including chemokine and cytokine production, which leads to decreased neutrophil recruitment to the lung (8, 13, 20, 154-156). While our data demonstrate increased neutrophil recruitment in response to ecSOD activity, this could be due to the differential production of ecSOD in the lung versus the liver, in addition to the differences attributed to a non-infectious inflammatory model compared to an infectious model. These findings could also result from the difference in regulation of inflammation between these two organs, since lung inflammation must be very tightly regulated. One study investigating the role of ecSOD during a lung *Escherichia coli* model showed ecSOD results in increased bacterial killing and decreased bacterial burden, however, this appeared to be primarily mediated by macrophage activity rather than neutrophils (157). Ultimately, the differences in the

model of inflammation or infection and the organ studied indicated that further studies would be required to elucidate the role of ecSOD activity.

The studies presented here have determined that the antioxidant enzyme, ecSOD, plays a significant role in regulating efficient neutrophil responses against *L. monocytogenes*. In the bone marrow, ecSOD activity leads to decreased concentrations of the chemokine CXCL12, which is important for binding to CXCR4 on neutrophils and retaining them in the bone marrow until they are fully mature. The mechanism by which ecSOD activity regulates CXCL12 concentrations remains unclear but may result from differences in G-CSF concentrations in the serum and the bone marrow. In addition, to the decreased retention signals from CXCL12/CXCR4 interactions, ecSOD activity leads to increased CXCL1 and CXCL2 concentrations in the serum. These neutrophil-attracting chemokines bind to CXCR2 on neutrophils and result in signals for neutrophils to exit the bone marrow. This combination of decreased retention factors and increased release factors leads to premature egress of immature neutrophils out of the bone marrow in ecSOD HI mice. This increase in immature neutrophils leaving the bone marrow leads to increased percentages of immature neutrophils in the blood. Furthermore, since granulopoiesis is not altered in these mice, the continuous generation of neutrophils to replace those recruited into circulation, leads to an increase in the percentage of immature neutrophils in the bone marrow.

These immature neutrophils that are now present in high percentages in the blood are recruited in large numbers into the liver, both in the presence and absence of infection, due to increased interactions with the ECM. Since ecSOD is protecting the ECM from degradation, there is more intact HA to bind CD44 and facilitate neutrophil extravasation into the liver. In

addition, increased concentrations of CXCL1 and CXCL2 in the liver of ecSOD HI mice enhance neutrophil trafficking into the liver during infection.

Unfortunately, due to the premature release of these neutrophils from the bone marrow, they are both phenotypically and functionally immature. During infection with *L. monocytogenes*, these neutrophils are recruited in large numbers into the liver but due to defects in functional ability, including impaired ROS generation and decreased TNF-α production, these neutrophils are unable to protect against the bacteria. Importantly, these defects are not a result of permanent cell-intrinsic defects in the neutrophils, but rather are a result from constant exposure to an environment with enhanced ecSOD activity. Ultimately, these studies provide insight into the relationship between antioxidants, such as ecSOD, and the immune response against pathogens. As therapies involving antioxidants, particularly ecSOD, progress it would be beneficial to account for the potential increased susceptibility to infection balanced against the protection from inflammatory damage.

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