Extracellular Superoxide Dismutase Inhibits Innate Immune Responses and Clearance of an Intracellular Bacterial Infection

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Reactive oxygen species and reactive nitrogen species (RNS) represent a first line of defense against microbial invasion and replication but can also cause collateral tissue damage. Extracellular superoxide dismutase (ecSOD) is the only enzyme that mediates the conversion of superoxide (O$_2^-$) into hydrogen peroxide (H$_2$O$_2$) in the extracellular matrix (ECM) of tissues (1). In this capacity, ecSOD is a potent antioxidant that can protect tissues from oxidative damage by regulating various ROS and RNS. H$_2$O$_2$ is also an important bactericidal agent and the precursor for hypochlorous acid, a major component of bleach. It, therefore, seems logical that ecSOD may contribute to pathogen clearance during acute infection.

Surprisingly, the role of ecSOD in immune responses and clearance of pathogens has not been investigated. There are conflicting views suggesting that ROS can have either a beneficial or detrimental impact on the host (2). In addition to its ability to control the relative concentrations of ROS, ecSOD can reduce neutrophil recruitment to the lung and dampen inflammatory responses during noninfectious insults (3–5). The product of ecSOD, H$_2$O$_2$, can directly induce neutrophil recruitment (6) but can also lead to cellular apoptosis (7). Notably, both H$_2$O$_2$ and O$_2^-$ have been shown to induce the production of proinflammatory cytokines and chemokines (8–10). Therefore, by directly influencing the oxidative environment in the ECM of various tissues, the level of ecSOD activity may impact pathogen survival and the function or recruitment of immune cells prior to, or during, infection.

The study of ecSOD is further warranted by the existence of polymorphisms in the human ecSOD gene that can lead to altered activity and localization of the enzyme. These polymorphisms have been associated with several inflammatory disorders including ischemic heart disease, chronic obstructive pulmonary disease, type 2 diabetes, preeclampsia, and acute lung injury (11, 12), although the molecular mechanisms are not fully understood. Importantly, the ability of humans with these polymorphisms to respond to pathogenic infections has not been investigated.

L. monocytogenes is a Gram-positive intracellular bacterium that is widely used to study host–pathogen interactions. In the murine model of L. monocytogenes infection, the bacteria primarily infect the spleen and liver, where macrophages, monocytes, and neutrophils facilitate early bacterial clearance (13). Our laboratory has recently shown that specific depletion of neutrophils with the anti-Ly6G (1A8) Ab reduces bacterial clearance and overall TNF-α production (14). Mice lacking TNF-α, or its receptor, are extremely susceptible to infection with L. monocytogenes and other pathogens (15–18). It has also been established that inducible NO synthase (iNOS) and components of the NADPH oxidase are required for efficient clearance of L. mono-
cytogenes (19–21). However, how ecSOD activity affects cytokine secretion, ROS and RNS levels, and the function of immune cells during bacterial infection has not been investigated.

To evaluate the importance of ecSOD in the acute response to bacterial infection, we took advantage of a novel experimental model. We used congenic mice that express high levels of ecSOD activity (ecSOD HI) (22), wild-type levels of ecSOD activity (ecSOD WT) (22), or lack ecSOD (ecSOD KO) (23, 24). All of these mice share the C57BL/6 (B6) background. The ecSOD HI mice express the ecSOD allele from the 129 strain of mice and are characterized by higher levels of this enzyme in most tissues, including the liver. These mice are congenic to the ecSOD WT mice, which express the ecSOD allele from B6 mice (22).

Contrary to our initial hypothesis, we report in this study that ecSOD has a detrimental impact during L. monocytogenes infection by decreasing host survival, bacterial clearance, TNF-α and peroxynitrite (NO3·−) production, and neutrophil function. Importantly, the current research identifies an exciting and novel role by which an extracellular enzyme decreases the ability of mice effectively to eradicate an intracellular pathogen.

Materials and Methods
Mice and L. monocytogenes infections
Mice that lack ecSOD (ecSOD KO) (24), have high levels of ecSOD activity (ecSOD HI, expressing the 129 allele of ecSOD) (22), and have wild-type levels of ecSOD activity (ecSOD WT, expressing the ecSOD allele identical to the founder B6) (22) were bred in-house. ecSOD KO mice were a kind gift from Dr. Cheryl L. Fattman (University of Pittsburgh). All of these mice were back-crossed to B6 mice. All studies used gender- and age-matched mice (2–4 mo old), which were housed with food and water ad libitum in sterile microisolator cages with stereo bedding at the University of North Texas Health Science Center American Association for the Accreditation of Laboratory Animal Care accredited animal facility. All animal experiments 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 10403 serotype 1 was grown on brain–heart infusion (BHI) agar plates (BD Bacto, Sparks, MD), and virulent stocks were maintained by repeated passage through B6 mice. For infection of mice, log-phase cultures of L. monocytogenes grown in BHI broth were washed twice and diluted in PBS to the desired concentration. Mice were inoculated i.v. with ∼109 L. monocytogenes for the experiments conducted at days 1 and 3 postinfection (p.i.). For experiments conducted at day 5 p.i., three days p.i., mice were sacrificed, and organ sections of frozen livers from L. monocytogenes-infected or uninfected ecSOD HI, ecSOD WT, and ecSOD KO mice were prepared for immunohistochemistry with anti-L. monocytogenes antibodies.

Preparation of organs for flow cytometry and in vitro culture
Peripheral blood from the lateral tail vein was collected in a heparin solution with HBSS plus 2% FCS. RBCs were lysed with TriS ammonium chloride. Splenocytes were obtained by grinding whole spleens, and RBCs were lysed with Tris ammonium chloride. Liver leukocytes were obtained as previously described (25). Briefly, cell pellets obtained from homogenized livers were resuspended in 35% Percoll media and layered upon 67.5% Percoll, after which the gradient was centrifuged at 600 × g for 40 min and low-density cells were collected from the gradient interface. Liver leukocytes were cultured in phenol red-free DMEM supplemented with 10% FCS, vitamins, l-glutamine, and penicillin/streptomycin. All supplements were from Invitrogen-Life Technologies (Carlsbad, CA).

Flow cytometry
For cell surface staining, the following Abs were obtained from BD Biosciences (San Diego, CA): anti-Ly6G PE or FITC (1A8), anti-CD11b PE–Cy7 (M1/70), anti-CD16/CD32 (2.4G2); eBioscience (San Diego, CA): anti–IFN-γ allophycocyanin (XMG1.2); BioLegend (San Diego, CA): NK1.1 PE (PK136); and Abcam (Cambridge, MA): anti-CD8 PE–TR (53-6.7). Cells were incubated at 4°C for 15 min with saturating amounts of the cell-surface Abs, and anti-CD16/CD32 to block Fc receptors, in staining buffer (PBS plus 2% FCS plus 0.1% sodium azide). Cells were fixed in BD Stabilization Fixative (BD Biosciences). To accomplish intracellular cytokine staining, GolgiPlug containing brefeldin A (BD Biosciences) was added 4 h before the harvest of the cell cultures. Cells were then fixed and permeabilized at 4°C for 20 min using an intracellular cytokine staining kit from BD Biosciences and incubated in saturating amounts of anti–IFN-α or anti–IFN-γ (BD Biosciences) at 4°C for 20 min. To measure apoptosis, annexin V PE was used according to the manufacturer’s instructions (BD Biosciences). Data were acquired and analyzed using a Beckman Coulter FC500.

Measurement of RNS
To measure NOn, liver leukocytes from L. monocytogenes-infected mice were cultured overnight with heat-killed L. monocytogenes, and the supernatant was analyzed using the NO: Quantitation Kit/Griess reagent (Active Motif). To determine the extent of NO3·− formation, protein nitrosylation levels were measured by Western blot. Livers from L. monocytogenes-infected mice were homogenized as previously described (22), and the protein concentration was determined using the Lowry assay. One hundred micrograms of liver protein was separated by 12% SDS-PAGE, transferred to polyvinylidene fluoride membranes, and immunoblotted with a nitrotyrosine-specific mAb (clone 1A6; Upstate Biotechnology, Lake Placid, NY). The extent of protein nitrosylation was normalized to β-actin protein levels. Detection and quantitation was performed using the FluoroChem FC2 Imaging system (Alpha Innotech, Santa Clara, CA).

Immunohistochemistry
Immunohistochemistry was performed as previously described (26). Briefly, 7-μm sections of frozen livers from L. monocytogenes-infected or uninfected ecSOD HI, ecSOD WT, and ecSOD KO mice were prepared for the Leica CM 1850 cryostat. Anti-Ly6G (1A8) (BD Biosciences) and Difco Listeria O polysaccharide (BD Biosciences) were used to detect neutrophils and L. monocytogenes, respectively. Anti-Ly6G Ab was developed with anti-rat Alexa Fluor 594 (Molecular Probes), and Difco Listeria O polysaccharide was developed with anti-rabbit Alexa Fluor 488 (BD Biosciences). Prolong Gold antifade reagent (Invitrogen) and a coverslip were added to the stained tissues. To view the stained tissue, an Olympus AX70 fluorescent microscope was used, and images were captured with an Olympus DP70 digital camera and analyzed with Image-Pro Plus software.

Quantification of TNF-α
ELISAs were performed on liver leukocyte culture supernatants using Ab pairs for TNF-α (eBioscience). Cytokine levels were determined by comparison with standard curves generated from recombinant TNF-α (eBioscience). Data were analyzed using a Biotek EL808 spectrophotometer.

Neutrophil depletions
To specifically deplete neutrophils, the anti-Ly6G (1A8) Ab (27) was used as previously described (14). Briefly, 750 μg anti-Ly6G or isotype control Ab (both from Bio X Cell, West Lebanon, NH) was injected i.p. 1 d prior to i.v. injection of L. monocytogenes. Three days p.i., mice were sacrificed, CFUs determined, and flow cytometry used to confirm depletion of neutrophils.

Statistical analyses
Analyses of variance were conducted on the data. Bonferroni t tests or Newman–Keuls t tests were used for post hoc analyses. L. monocytogenes CFU data were log transformed prior to analysis and are represented as such in the figures. Kaplan–Meier plots and log-rank tests were used to compare the survival curves between groups. A p value <0.05 was considered significant in all cases.

Results
ecSOD activity increases susceptibility to L. monocytogenes infection
To determine how ecSOD activity affects the susceptibility of mice to infection, ecSOD HI, ecSOD WT, and ecSOD KO mice were infected with L. monocytogenes, and survival was monitored (Fig. 1). Surprisingly, mice lacking ecSOD were 100% resistant to L. monocytogenes infection, regardless of gender. Mice that express...
the highest ecSOD activity were most susceptible to infection, whereas ecSOD WT mice demonstrated intermediate susceptibility to infection. Because most of the ecSOD WT and ecSOD HI mice succumbed to infection early, bacterial burdens in the spleens and livers of the three groups of mice were determined at days 1, 3, and 5 p.i. with sublethal doses of *L. monocytogenes* (Fig. 2). Interestingly, there were no differences in *L. monocytogenes* CFUs in the liver among the three groups of mice at day 1 p.i., although the ecSOD HI mice had slightly higher CFUs in the spleen compared with the other mice (Fig. 2A). Bacterial burdens in the spleen and liver at days 3 (Fig. 2B) and 5 (Fig. 2C) p.i. were significantly decreased in mice lacking ecSOD relative to the other groups. Conversely, mice expressing the highest activity were least efficient in clearance of the bacteria at both time points. It was also confirmed that B6 mice have the same bacterial burden as ecSOD WT mice in the spleen and liver at day 3 p.i. (data not shown). Collectively, these data indicate that resistance to *L. monocytogenes* infection in mice is inversely related to the level of ecSOD activity.

Lack of ecSOD does not enhance NK or CD8\(^+\) T cell responses in the liver

Previous publications have established that the production of IFN-γ, primarily from NK and CD8\(^+\) T cells, is critical for innate clearance of *L. monocytogenes* (25, 28–30). Therefore, we investigated how ecSOD regulates the recruitment and activity of NK and CD8\(^+\) T cells during *L. monocytogenes* infection. ecSOD HI, ecSOD WT, and ecSOD KO mice were infected with *L. monocytogenes*. At day 1 p.i., splenocytes and liver leukocytes were analyzed to determine the percentage of NK and CD8\(^+\) T cells. ecSOD activity did not alter the percentage of NK1.1\(^+\) cells (Supplemental Fig. 1A) but did lead to a slight enhancement of the percentage of CD8\(^+\) T cells in the spleen and liver (Supplemental Fig. 1B). Notably, ecSOD activity enhanced the percentage of NK1.1\(^+\) cells secreting IFN-γ (Supplemental Fig. 1C) but did not significantly alter the percentage of CD8\(^+\) T cells secreting IFN-γ (Supplemental Fig. 1D), although the trend followed that of the NK1.1\(^+\) cells. Collectively, these data indicate that mice lacking ecSOD, which are able efficiently to clear early *L. monocytogenes* infection, do not show enhanced NK1.1\(^+\) or CD8\(^+\) T cell responses in the spleen or liver.

ecSOD activity enhances the percentage and total number of neutrophils in the liver

It has been observed that ecSOD activity can decrease neutrophil recruitment to the lung during inflammatory insults (4, 5, 31). However, it is not known if ecSOD activity affects the recruitment of neutrophils to other organs. To investigate this possibility, the percentages and numbers of neutrophils in the spleen, liver, and blood of uninfected ecSOD HI, ecSOD WT, and ecSOD KO mice were quantified via flow cytometry (see Supplemental Fig. 2 for

**FIGURE 1.** ecSOD activity enhances susceptibility of mice to *L. monocytogenes* infection. ecSOD HI, ecSOD WT, and ecSOD KO mice were infected with *L. monocytogenes*, and survival was monitored in female (A) and male (B) mice. A log-rank analysis detected a significant difference between survival curves as indicated in the graphs (p < 0.05). The data are combined from two independent experiments (n = 10–12/group).

**FIGURE 2.** ecSOD activity decreases clearance of *L. monocytogenes* from the spleen and liver. ecSOD HI, ecSOD WT, and ecSOD KO mice were infected with *L. monocytogenes*. Spleen and liver CFUs were determined at day 1 p.i. (A), day 3 p.i. (B), and day 5 p.i. (C). Two-way ANOVAs detected significant differences between groups. These data are representative of two independent experiments. All data are expressed as the mean ± SEM (n = 4–5/group). *p < 0.05, **p < 0.01, ***p < 0.001 (groups differ).
gating strategy). Surprisingly, there was an increased percentage of neutrophils in the livers of ecSOD HI mice compared with the ecSOD KO mice, with ecSOD WT mice having intermediate percentages (Fig. 3A). Unlike the liver, the neutrophil populations did not differ in the blood or spleen of the three groups of mice prior to infection with *L. monocytogenes*. Neutrophil percentages were also examined at days 1, 3, and 5 p.i. to determine if ecSOD activity affects infection-induced neutrophil recruitment. It was observed that the ecSOD HI mice continued to have higher percentages of neutrophils in the liver, as well as the blood, compared with the ecSOD KO mice, with ecSOD WT mice having intermediate percentages (Fig. 3B–D). The total number of neutrophils in the livers of the three groups of mice correlated with the percentages at each time point before, and after, infection (Fig. 3E–H). Given that neutrophils are required for resistance to *L. monocytogenes* infection in B6 mice (14), the fact that ecSOD activity results in decreased resistance yet increased neutrophil recruitment is counterintuitive and deserves further investigation.

**ecSOD activity regulates the localization of neutrophils to sites of *L. monocytogenes* lesions**

To determine how ecSOD affects the ability of neutrophils to localize with *L. monocytogenes* lesions, immunohistochemistry was performed. At day 1 p.i., there were no differences in the ability of neutrophils to localize with *L. monocytogenes* lesions in the livers of the three groups of mice (Fig. 4A–C). At day 3 p.i., however, ecSOD activity clearly affected the ability of neutrophils to localize with *L. monocytogenes* lesions. Numerous *L. monocytogenes* lesions appeared to be completely devoid of neutrophils in the ecSOD HI mice, whereas fewer such neutrophil-free lesions were observed in the ecSOD WT mice. In the ecSOD KO mice, there were very few *L. monocytogenes* lesions, but neutrophils localized to these lesions 100% of the time (Fig. 4D–F). In the livers of ecSOD KO mice, neutrophils colocalized with *L. monocytogenes* lesions on every occasion, whereas fewer of the lesions in the ecSOD HI mice had associated neutrophils (Fig. 4G). Additionally, the ecSOD KO mice had the greatest number of neutrophils associated with individual *L. monocytogenes* lesions compared with the other groups of mice (Fig. 4H). These data suggest that ecSOD activity may be negatively affecting the ability of neutrophils effectively to combat the infection due to the lack of neutrophil/*L. monocytogenes* colocalization.

**ecSOD activity increases neutrophil apoptosis in the liver**

It has been shown that exogenous superoxide dismutase can induce neutrophil apoptosis (32) and that apoptotic neutrophils are anti-inflammatory in nature (33, 34). To determine if ecSOD regulates neutrophil apoptosis, liver leukocytes from the three groups of mice were stained for Ly6G, CD11b, and annexin V. ecSOD activity did not preferentially increase the percentage of neutrophils undergoing apoptosis in uninfected mice or mice infected for 1 d (Supplemental Fig. 3A). However, at day 3 p.i. with *L. monocyto-

![FIGURE 3. ecSOD activity enhances the percentage and total number of neutrophils in the liver. ecSOD HI, ecSOD WT, and ecSOD KO mice were left uninfected (A, E) or infected for 1 d (B, F), 3 d (C, G), or 5 d (D, H) with *L. monocytogenes*. Percentages of neutrophils in the spleen, liver, and blood (A–D) and total numbers (E–H) of neutrophils in the liver were determined by flow cytometry. Two-way (A–D) and one-way (E–H) ANOVAs detected significant differences between groups. All data are representative of two independent experiments. All data are expressed as the mean ± SEM (n = 4–5/group). *p < 0.05, **p < 0.01, ***p < 0.001 (groups differ).
ocytogenes, there was a higher percentage of neutrophils undergoing apoptosis in the ecSOD HI mice (Supplemental Fig. 3A). Importantly, due to the fact that the ecSOD HI mice have a higher percentage of neutrophils in the liver, there was a higher overall percentage of apoptotic neutrophils in the livers of uninfected ecSOD HI mice compared with ecSOD WT and ecSOD KO mice (Fig. 5A). Furthermore, when mice were infected with L. monocytogenes for 1 d (Fig. 5B) or 3 d (Fig. 5C), the ecSOD HI mice had a higher percentage of apoptotic neutrophils than the ecSOD KO mice, with ecSOD WT mice having intermediate values. A similar trend was observed for the total numbers of neutrophils undergoing apoptosis in the liver (Supplemental Fig. 3B), once again due to the fact that the ecSOD HI mice contain increased percentages and numbers of neutrophils. These data suggest that ecSOD activity results in more apoptotic neutrophils, which may ultimately be detrimental to the immune response to L. monocytogenes infection.

determine if ecSOD was affecting the production of TNF-α in the liver, the three groups of mice were infected with L. monocytogenes. Liver leukocytes were isolated at 1 d (Fig. 6A) or 3 d (Fig. 6B) p.i. and cultured with heat-killed L. monocytogenes. Liver leukocytes from ecSOD KO mice produced higher concentrations of TNF-α compared with those from ecSOD HI mice. Liver leukocytes from ecSOD WT mice produced intermediate concentrations of TNF-α (Fig. 6A, 6B). These data indicate that ecSOD activity decreases TNF-α production in the liver during L. monocytogenes infection.

Liver leukocytes from the three groups of mice infected for 1 d with L. monocytogenes were also stained for intracellular TNF-α. The percentage of neutrophils secreting TNF-α was higher in the ecSOD KO mice compared with ecSOD HI mice, with an intermediate percentage of neutrophils from ecSOD WT mice producing TNF-α (Fig. 6C). Furthermore, it was observed that neutrophils from ecSOD KO mice had a higher mean fluorescence intensity (MFI) for TNF-α (Fig. 6D), indicating that increased amounts of TNF-α were being produced on a per-cell basis. These data demonstrate that ecSOD activity decreases overall and neutrophil-specific TNF-α production in the liver, which may partially explain how ecSOD activity influences susceptibility to L. monocytogenes infection.
ecSOD activity reduces the extent of protein nitrosylation

It is known that ROS and RNS are important for clearance of L. monocytogenes (19–21). Although ecSOD directly regulates the extracellular concentrations of O$_2^·$ and H$_2$O$_2$, it is also known to regulate RNS levels indirectly (35). To investigate how ecSOD alters RNS levels, the three groups of mice were infected for 1 d with L. monocytogenes. Our data indicate that ecSOD did not alter the production of NO$^·$ from liver leukocytes (Fig. 7A). Next, the extent of protein nitrotyrosine formation, as a measure of NO$_3^·$ activity, was determined in liver homogenates after infection. Importantly, mice lacking ecSOD contained significantly increased levels of protein nitrotyrosine compared with ecSOD HI mice, with ecSOD WT mice showing intermediate levels (Fig. 7B). Therefore, ecSOD activity reduces the amount of protein nitrosylation, suggesting lower production of NO$_3^·$, which is known to be important for killing L. monocytogenes (36).

ecSOD-induced differences in liver CFUs are partially abrogated by neutrophil depletion

Because ecSOD activity decreases NO$_3^·$ levels and the ability of neutrophils to colocalize with L. monocytogenes, survive, and secrete TNF-$\alpha$, it is reasonable to suggest that these decreases may influence the ability of neutrophils to clear infection. To test this possibility, ecSOD HI, ecSOD WT, and ecSOD KO mice were injected with the anti-Ly6G (1A8) neutrophil-depleting Ab or an isotype control and were infected with L. monocytogenes the following day. Neutrophil depletion was confirmed by staining of blood leukocytes for neutrophil-specific cell surface molecules (Supplemental Fig. 4). In agreement with Fig. 2, isotype-treated ecSOD HI mice had the highest bacterial burden, ecSOD WT mice had intermediate bacterial burdens, and ecSOD KO mice had the lowest bacterial burden (Fig. 8A). With depletion of neutrophils, ecSOD HI mice actually showed a slight decrease in CFUs compared with isotype-treated mice. Conversely, both the ecSOD WT and ecSOD KO mice had increased CFUs after depletion of neutrophils. This increase in CFUs was significant in the ecSOD KO mice (Fig. 8A). To visualize these differences better, the log change in CFUs was also determined. Fig. 8B shows a slight decrease in CFUs with depletion of neutrophils in the ecSOD HI mice. However, equivalent increases in CFUs in both the ecSOD WT and ecSOD KO mice were observed upon neutrophil depletion. These data suggest that neutrophils in the three groups of mice may be functioning differently. The neutrophils in the ecSOD HI mice may be ineffective, as depletion of these
Experiments. All data are expressed as the mean between groups. These data are representative of two independent experiments. All data are expressed as the mean ± SEM (n = 3–5/group). *p < 0.05 (groups differ).

Neutrophils led to a slight decrease in CFUs. Conversely, neutrophils in ecSOD WT mice function normally, as depletion increased CFUs, as previously observed (14). Finally, neutrophils in ecSOD KO mice function at a higher efficiency, as fewer neutrophils provide protection equivalent to that seen by a greater number of ecSOD WT neutrophils.

Discussion

It has been previously established that the NADPH oxidase and iNOS (which produce O$_2^-$ and NO, respectively) are important for eliminating bacteria, including *L. monocytogenes* (19–21). Additionally, it was recently shown that mitochondrial production of ROS is important for bacterial clearance (37). Our data now establish that ecSOD actually impairs the host's ability to mount an effective innate immune response to bacterial infection. ecSOD HI mice were less resistant to *L. monocytogenes* infection, whereas ecSOD KO mice were more resistant than ecSOD WT mice. Surprisingly, ecSOD enhanced the mobilization of neutrophils to the livers, although this increase in neutrophils in the ecSOD HI mice appears to be detrimental to clearance of *L. monocytogenes*. In support of this notion, ecSOD activity was associated with decreased neutrophil colocalization with *L. monocytogenes* lesions in the liver. Moreover, the extent of ecSOD activity correlated with increased neutrophil apoptosis in the liver, which may, in turn, suppress the generation of an effective immune response. Overall NO$_3^-$ levels, as well as TNF-α production by liver leukocytes, was also decreased with increasing ecSOD activity during *L. monocytogenes* infection. Similarly, ecSOD activity reduced both the percentage of liver neutrophils producing TNF-α and the amount of TNF-α being produced on a per-cell basis, suggesting that ecSOD may alter the functional activity of neutrophils.

Neutrophil depletion studies showed that neutrophils in the ecSOD HI mice did not contribute to bacterial clearance, as their removal resulted in a slight decrease in CFUs. In contrast, depletion of neutrophils from ecSOD WT and ecSOD KO mice resulted in equivalent increases in liver CFUs, indicating their importance in bacterial clearance in these mice. Moreover, the functional activity of the ecSOD KO neutrophils, relative to that of neutrophils from ecSOD WT mice, appears to be increased, as similar protection is achieved by fewer neutrophils. Therefore, of the three groups, the neutrophils in the ecSOD KO mice are most efficient at controlling the *L. monocytogenes* infection. Our data suggest that ecSOD activity determines the contribution of neutrophils to clearance of *L. monocytogenes* from the liver.

These data further support our previous publication showing that neutrophils are required for protection against *L. monocytogenes* (14). Notably, a recent publication used the same neutrophil-depleting Ab and concluded that neutrophils are not required for clearance of *L. monocytogenes* (38). However, there are several experimental differences (source of B6 mice, dosage of *L. monocytogenes*, amount of depleting Ab used, timing of injection of depleting Ab, etc.) between the two studies that could have led to the discordant conclusions. One possibility is that the different B6 mice used in the studies expressed different amounts of ecSOD activity, which according to our current data can have a profound impact on the ability of neutrophils to provide protection against *L. monocytogenes*. Importantly, in both of our studies utilizing the anti-Ly6G (1A8) Ab, we confirmed that neutrophils, but not monocytes, were effectively depleted (Ref. 14 and data not shown).

The altered activity of the neutrophils in mice with varying levels of ecSOD activity may be related to the degree of apoptosis. We provide data demonstrating that ecSOD activity increases the percentage of apoptotic neutrophils in the livers of uninfected and...
infected mice. Previous research suggests that increased apoptosis can suppress immune responses to L. monocytogenes (33). However, ecSOD may also be influencing the effector functions of neutrophils independent of the extent of apoptosis. Recent research has identified a population of cells that express Ly6G but are characterized as myeloid-derived suppressor cells (MDSCs). These MDSCs suppress immune responses by secreting anti-inflammatory cytokines and decreasing the bioavailability of L-arginine (39–41). Studies regarding MDSCs have shown a strong correlation with cancer and suppression of the immune response against tumors. However, it has also been shown that MDSC numbers are increased during bacterial and parasitic infections (41). It is possible that increased ecSOD activity leads to an alteration in the oxidative environment that is conducive to the induction of MDSCs, which could account for the differences in neutrophil function observed in the three groups of mice.

Although it has been previously shown that ecSOD can alter RNS levels, including NO· (35), our data indicate that ecSOD activity did not alter the production of NO· from liver leukocytes isolated from infected mice. It is possible that ecSOD regulates NO· availability in the vicinity of endothelial NO· synthase (in the vasculature), but not NO· produced by iNOS-expressing leukocytes. It is well-established that ecSOD mediates the conversion of O2− into H2O2 in the ECM of tissues (1). Therefore, mice lacking ecSOD likely generate increased amounts of O2− in the ECM, which scavenge NO· to produce increased NO3− levels. Indeed, our data show that ecSOD KO mice have increased levels of nitrosylated proteins, indicative of increased NO3− levels, compared with mice expressing ecSOD. It is known that NO3− is a potent antimicrobial molecule that can directly kill L. monocytogenes (36). Therefore, it is possible that one mechanism contributing to enhanced clearance of L. monocytogenes in the ecSOD KO mice is increased NO3− production.

The lack of a complete abrogation of differences in liver CFUs in the three groups of mice depleted of neutrophils indicates that there are other factors affected by ecSOD activity. One way in which ecSOD may alter immune responses is through the suppression of proinflammatory cytokine secretion, which is supported by our TNF-α data. It is known that ecSOD has a high affinity for binding to collagen and heparin sulfate in the ECM and plays an important role in protecting tissues from oxidative damage and inflammation. Oxidative stress and the resulting degradation of the ECM can lead to increased inflammation (42, 43). The increased levels of extracellular O2− in the ecSOD KO mice may lead to increased degradation of the ECM and release of hyaluronan. Degraded hyaluronal fragments have been shown to bind to TLR-4 and TLR-2, thus inducing inflammatory responses (44). Therefore, the increased fragmentation of hyaluronan in the ecSOD KO mice may lead to enhanced signaling through TLR-4 or TLR-2, accounting for the increased neutrophil activity, TNF-α secretion, and clearance of L. monocytogenes observed in our model.

It has been previously shown that 129 mice are more susceptible to L. monocytogenes infection than B6 mice (45, 46). Our survival and CFU data obtained using ecSOD congenic mice may explain the observed differences in L. monocytogenes susceptibility between B6 and 129 mice. The 129 mice are genetically distinct from B6 mice in a variety of ways, with the difference in the ecSOD allele being just one example (22). Our current data establish that the ecSOD HI mice (expressing the 129 allele of ecSOD) are more susceptible to L. monocytogenes infection than ecSOD WT mice (expressing the B6 allele of ecSOD). This finding would support the idea that the increased susceptibility of 129 mice to L. monocytogenes infection is due to increased ecSOD activity.

Collectively, our data indicate that ecSOD activity is detrimental during acute bacterial infection. In a clinical setting, our findings could potentially lead to treatments aimed at eradicating pathogens. We would anticipate that suppressing ecSOD activity would increase proinflammatory responses, thus providing a useful therapeutic option to treat infections in conjunction with antibiotics or for antibiotic-resistant strains of bacteria.

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Disclosures
The authors have no financial conflicts of interest.

References


Supplemental Figure 1. A lack of ecSOD does not enhance NK1.1\(^+\) and CD8\(^+\) T cell percentages or production of IFN-\(\gamma\) during LM infection. ecSOD HI, ecSOD WT, and ecSOD KO mice were infected with LM for 1 day. Splenocytes and liver leukocytes were stimulated with HKLM for 4 hours, and the percentages of NK1.1\(^+\) (A) and CD8\(^+\) T (B) cells were determined with flow cytometry. The percentage of NK1.1\(^+\) (C) and CD8\(^+\) T (D) cells producing IFN-\(\gamma\) in the spleen and liver is depicted. Two-way ANOVAs detected significant differences between groups. An ** or *** indicates that the groups differ at \(p < 0.01\) or \(p < 0.001\) respectively. The data are expressed as the mean +/- SEM (n = 3-5/group). These data are representative of two independent experiments.
Supplemental Figure 2. Identification of neutrophils based on expression of Ly6G and CD11b. Liver leukocytes from uninfected ecSOD HI, ecSOD WT, and ecSOD KO mice were stained for Ly6G and CD11b to identify the neutrophil population. Neutrophil percentages were determined by gating on the Ly6G\textsuperscript{+}CD11b\textsuperscript{+} population. The flow plots depicted are representative of 5 individual mice per group. These data are representative of two independent experiments.
**Supplemental Figure 3.** ecSOD activity increases neutrophil apoptosis in the liver at day 3 p.i. with LM. ecSOD HI, ecSOD WT, and ecSOD KO mice were left uninfected or infected with LM for 1 or 3 days. The percentage of apoptotic neutrophils (Annexin V⁺) out of all neutrophils (A) and the total number of neutrophils undergoing apoptosis (B) were determined by flow cytometry in the liver. Two-way ANOVAs detected significant differences between groups. An * or *** indicate that the groups differ at p < 0.05 or p < 0.001 respectively. All data are expressed as the mean +/- SEM (n = 5/group).
Supplemental Figure 4. Administration of anti-Ly6G (1A8) antibody effectively depletes neutrophils. ecSOD HI, ecSOD WT, and ecSOD KO mice were injected with isotype control antibody (A-C) or anti-Ly6G antibody (D-F) one day prior to infection with LM. Three days p.i., neutrophil depletions were confirmed by staining blood leukocytes for Ly6G and CD11b (G). The dot plots depicted are representative of 5 individual mice per group. A one-way ANOVA detected significant differences between groups. An *** indicates that the group differs from isotype control at p < 0.001. The data in (G) are expressed as the mean +/- SEM (n = 5/group). These data are representative of two independent experiments.