Sepsis is a major cause of mortality worldwide. Acute or chonic ethanol exposure typically suppresses innate immunity and inflammation and increases the risk of mortality in patients with sepsis. The study described here was designed to address the mechanism(s) by which acute ethanol exposure alters the course of sepsis. Ethanol administered to mice shortly before Escherichia coli (injected ip to produce sepsis) decreased production of proinflammatory cytokines and chemokines for several hours. Bacteria in the peritoneal cavity decreased over time in control mice and were mostly cleared by 21 h, but in ethanol-treated mice, bacteria increased over time to more than $2 \times 10^8$ at 21 h. Killing of bacteria in macrophages and neutrophils was apparently compromised by ethanol, as the percentage of these cells that had cleared phagocytosed bacteria increased over time in control mice but not in ethanol-treated mice. The roles of TLR4, MyD88, and myeloperoxidase (MPO) were evaluated using mutant or knockout mice, and these experiments indicated that mice with hyporesponsive TLR4 survived better than those with normal TLR4. Lack of MyD88 or MPO did not significantly alter survival in the presence or absence of ethanol. Ethanol decreased survival in all groups. This indicates that the antimicrobial activities induced though TLR4 are dispensable for survival but contribute to lethality late in the course of sepsis. Thus, the effects of ethanol responsible for lethal outcome in sepsis are not dependent on inhibition of TLR4 signaling, as we and others had previously suspected.

Key Words: macrophages; neutrophils; bacterial infection; cytokines; lipopolysaccharide; inflammation.

Sepsis is a complex process involving interactions between the host and the causative microbe. The mortality rate for sepsis ranges from about 12% (Lin et al., 2009) to more than 60%, depending on a variety of factors. In spite of antibiotic and supportive therapy, sepsis is the 10th leading cause of death in the United States (Anonymous, 2007). Acute ethanol consumption is a significant risk factor for mortality in patients with sepsis (Huttunen et al., 2007; McGill et al., 1995), and our mouse model produces similar results (Pruett et al., 2004c). Animal models have also revealed that proinflammatory mediators are important contributors to lethality in sepsis (Lally et al., 2000). However, inhibition of these mediators in clinical trials has typically not improved the outcome significantly for sepsis patients (Marshall, 2008). Characterizing the increase of lethality in sepsis caused by ethanol in a mouse model is a unique approach to identify mechanisms of lethality in sepsis that may be, at least in part, functionally redundant, thereby explaining the ineffectiveness of inhibition of any single inflammatory mediator. The study described here was designed to characterize some of the inflammatory mechanisms associated with lethality in sepsis as an initial step in identifying such mechanisms, which would be potential targets for combination therapies to neutralize more than one inflammatory mediator. Lethality was increased by treating mice with ethanol in a binge-drinking model.

Chonic and acute ethanol exposure inhibits a variety of immunological parameters and decrease resistance to infection (Bagby et al., 2006; Brown et al., 2006). Suppression of inflammatory responses by acute exposure to ethanol is particularly striking in both humans (Gluckman and MacGregor, 1978; Szabo et al., 1999) and animal models (Fitzgerald et al., 2007; Pruett et al., 2004b). The model used in the present study is intended to represent sepsis caused by microbial contamination of the peritoneal cavity (e.g., following penetrating abdominal trauma, ruptured appendix, cirrhosis of the liver, or other conditions that interfere with the normal barrier function of the mucosa). Increased rates of infection have been reported in patients with penetrating abdominal trauma following acute ethanol exposure (Gentilello et al., 1993). Similar results have been reported in burn patients (Germann et al., 1997). Sepsis caused by indigenous bacteria may begin as a polymicrobial infection, but in most cases. only one bacterium is isolated, and Escherichia coli is one of the most frequent (Huttunen et al., 2007). Decreased resistance to infection has also been reported in animal models (D’Souza et al., 2004b).
et al., 1995; Pruett et al., 2004b), but this has not been investigated in as much detail as inhibition of production of particular inflammatory mediators. Studies in which inhibition of inflammatory responses has been implicated as a cause of decreased resistance to microbes have been relatively rare. However, such studies can potentially reveal much about the normal mechanisms by which inflammation promotes bacterial clearance and host survival because the effects of inhibiting a number of those mechanisms simultaneously are revealed by ethanol. In this instance, inhibiting production of several mediators at the same time is beneficial because it will provide an indication of a set of mediators that contribute to lethality. In the present study, evidence is presented indicating that mice treated with ethanol exhibit suppression of several mediators and processes of inflammation early, which is followed by an overgrowth of bacteria and possibly a lethal systemic inflammatory response.

Responses triggered though TLR4 and other Toll-like receptors are inhibited by acute ethanol exposure (Goral and Kovacs, 2005; Pruett et al., 2004b), and many investigators (including the present authors) have suggested that this contributes to decreased resistance to infection. To test this assumption, effects of ethanol in wild-type mice were compared with its effects in mice lacking MyD88, myeloperoxidase (MPO), or with a mutant hyporesponsive TLR4. The results unexpectedly demonstrated that inhibition of TLR4 signaling by ethanol is probably not a major cause of decreased resistance to sepsis in the experimental system used here.

MATERIALS AND METHODS

**Mice, treatments, and procedures.** For most experiments, female C57Bl/6 × C3H F1 mice were used. Female mice were used because males fight when group housed and this causes stress, which can affect the results, and single housing can also cause stress. These mice were obtained though the National Cancer Institute’s Animal Program. They were allowed to recover from shipping stress for at least 2 weeks before beginning experiments, and they were 12–16 weeks old when used. In some experiments, C3H/HeJ mice were used because they have a mutant TLR4 gene, which yields a protein that is almost nonresponsive to bacterial lipopolysaccharide (LPS; the major naturally occurring ligand for TLR4). These mice and a strain matched as closely as possible at every locus other than TLR4, C3H/HeOuJ, were obtained from Jackson Labs (Bar Harbor, ME). These mice were female and were used at 12–16 weeks of age after at least 2 weeks of recovery from shipping stress. Transgenic MyD88-knockout mice on a C57Bl/6 background were kindly provided by Dr Shizuo Akira (Hyogo College of Medicine, Hyogo, Japan) treated as the other strains and used at the same age range. An equal number of C57Bl/6 wild-type mice were purchased from Jackson Labs as controls. MPO-deficient mice (obtained from Jackson Labs) also had a C57Bl/6 background, and C57Bl/6 controls were used. Because of decreased resistance to some microbes, food, water, and bedding were autoclaved before use for both the knockout and wild-type C57Bl/6 mice. Mice were housed in filter top shoebox cages with five mice per cage in a temperature (70–78°F) and humidity (40–60%)-controlled environment. The laboratory animal facility and animal research program at Mississippi State University are accredited by the American Association for Accreditation of Laboratory Animal Care. Mice were housed and used in accord with the National Institutes of Health and Mississippi State University regulations. Sentinel mice housed in the same room as the mice used in this study were negative for all common pathogens of mice during the period of this study.

Mice in various groups were treated as follows: ethanol was administered by gavage at 4 or 6 g/kg using a 32% (vol/vol) solution in tissue culture grade water or water alone as a control; viable E. coli, log phase, grown in Luria Bertani broth, were administered ip. Both dosages of ethanol yield blood ethanol levels that occur in humans (Usoro et al., 1981), and this mouse model for binge drinking developed in this laboratory has been thoroughly characterized (Carson and Pruett, 1996) and used by a number of other investigators (Park et al., 2005). The dosage of E. coli was 1.5 × 10^8 per mouse, which was similar to dosages used by other investigators (Roget et al., 2009).

Mice were observed at least every 6 h after challenge, and mice that were moribund and those with a body temperature less than 27°C were euthanized and counted as dead at each time point indicated. Body temperature was measured by an electronic thermometer with a mice rectal probe (Physitemp, Clifton, NJ). Mice were restrained manually, the probe was lubricated using sterile glycerol and inserted for 5 s (or until the temperature reading stabilized), and results were recorded. In this model of sepsis (as in some cases of sepsis in humans), profound hypothermia is observed. However, in mice that will ultimately survive, hypothermia is less severe (Wei and Pruett, unpublished observations). It should be noted that severe illness typically proceeded to death very quickly, such that most deaths occurred between the 6-h observation points, and only a few cases of moribund mice or mice with very low body temperatures were removed from the study.

Experiments were designed with a group size of 5 for all experiments except survival studies, for which the group size was 10. In survival studies, mice were observed for 72 h (in previous studies, no deaths were observed after 72 h). Mice were treated by gavage with vehicle (water) or ethanol, and 30 min later, they were challenged ip with E. coli (1.5 × 10^8 per mouse). In most experiments, one group of mice, referred to as naive, remained untreated and was not challenged with E. coli. This group served to confirm that the anticipated inflammatory changes were induced by E. coli. Different groups of mice were anesthetized by inhalation of halothane at various times from 1 to 21 h after E. coli challenge and were bled (from the retro-orbital plexus), and peritoneal lavage was performed. Samples of this fluid were used to quantify bacteria by making serial dilutions in LB agar (held at 45°C to prevent solidification, plating, and performing plate counts). Samples were also used for cytospin preparations followed by Wright-Giemsa staining and differential cell counts at 600× magnification with an oil immersion lens. Cells with three or more bacteria associated were referred to as cells with E. coli, and cells with less than three bacteria were referred to as cells without E. coli. This was designed to account for the possibility that some of the bacteria that appeared to be intracellular might actually be on the cell surface. The number of nucleated cells was determined using a sample of peritoneal lavage fluid with cells suspended. Manual lysing reagent was added to lyse the cytoplasmic membrane, leaving nuclei only to be counted. Counts were determined using a Coulter Z1 particle counter (Hialeah, FL).

**Bacteria.** The E. coli strain used in this study was isolated from the colon of one of the mice in our specific pathogen-free colony. It was characterized by the College of Veterinary Medicine Clinical Microbiology Lab as nonpathogenic E. coli. As expected for nonpathogenic bacteria, mice can clear a large number without mortality. However, 1.5 × 10^8 per mouse routinely yields 10–20% mortality, indicating that this is a sufficient dosage to identify decreased resistance to sepsis, which would cause higher mortality. Bacteria for each experiment were prepared starting with a frozen vial, which was one of a set frozen at the same time from the same culture. Bacteria were in log growth phase (as indicated by optical density [OD] at 650 nm), and dosages were estimated using OD measurements and a standard growth curve. This number was verified by serial dilutions and plate counts, and values were always within 10% of the nominal count. This model is expected to be representative of sepsis in humans that begins with loss of gastrointestinal barrier function (caused, e.g., by trauma, appendicitis, diminished liver function, or other conditions). In human peritonitis, a single species of bacteria often predominates, and in
approximately half of cases, E. coli is the species isolated in blood cultures (De Waele et al., 2008). Thus, administration of a single strain of indigenous E. coli in our model allows more controlled conditions than cecal ligation and puncture but yields peritonitis and sepsis similar to that observed in humans.

Cytokine and chemokine assays. Concentrations of cytokines and chemokines in serum were quantified using multiplexed bead array kits (with standards for each cytokine or chemokine) from Bio-Rad (Hercules, CA) with a BioPlex analyzer (Bio-Rad).

Statistical analysis. The log-rank test, as implemented by Prism 4.0 software (GraphPad Software, LaJolla, CA), was used to compare survival data. The same software was used to perform one-way ANOVA, and the means of all groups were compared using the Newman-Keuls post hoc test. A p value < 0.05 was considered significant.

RESULTS

Acute administration of ethanol increased mortality in a mouse model for peritonitis/sepsis. This was a consistent finding, but the exact percentages of survival in control and ethanol-treated animals varied to some degree, as typical for infectious disease models. The results shown in Figure 1 indicate survival results using 10 mice per group that were gavaged with vehicle or ethanol then challenged with E. coli. In each experiment, ethanol significantly decreased survival at 72 h following challenge with the same dosage of non-pathogenic E. coli. The mortality at 72 h in this model ranged from 10 to 30% in control animals in three independent experiments with B6C3F1 mice and from 50 to 100% in ethanol-treated mice (6 g/kg 30 min before bacterial challenge).

Results shown in Figure 2 indicate the number of viable E. coli isolated at various times after challenge from the peritoneal cavity of control and ethanol-treated mice. The results demonstrate that after a brief increase in number, bacteria are progressively cleared over time in control mice. In contrast, the bacteria continue to grow though 21 h in ethanol-treated mice. The SEM increases at the later time points in ethanol-treated mice. This very likely reflects the fact that some of the mice in the ethanol-treated group will survive (as in Fig. 1). Survivors may have begun clearing bacteria by 21 h, whereas nonsurvivors would not be expected to do this.

As we reported previously, most of the cells in the peritoneal cavity of normal B6C3F1 female mice are macrophages (Fig. 3A). In contrast, 18 h after challenge with E. coli, the predominant cell type was the neutrophil in control mice and very few bacteria remained (Fig. 3B). In contrast, bacteria were remarkably abundant in mice treated with ethanol before E. coli challenge (Fig. 3C) due to either increased phagocytosis or decreased clearance of the bacteria. There was also a lower percentage of neutrophils than in the control group (shown in detail in Fig. 4), indicating impaired chemotaxis or migration of neutrophils to the initial site of infection or increased death of neutrophils.

Although the numbers of bacteria phagocytosed by each macrophage or neutrophil could not be determined precisely by the type of microscopic analysis done here, quantifying the overall process of phagocytosis and clearance was accomplished by discriminating between neutrophils/macrophages with associated bacteria and the ones without bacteria. Because it was
possible that some of the bacteria observed were associated with phagocytes on the cell surface and not phagocytosed, a criterion of three bacteria per cell was used to classify a cell as positive for bacteria. Cells with less than three associated bacteria were classified as negative. This is not intended as precise quantitation of phagocytosis but was done just to give a general impression of how the phagocytosis of bacteria by macrophages/neutrophils after \textit{E. coli} challenge is affected by ethanol. Results of such an analysis are shown in Figure 4. The percentage of macrophages with bacteria increased from 1 to 4 h in control mice, probably reflecting continued phagocytosis. After 4 h, the percentage of macrophages with bacteria decreased in control mice as bacteria were killed and digested by the phagocytic cells. Ethanol increased the percentage of macrophages with bacteria throughout the time course. At the early time points (1–4 h), this could be due to increased phagocytosis or decreased clearance because the number of cells with bacteria depends on the rate of phagocytosis and the rate of clearance. However, at later times, it seems unlikely that enhanced phagocytosis could explain the increased number of bacteria because bacteria had mostly been phagocytosed by then in the ethanol-only group. Thus, it seems more likely that the increased number of cells with bacteria (which corresponds with the increased bacterial number overall shown in Fig. 2) is due to macrophage and/or neutrophil dysfunction leading to inefficient bacterial killing. In control mice, the percentage of neutrophils in the peritoneal cavity increases over time, but this increase was inhibited by ethanol, which is consistent with inhibition of some of the chemokines that attract neutrophils (see next paragraph).

The effects of ethanol on cytokine and chemokine production at various times after \textit{E. coli} challenge are indicated in Figure 5. Most of the cytokines and chemokines tested here were significantly decreased (all but interleukin [IL]-10 and IL-1\(\beta\)) at one or more time points by one dose of ethanol 30 min before challenge with \textit{E. coli}. It is particularly interesting that for IL-1\(\beta\) and IL-6, two cytokines strongly associated with inflammation and acute phase responses, the concentration was increased at 21 h, even if it was significantly decreased at earlier times. This is consistent with the much greater bacterial load in the ethanol-treated mice at 21 h than at any other time for any other group (Fig. 2). It is not clear why this was not the case for other cytokines, but there may be a regulatory mechanism that prevents excessive production of these cytokines. The results are also consistent with the known sequence of cytokine production with peak concentrations of tumor necrosis factor-\(\alpha\) produced early, interferon (IFN)-\(\gamma\) considerably later, and most others at an intermediate time.

To examine the role of LPS acting through TLR4 and the role of other TLRs in this system, mutant and transgenic mice were used. The C3H/HeJ mouse strain has a mutation in TLR4 that renders it hyporesponsive to LPS (Cross \textit{et al.}, 1995). Therefore, this strain was used to evaluate the role of TLR4 in this experimental system. The adapter molecule MyD88 can initiate TLR signaling for all TLRs except TLR3 (Johnson \textit{et al.},
Thus, MyD88-knockout mice were used to assess the role of TLRs in general (except TLR3) in this experimental system. The results shown in Figure 6 demonstrate that TLR4 is required for lethality but apparently is dispensable for survival (and presumably for bacterial clearance). Ethanol decreased survival in both TLR4-mutant C3H/HeJ mice and the corresponding wild-type strain. This suggests that the inhibition of TLR4 signaling by ethanol is probably not a major cause of increased mortality because C3H/HeJ mice, which have even less TLR signaling though LPS than ethanol-treated C3H/HeOuJ mice, survive better than C3H/HeOuJ mice with or without ethanol treatment. Survival of C3H/HeOuJ mice was less than noted in Figure 1 for B6C3F1 mice. This is probably due to genetic differences between resistance-related genes in the C3H and C57Bl/6 (the wild-type mice for MyD88 knockouts and MPO knockouts were C57Bl/6). This suggests that complementary genes enhance survival in the B6C3F1 hybrid and that resistance is less effective in C57Bl/6 mice. Absence of MPO did not significantly decrease survival but caused a nonsignificant increase in survival in control groups (Fig. 6). Ethanol significantly and similarly decreased survival in both wild-type and MPO-knockout mice, suggesting that MPO is not critical for survival in this model and that MPO does not have a major role in the inhibition of survival caused by ethanol.

The results shown in Figure 7 indicate that LPS is a major contributor to the induction of cytokines and chemokines by E. coli. The production of cytokines and chemokines was ~2- to 15-fold greater in C3H/HeOuJ mice than in the TLR4-mutant C3H/HeJ mice. Thus, the poor response of C3H/HeJ mice to LPS substantially decreases the response of these mice with regard to all tested cytokines and chemokines, even though gram-negative bacteria have components that can activate many receptors in addition to TLR4. The results shown in Figure 7 also indicate that ethanol alone (at 6 g/kg) does not induce these cytokines or chemokines to concentrations significantly greater than found in control mice and that ethanol at 4 g/kg significantly modulates production of most cytokines and chemokines. This is of interest because, in this mouse model for binge drinking, a dosage of 4 g/kg produces a peak blood ethanol concentration of ~65 mM (Carson and Pruett, 1996), a concentration not uncommon in humans (Jones and Holmgren, 2009; Urso et al., 1981).

DISCUSSION

The results presented here demonstrate that ethanol-treated mice challenged ip with nonpathogenic E. coli exhibit decreased production of most proinflammatory cytokines and chemokines at early time points, decreased attraction of neutrophils to the peritoneal cavity, decreased clearance of bacteria by macrophages and neutrophils in the peritoneal cavity, and increased mortality. This suggests a scenario in which ethanol inhibits the initial inflammatory response to E. coli, which decreases the clearance of bacteria in the first few hours after challenge. After ethanol has been cleared (~6 h after dosing) (Carson and Pruett, 1996), the increased number of bacteria induces an inflammatory response that probably contributes to the lethal outcome observed. Further studies are needed to determine the role of particular inflammatory mediators in lethal outcome in this experimental system.

Some portion of the inhibition of production of these inflammatory cytokines by ethanol could be mediated by stress hormones such as corticosterone that can be induced by ethanol. However, our recent studies indicate that ethanol-
FIG. 5. Effect of ethanol on *Escherichia coli*-induced concentrations of selected cytokines and chemokines in serum. Values shown are means ± SEM, and the group size was 5. Significant differences between control and ethanol-treated groups at each time point (as determined by one-way ANOVA followed by Newman-Keuls post hoc) are indicated by *p < 0.05, **p < 0.01, ***p < 0.001.
induced glucocorticoids and catecholamines do not contribute to the inhibition of cytokine production induced through TLR3 or TLR4, with the possible exception of a partial role in inhibition of IL-6 production (Glover and Pruett, 2006; Glover et al., 2009). Thus, it seems unlikely that the stress response is critical to the effects of ethanol on the pathogenesis of sepsis in this model system.

Several investigators have reported that ethanol inhibits TLR signaling (Dai et al., 2005; Szabo et al., 2007), and it seemed likely that this was involved in the decreased resistance to infection associated with acute ethanol exposure. However, the results presented here indicate that survival was enhanced in the absence of fully functional TLR4, so inhibition of TLR4 signaling is unlikely to be the major mechanism by which ethanol suppresses resistance to lethality in this experimental system. This is also suggested by the observation that ethanol decreases survival time and/or percentage in TLR4-knockout mice to a similar degree as in wild-type mice, indicating that targets other than TLR4 are involved in lethality. There are cytoplasmic receptors (Cartwright et al., 2007) as well as membrane-bound receptors that respond to LPS and other TLRs that respond to other components of gram-negative bacteria. It is possible that in the absence of TLR4, these receptors mediate sufficient response to lead to bacterial clearance but not to a lethal overproduction of inflammatory mediators. Our results with regard to cytokine and chemokine production in TLR4-mutant and wild-type mice support this idea (Fig. 7). However, it is also possible that decreased cytokine responses in the first few hours after challenge in mice treated with ethanol allow overgrowth of bacteria which becomes lethal after the ethanol has been eliminated. This lethality may be related to TLR4-induced overproduction of inflammatory mediators, but this cannot be the only mechanism because TLR4-mutant mice treated with ethanol have a similar mortality profile as wild-type mice treated with ethanol. This strongly suggests that lethal effects of sepsis are mediated through other receptors and may not involve well-recognized inflammatory mediators because many of these are decreased in concentration in TLR4-mutant mice. Other receptors that may be involved in the pathogenesis include TLR3 and TLR2. There are reports that TLR3 contributes to lethality in sepsis by sensing the RNA released from necrotic cells during sepsis and amplifies the secondary inflammatory responses (Cavassani et al., 2008). Similarly, TLR2-dependent signaling has been shown to be critical for host resistance to bacterial infection both in animal studies and humans (Alves-Filho et al., 2009; Ferwerda et al., 2009; Mancuso et al., 2004; Murphey et al., 2008). Interestingly, ethanol also suppresses TLR3 and TLR2 signaling as reported in our previous study (Pruett et al., 2004a,c).

It remains possible that inhibition of TLR4 signaling by ethanol (or TLR4 mutation) does play an important role in decreased resistance to lower dosages of bacteria, as reported for C3H/HeJ mice (Alves-Filho et al., 2006), and that clearance of higher dosages of bacteria could be delayed (van Westerloo et al., 2005) by inhibition or lack of TLR4, even if ultimately effective. Results for enteropathogenic E. coli indicate that C3H/HeJ mice do not survive as well as wild-type mice when a low dose of bacteria is administered (Cross et al., 1995). Similar results were obtained by another group when mice were treated with a sublethal challenge dose of bacteria (Alves-Filho et al., 2006). However, when mice were treated with a greater dose of bacteria (lethal for a major percentage of wild-type mice), a much higher percentage of C3H/HeJ mice survived than wild type, as noted in our study. Another group
very recently reported similar results, indicating that TLR4-mutant mice have increased resistance to a lethal outcome in *E. coli* sepsis caused by a high dosage of *E. coli* (Roger et al., 2009). Thus, it seems that the role of TLR4 in resistance to sepsis and lethality in sepsis depends on the initial challenge dose of bacteria.

In contrast to the clear protective effect of the absence of functional TLR4 in the C3H/HeJ mice, the lack of MyD88 in MyD88-knockout mice did not significantly improve survival (although there was a tendency in that direction). This leaves open the interesting possibility that the lethal effects of sepsis involve signals transmitted through the alternate adaptor molecule used by TLR4 (and by TLR3 as its only adaptor), TIR-domain-containing adaptor inducing interferon-beta (TRIF). Results indicating that mice lacking TRIF survive sepsis better than wild-type mice are consistent with this possibility (Weighardt and Holzmann, 2007). Also consistent with this idea is a recent report indicating that IFN-β (the production of which is mediated by the TRIF pathway) is required for release of high-mobility group box 1 protein and lethality in sepsis (Kim et al., 2009). We previously reported that IL-12 and IL-10 production in response to LPS is decreased almost to the lower limit of detection (Pruett et al., 2005). The finding that MyD88 is dispensable in survival of

![Graphs showing cytokine concentrations in serum](https://academic.oup.com/toxsci/article-abstract/117/2/314/1642730)
E. coli infection is consistent with findings in humans, in which individuals with a genetic defect in MyD88 were less resistant only to pyogenic bacterial infections, not other to other types of infections (von Bernuth et al., 2008). In apparent contrast to these findings, Peck-Palmer et al. (2008) reported that MyD88-knockout mice do not survive as well as wild-type mice in a cecal ligation and puncture model of sepsis. Mortality in that study was 40% in wild-type C57Bl/6 mice, whereas it was 100% in our study (Fig. 6). Thus, the difference in results may simply reflect different outcomes with high versus lower dosages of bacteria, as reported by other investigators (Alves-Filho et al., 2006; Mancuso et al., 2004) or differences between polymicrobial sepsis and E. coli sepsis.

The observation that mice lacking MPO were not significantly more susceptible to sepsis-induced mortality than wild-type mice was not entirely unexpected because similar results have been reported previously (Brovkovych et al., 2008). However, the finding that wild-type and MPO-knockout mice are similarly and significantly susceptible to ethanol-induced mortality in sepsis indicates that ethanol does not act primarily by inhibiting expression or function of MPO. We had considered this as a potential mechanism because our microarray analysis indicated that early after E. coli challenge MPO expression was decreased by ethanol (data not shown).

The effects of ethanol on survival were similar for wild-type mice, MyD88-knockout mice, TLR4-mutant mice, and MPO-knockout mice. Significantly decreased survival percentage or survival time was noted in all cases. These results indicate that inhibition of TLR4 signaling through MyD88 (which does occur) is not the major mechanism by which ethanol decreases host resistance. They also demonstrate that inhibition of MPO by ethanol is not a major mechanism for decreased resistance to sepsis in this system.

The cellular targets for decreased antibacterial effectiveness seem to be macrophages and/or neutrophils. Although the results reported here indicate that phagocytosis occurred in ethanol-treated mice (Fig. 3), it was evident that killing and degradation of the bacteria in both macrophages and neutrophils were substantially decreased (Fig. 4). Microarray results from this experimental model indicate an inhibition by ethanol of a variety of genes coding for proteins involved in antibacterial effects of phagocytic cells (manuscript in preparation). Others have reported that acute ethanol exposure decreases host resistance by inhibiting upregulation of key antimicrobial effectors (e.g., nitric oxide synthase 2) (Greenberg et al., 1999). Future studies will be conducted to determine which of these are involved in the inhibition of phagocyte antimicrobial function.

There are at least two obvious potential applications suggested by these results for treatment of sepsis in humans. For E. coli-mediated sepsis, inhibition of TLR4 signaling may be a useful therapeutic approach because decreased response to TLR4 in a mutant mouse strain improved survival substantially (Fig. 6). This conclusion is consistent with another recently published report (Roger et al., 2009). This approach has the advantage of decreasing responses of several mediators of inflammation, not just one. Also, greater efficacy of therapy in sepsis might be obtained using treatments that inhibit combinations of the cytokines overproduced in the later stages of sepsis (e.g., IL-1β and IL-6) (Fig. 5) and that augment key mediators that are not known to contribute to lethality at intermediate stages of sepsis (e.g., granulocyte-macrophage colony-stimulating-factor) (Fig. 5). It is clear that this animal model is associated with rapid lethality, which is different from the typical course of sepsis in humans, which lasts 15–17 days, possibly due to antibiotic therapy that is not included in the mouse model (Angus et al., 2001). However, there is evidence that intervention of various types early after the diagnosis of sepsis is more effective than later intervention (Moore et al., 2009). Thus, the patterns noted in the animal model may be useful, but the results reported here would suggest that the timing of intervention is critical and will need to be determined in studies with human subjects.

In summary, the results presented here demonstrate conclusively that inhibition of TLR4 signaling, MPO expression, and MyD88 expression or function do not represent major mechanisms by which ethanol inhibits resistance to sepsis. The results instead suggest that the primary defect caused by ethanol is in the killing of phagocytosed bacteria by macrophages and neutrophils. Ethanol inhibits cytokine production early, but similar decreases in mice with a defective TLR4 did not decrease resistance to sepsis. These findings highlight how little is known about the quantitative relationships and time dependence of cytokines and chemokines with regard to host resistance to sepsis.

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