Published in final edited form as:

Shock. 2011 April ; 35(4): 403–410. doi:10.1097/SHK.0b013e31820217c9.

PROLONGED CHEMOKINE EXPRESSION AND EXCESSIVE NEUTROPHIL INFILTRATION IN THE LUNGS OF BURN-INJURED MICE EXPOSED TO ETHANOL AND PULMONARY INFECTION

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Abstract

Pulmonary infections are a major cause of mortality in the critically ill burn patient. Alcohol consumption before burn increases the risk of pulmonary infection. Previously, we have shown an elevated mortality and lung pathology in mice given ethanol before burn and intratracheal infection relative to controls. Here we examine the cellular composition at 24 and 48 h in the circulation and the alveoli of infected mice given alcohol and burn. At 24 h after injury, blood neutrophils obtained from mice exposed to ethanol before burn and infection were 2-fold above those of the experimental controls ($P < 0.05$). By 48 h, the number of circulating neutrophils decreased and was comparable to levels found in untreated animals. Moreover, at 24 h, bronchoalveolar lavage cells obtained from all treatment groups had similar frequencies and contained 80% neutrophils regardless of treatment. In contrast, the following day, neutrophils were elevated 2-fold only in the alveoli of infected burn animals and 5-fold when ethanol preceded the injury ($P < 0.05$). These data were confirmed by immunofluorescence microscopy using a neutrophil-specific marker ($P < 0.05$). Levels of neutrophil chemoattractants, KC and macrophage inflammatory protein 2, and the cytokine, IL-1 β , were 2-fold greater in the lungs of infected mice given burn, regardless of ethanol exposure, relative to infected sham injured animals ($P < 0.05$). Like the number of neutrophils, by the second day after injury, KC and macrophage inflammatory protein 2 remained 5-fold higher in the animals given ethanol, burn, and infection, when compared with other groups ($P < 0.05$). A similar pattern was seen for pulmonary levels of IL-1 β ($P < 0.05$). Additionally, a reduction in neutrophil apoptosis was observed at the 24-h time point in infected mice exposed to ethanol and burn ($P < 0.05$). Targeting proinflammatory mediators in mice exposed to ethanol before burn and infection may help alleviate prolonged neutrophil accumulation in the lungs.

Keywords

Alcohol; inflammation; KC; macrophage inflammatory protein 2; cytokines; apoptosis; *Pseudomonas aeruginosa*; acute lung injury

INTRODUCTION

Each year, about 2 million burn injuries occur in the United States (1). There are 300,000 hospitalizations resulting from these injuries, as reported by the American Burn Association (1), and despite improvements in burn care, more than 8,000 patients die annually (1). Prevention of burn wound infections with early excision, rapid wound closure, better resuscitation techniques, and aggressive antibiotic therapies has been the main contributor to the observed decline in mortality (2). However, although advancements in the prevention of burn wound infections have been made, infections at remote organ sites, such as the lungs, have remained constant (3, 4). Susceptibility to nosocomial infections, which are often antibiotic resistant and can develop into bacteremia, pneumonia, and eventually sepsis-related multiple organ failure, is still prevalent and is the leading cause of mortality in burn patients (1).

The consumption of alcohol before trauma is considered a risk factor contributing to increased morbidity and mortality (5). Nearly 50% of adult patients admitted to the hospital for burn injury sustain their injuries under the influence of alcohol (6). Burn patients with detectable blood alcohol level at the time of injury are more susceptible to infectious complications than patients with comparable injuries who have not been drinking (5, 7, 8). Furthermore, severely injured trauma patients, or some with the combined insult of alcohol exposure and traumatic injury, are particularly susceptible to pulmonary infections because of their suppressed immune state and frequent need for mechanical ventilation. Interestingly, among the burn patients, those with prior alcohol exposure have higher incidence of ventilator support as well as prolonged duration on the machine (9).

Hospital-associated pneumonia accounts for 15% of all hospital-acquired infections and for 25% of all infection in the intensive care unit (10). According to Fagon et al., the gram-negative bacilli *Pseudomonas* and *Acinetobacter* account for 73% of nosocomial pneumonia in mechanically ventilated patients (11). Accounting for the high morbidity and mortality of *Pseudomonas aeruginosa*-infected burn patients and the emergence of resistant bacterial strains, it is important to understand the innate immune response to be able to improve treatment, in addition to traditional antibiotic regimens.

As neutrophils are part of the first line of the body's defense against infections, their proper recruitment to sites of injury or infection, bacterial killing, and their clearance from the site of infection are critical steps in the process of pathogen elimination and disease resolution. Hence, in the studies described here, the early pulmonary inflammatory response in ethanol-exposed and burn-injured mice given an intratracheal bacterial challenge with *P. aeruginosa* was examined. Previous studies in our laboratory demonstrated an increased susceptibility to a *P. aeruginosa* pulmonary infection after mice were exposed to ethanol and burn injury (12). To explain this increased susceptibility to lung infections in mice given ethanol and burn injury, our current study investigated pulmonary neutrophil infiltration and neutrophil apoptosis at the infection site. Gaining an understanding of the mechanisms that contribute to decreased pulmonary bacterial clearance in infected mice given ethanol and burn injury may uncover novel therapeutic targets that will help to improve survival in all burn patients with infectious complications of the respiratory system.

MATERIAL AND METHODS

Animals

Male C57BL/6 mice, 8 to 10 weeks old, were obtained from Harlan Laboratories (Indianapolis, Ind) at least 7 days before experimentation. They were housed with food and water available *ad libitum* at the Loyola University Medical Center Animal Facility in rooms that were temperature and humidity controlled on a 12-h light-dark (7:00 AM to 7:00 PM) cycle. All animal studies described here were performed according to the Animals Welfare Act and the Guide for the Care and Use of Laboratory Animals, National Institutes of Health. The following studies were also carried out and completed with strict accordance to the rules and regulations set by Loyola University Chicago Animal Care and Use Committee.

Induction of ethanol exposure, burn injury, and pulmonary infection

Before each experiment, mice were weighed, and those weighing 22 to 27 g were used in all studies. Animals were given ethanol (1.2 g/kg or saline vehicle) at a dose designed to elevate the blood alcohol concentration to 150 mg/dL at 30 min after i.p. injection, as previously described (12, 13). Mice were anesthetized with nembutal (50 mg/kg i.p.), the dorsum shaved, and the animal was placed into a plastic template designed to expose 13% to 15% of the total body surface area of the animal's back as previously described (14). Full-thickness scald injury was achieved by immersing the opening of the plastic template with the animal's dorsum in a 94°C to 96°C water bath for 8 s according to a modified protocol (15). As a control, sham animals were anesthetized, shaved, and immersed in room temperature water. To compensate for fluid loss and prevent circulatory shock, all animals received 1 mL of body temperature saline i.p. immediately after burn injury (16). While still under anesthesia, animals received an intratracheal inoculation with *P. aeruginosa*, as described previously (17, 18) with minor modifications (12). Briefly, the mice were placed in the supine position and were inoculated with 100 µL of *P. aeruginosa* (2,000 colony-forming units) followed by 100 µL of air using polyethylene tubing 60 attached to a 1-mL syringe. The animals were then placed on their abdomen on an incline in their cage. Body temperature was maintained at physiological levels by placing the cages on heating pads until the animals were awake from anesthesia. Because hormones, such as corticosterone, are differentially produced during sleeping and waking hours and many of these hormones affect the inflammatory response, all experiments were carried out between 8 and 10 AM, when nocturnal animals have low serum levels of corticosterone. The studies included four experimental groups: sham vehicle, sham ethanol, burn vehicle, and burn ethanol; all four groups were infected intratracheally after sham or burn injury. No other therapeutic intervention was provided, as administration of anti-inflammatory or analgesic medication may introduce confounding factors into the assessment of inflammatory responses. At any of the given time points before collection of tissues and fluids, mice were killed using carbon dioxide (CO₂) inhalation and cervical dislocation.

Measuring peripheral blood neutrophils

Peripheral blood neutrophils were measured in whole blood obtained by cardiac puncture from the left ventricle. Immediately after killing, 100 µL of blood was collected in an EDTA tripotassium, salt-coated capillary microvette (Sarstedt, Nümbrecht, Germany). Blood mixing occurred for 10 min on a slow-speed shaker, after which the blood was analyzed on a Hemavet HV950FS (multispecies hematology system) instrument (Drew Scientific, Oxford, Conn). Calculations of total and differential white blood cell (WBC) counts were obtained.

Detecting leukocytes in lung tissue by immunofluorescence

Lungs were removed at the time of killing, inflated with 25% O.C.T. freezing medium (Tissuetech INC, Miami, Fla), and embedded for frozen sectioning. The lung sections were fixed in acetone and blocked with normal goat serum (19). Sections were first incubated with 1 µg/mL of rat anti-Ly6C/6G (Gr-1) antibody (Invitrogen, Carlsbad, Calif) followed by 4 µg/mL of goat anti-rat IgG conjugated to Alexa Fluor 488 (Invitrogen). Because monocytes and immature macrophages express part of the Gr-1 (Ly6C) molecule (20), the sections were dual stained with biotinylated anti-MOMA-2 antibody (0.2 µg/mL; BMA Biomedicals, Augst, Switzerland), a pan-macrophage marker, and detected with Cy3 streptavidin (2 µg/mL; Invitrogen). The macrophage antibody MOMA-2 was selected for this experiment as it has been shown previously by our laboratory and others to work very well on fresh frozen tissue sections (19). Using fluorescent microscopy, total pixel fluorescence of neutrophils (designated as Ly6C/6G [Gr-1]⁺, MOMA-2⁻ cells) and macrophages (designated as Ly6C/6G [Gr-1]⁻, MOMA-2⁺) was calculated using the Axio Vision Rel 4.5 (Zeiss, Thorndale, Calif) software. Total pixel fluorescence was collected across 10 low-power field images per sample, after which average pixel fluorescence was determined for each sample. To verify the specificity of the primary antibodies, and to determine the background fluorescence due to unspecific binding, both secondary antibodies (goat anti-rat IgG conjugated to Alexa Fluor 488 and Cy3 streptavidin) were incubated on separate lung sections individually without primary antiserum.

Performing bronchoalveolar lavage

To characterize the cell populations in the alveolar space, bronchoalveolar lavage (BAL) was performed at 24 and 48 h after injury, and cells were collected from the lungs as previously described (21). In brief, immediately after killing, the animals were placed in the supine position, the trachea exposed, and the lungs lavaged with cold phosphate-buffered saline. The procedure was repeated six to seven times, for a total collection of BAL fluid of about 5 mL per animal. Lavage fluid containing cells was centrifuged at 250g for 3 min, and the cells were resuspended in the appropriate buffer for subsequent experiments.

Measuring alveolar leukocyte counts by flow cytometry

Flow cytometry was performed as previously described (22). Briefly, cells obtained by BAL were resuspended in 1 mL of flow buffer (phosphate-buffered saline + 1% bovine serum albumin + 0.1% sodium azide) (23), counted using trypan blue for the exclusion of dead cells, and the concentration was adjusted to 1×10^6 cells/mL. To block unspecific binding to the Fcγ II/III receptor, cells were incubated for 20 min at 4°C with anti-CD16/32 antibody. After blocking, cells were stained using anti-mouse antibodies: phycoerythrin (PE)-conjugated rat anti-mouse Ly6C/6G (Gr-1) (2 µg/mL, clone RB6-8C5; BD Pharmingen, San Diego, Calif) and allophycocyanin (APC)-conjugated rat anti-mouse F4/80 (clone BM8) (1 µg/mL; eBioscience, San Diego, Calif). The rat anti-mouse F4/80, clone BM8, was used for this experiment because it is highly specific for resident macrophage populations and works very well when staining isolated cells for flow cytometry (24). Antibody staining was carried out for 30 min at 4°C protected from light, after which cells were washed twice with flow buffer and analyzed within 1 h or fixed with 1% paraformaldehyde for an overnight analysis. Each experiment included an unstained sample and a single-color stain for each antibody used in the experiment. Fluorescence was measured by flow cytometry using the BD FACS Canto 6-color flow cytometer or BD FACS Canto II 8-color flow cytometer (BD Biosciences, San Jose, Calif), and data were analyzed using the TreeStar Flow-Jo 8.3 software (TreeStar Inc, Ashland, Ore).

Detecting macrophage inflammatory protein 2 and KC levels in the lung

To determine the amount of macrophage inflammatory protein 2 (MIP-2) and KC in the lungs, in separate sets of animals, the entire lung was homogenized in protease inhibitor cocktail (Roche Applied Sciences, Indianapolis, Ind) while kept on ice. After homogenization, the samples were sonicated, aliquoted, and flash frozen in liquid nitrogen (15, 25). Aliquoted sample of the homogenates was used to measure the levels of MIP-2 and KC by enzyme-linked immunosorbent assay according to the manufacturer's specifications (R&D Systems, Minneapolis, Minn). Total protein content in the lung homogenate used for the experiment was determined by BioRad protein assay (BioRad Laboratories, Hercules, Calif) as previously described (26). Final concentration of each chemokine is expressed in picograms per milligram of protein.

Assessment of apoptosis in neutrophil isolated by BAL

Bronchoalveolar lavage cells were isolated at 24 h after injury, washed, and counted on a hemocytometer using trypan blue for the exclusion of dead cells. The cell concentration was adjusted to 1×10^6 cells/mL. Active caspase 8 and active caspase 9 were measured using CaspaTag Caspase 8 or CaspaTag Caspase 9 (Chemicone, Temecula, Calif). A fluorochrome inhibitor of either caspase 8 or caspase 9 covalently binds cysteine residues on the large subunit of the active caspase heterodimer, thereby inhibiting further enzymatic activity. The fluorescent signal is then a direct measurement of the amount of active caspase (27).

The active amount of each caspase in the samples was established using flow cytometry as follows. To each sample, 10 μ L of fluorochrome inhibitor of caspase (FLICA) was added to 300 μ L of cells. The cells were incubated for 1 h at 37°C and 5% CO₂ protected from light. After incubation, the cells were washed twice with a wash buffer provided in the assay kit to remove unbound FLICA. To distinguish between apoptotic and dead cells, propidium iodine (PI) was added to each sample. Unstained, single-color and bicolor cell suspensions were used as controls. Apoptosis was induced in cells by adding phorbol myristate acetate (PMA) (100 ng/mL) to a cell suspension. This sample was used as a positive control. All samples and controls were immediately analyzed using an 8-channel BD FACS Canto flow cytometer (BD Biosciences), and fluorescence was recorded using the fluorescein isothiocyanate channel.

Statistical analysis

Statistical comparisons were made between infected animals in the sham vehicle, sham ethanol, burn vehicle, and burn ethanol groups. Differences were noted among treatments, and multiple comparison tests were conducted using ANOVA. Tukey-Kramer multiple-comparisons post hoc test was used following significant results at the $P < 0.05$ level after ANOVA analysis.

RESULTS

WBC analysis

To determine whether total WBC counts and total neutrophil counts were elevated at 24 and 48 h after ethanol burn and infection, whole blood was obtained by cardiac puncture and analyzed on a veterinary, whole-blood cell counter. In our hands, total WBC counts in unmanipulated animal were about 8,000 cells/ μ L (Fig. 1). Normal range of murine WBC counts can range from 2,000 to 10,000 cells/ μ L (28). In the first 24 h after injury, the total WBC count was elevated 2-fold in the blood of mice exposed to ethanol before burn and infection when compared with the other treatment groups ($P < 0.05$). By 48 h, the circulating WBCs in all treatment groups were not significantly different from WBC levels

established in unmanipulated animals (Fig. 1). The increased number of WBCs observed at the 24-h time point in infected mice exposed to ethanol before burn was predominantly due to elevation in circulating neutrophils (Fig. 2). There was a 3-fold increase in neutrophils in animals treated with ethanol before burn and infection when compared with unmanipulated animals, as well as a 2-fold increase relative to infected sham animals regardless of ethanol exposure, and infected burn animals given saline vehicle ($P < 0.05$) (Fig. 2). Similar to the decline in WBCs observed at 48 h after injury, peripheral blood neutrophils returned to normal levels by 48 h in all treatment groups. Other leukocyte subsets were not found to be significantly different between treatment groups at these time points (data not shown).

Leukocyte infiltration in the lung

To characterize the inflammatory leukocytes in the lungs of infected mice exposed to ethanol before burn, frozen lung sections were immunostained with a rat anti-Ly6C/6G (Gr-1) antibody (29). Because peripheral blood monocytes are Ly6C⁺, and recent studies have described that Ly6C⁺ monocytes enter inflammatory tissues retaining this marker (30), we coimmunostained frozen lung sections with a rat anti-MOMA-2 antibody, a pan-monocyte/macrophage marker. Cells expressing only Ly6C/6G (Gr-1) and negative for MOMA-2 were designated as neutrophils. Representative images for all treatment groups are shown (Fig. 3).

In parallel with the hematoxylin-eosin analysis showing an increased leukocyte infiltration at 48 h in the lungs of ethanol-exposed mice given burn and infection (12), we found that there was a 2-fold increase in the number of Ly6C/6G (Gr-1)⁺ and MOMA-2⁻ cells (neutrophils) in the lungs of infected mice given ethanol exposure and burn injury when compared with the lungs of infected, burn-injured mice, and 5-fold higher than seen in infected, sham mice regardless of ethanol exposure ($P < 0.05$) (Fig. 4A). The resident macrophage population designated MOMA-2⁺ and Ly6C⁻ was not affected by ethanol, burn, or the combined injury, and no significant differences were observed between groups (Fig. 4B).

Neutrophil accumulation in pulmonary airway

Neutrophils migrate into the airway in response to a bacterial pathogen (31). In this study, the inoculum of *P. aeruginosa* was instilled intratracheally. To determine if neutrophils were accumulating in the alveolar space, BAL was performed at 24 and 48 h after ethanol exposure, burn, and pulmonary infection. Using flow cytometry, the BAL of healthy animals, in the absence of injury or infection, was found to consist of greater than 95% alveolar macrophages (data not shown). As expected, at 24 h after the bacterial challenge, the frequency of neutrophils (Gr-1⁺ F4/80⁻ cells) in the alveolar space increased to 70% to 85% of the total BAL cell population (Fig. 5). There was no difference in neutrophil accumulation in the lungs at this time point, regardless of ethanol and burn injury. In contrast, at 48 h after injury, the number of neutrophils in the alveoli remained elevated only in infected mice exposed to burn (Fig. 6A). Neutrophils in the BAL of infected burn-injured mice were 2-fold greater than that of infected sham mice regardless of ethanol exposure ($P < 0.05$) (Fig. 6B). Furthermore, neutrophils from animals exposed to ethanol before burn and infection were elevated an additional 3-fold when compared with infected burn-injured mice ($P < 0.05$) (Fig. 6B).

Chemokine and cytokine levels in lung homogenates

In the first 24 h after injury, the neutrophil chemoattractant chemokines, KC and MIP-2, were significantly elevated after infection in both burn groups when compared with infected sham groups, regardless of ethanol exposure (Fig. 7). At this time point, there was a 3-fold increase in the level of both mediators in infected burn animals regardless of ethanol

exposure and when compared with either sham-injured group with lung infection. However, at 48 h after injury, chemokine levels were elevated only in infected animals exposed to ethanol before burn (Fig. 7A) ($P < 0.05$). This magnitude of KC and MIP-2 was comparable to what was seen in the infected burn-injured groups at 24 h, 3-fold greater than that of all other groups.

At 24 h after injury and infection, there was a 3-fold increase in the pulmonary level of the proinflammatory cytokine, IL-1 β , which in the ethanol and burn group returned to sham levels by 48 h (Fig. 8A). In contrast, the IL-1 β content remained elevated in the lungs of ethanol-exposed and burn-injured mice remained elevated at 48 h ($P < 0.05$). In this model, at 24 h after injury, lung TNF- α levels were 2-fold higher in burn groups regardless of ethanol exposure (Fig. 8B). However, there were no differences in TNF- α levels between groups at 48 h after ethanol, burn, and infection.

Neutrophil apoptosis in pulmonary airway

To attempt to explain the persistent neutrophil numbers in the lung of animals exposed to ethanol before burn and intratracheal infection, two independent apoptotic pathways were analyzed in the neutrophil. The activity of caspase 8 (downstream of death receptor activation) and caspase 9 (downstream of cytochrome *c* leakage from mitochondria) was measured. Both pathways were assessed at 24 h after injury in neutrophils obtained by BAL. This time point was chosen because all four treatment groups showed an increase in neutrophil numbers at 24 h (Fig. 5), allowing us enough cells to perform the studies with samples from individual mice. We elected not to conduct this investigation at 48 h, as neutrophils were present in large numbers only in infected burn mice exposed to ethanol and to a lesser degree in burn mice given infection. As previously shown by others, burn injury induces a delay in neutrophil apoptosis regardless of ethanol exposure (27). At 24 h, we were able to detect an apparent, but not significant, decrease in neutrophil apoptosis, measured by caspase 8 activation, when the cells were isolated by BAL from infected burn animals (Fig. 9A). Exposure to ethanol before burn and infection resulted in decreased active caspase 8 relative to the infected burn group and to all other groups ($P < 0.02$). Caspase 9 was also measured in neutrophils isolated by BAL. Similar to caspase 8, caspase 9 decreased when neutrophils were isolated from the lungs of ethanol-exposed, burn-injured, and infected animals relative to the other treatment groups ($P < 0.05$) (Fig. 9B).

DISCUSSION

To our knowledge, this is the first clinically relevant animal model that describes neutrophil infiltration and apoptosis to the lungs of infected mice exposed to ethanol and burn injury. Although it has been known for at least a decade that alcohol consumption is a risk factor for trauma patients and the development of infections (32), few clinical studies have addressed the effects of alcohol on the susceptibility to pulmonary infection after burn. Our laboratory has tried to address this issue in an animal model.

In the current study, the pulmonary chemokines specific to neutrophil chemotaxis were elevated in the lungs of infected mice exposed to ethanol before burn for a longer period than when compared with the other treatment groups. Therefore, the increased, prolonged infiltration of neutrophils could be explained by the sustained presence of chemoattractants seen in the lungs. This is consistent with the work of others, showing that neutrophil accumulation in the lung and in other organs is directed by chemokines (33, 34). Increased concentration of chemokines in the inflammatory environment is correlated positively with increased neutrophil infiltration (34). Previous studies from our laboratory, using the combined model of ethanol exposure and burn injury, have shown that even in the absence of pulmonary infection, MIP-2 is elevated for a prolonged period in lung homogenates from

mice exposed to ethanol before burn when compared with burn animals given vehicle saline (26). As such, we propose that the combined injury of ethanol and burn stimulates alveolar macrophages and surrounding parenchymal cells (lung epithelial cells and fibroblasts) to produce higher levels of chemokines in response to an infectious challenge with *P. aeruginosa*.

The rate of neutrophil clearance contributes to the balance of inflammation. The primary mechanism of neutrophil cell death is apoptosis, and a delay in neutrophil apoptosis correlates with the degree and resolution of inflammation. In this study, neutrophil accumulation in the lungs was greatly elevated in mice exposed to ethanol before burn and infection. Others have shown that burn injury delays apoptosis in neutrophils, in both clinical and animal studies (35). The delay in neutrophil apoptosis seen after burn injury can affect both apoptotic pathways: death receptor induced apoptosis and mitochondrial membrane leakage (35, 36). In most cases, as shown by others, the programmed cell death pathway is interrupted by upregulation of antiapoptotic genes such as Bcl-X_L and downregulation of proapoptotic genes such as Bad and Bax (36). Furthermore, the prosurvival pathways PI₃K and nuclear factor κB are activated, leading to the upregulation of prosurvival genes. In other studies, apoptosis via the death receptor (TNF receptor) pathway was not stimulated even in the presence of TNF-α, and an increase in serum granulocyte-macrophage colony stimulating factor prolonged survival of neutrophils after burn (35, 36). In our model, the lack of neutrophil clearance from the lungs seemed to be compounded in the presence of ethanol before burn injury. Although prosurvival pathways were not measured in this study, ethanol exposure before burn decreased the activation of both caspases, caspase 8 (activated by death receptor signaling) and caspase 9 (downstream of mitochondrial leakage), suggesting that neutrophil survival is prolonged with the combined injury.

Conclusions from these studies indicate that burn injury elevates levels of proinflammatory mediators in the lungs after infection with an opportunistic pathogen. These effects seem to be prolonged by ethanol exposure before the injury. The results of which are increased accumulation and infiltration of neutrophils in the airway. Furthermore, the combined injury of ethanol exposure and burn induces a delay in neutrophil apoptosis. To our knowledge, there are no published studies that have attempted to describe the effect of ethanol exposure on neutrophil migration and function after burn. Similar to other reports, neutrophil apoptosis was delayed by burn in our model, whereas ethanol exposure before burn further prolonged neutrophil survival. Here we suggest that burn injury results in increased neutrophil accumulation in the lungs in response to infection with subsequent delay in apoptosis.

In summary, we have demonstrated that neutrophils accumulate in the lungs of mice exposed to ethanol before burn and intratracheal infection in greater numbers for a longer period than seen in the other treatment groups. The accumulation of neutrophils in the alveoli parallels a prolonged elevation in pulmonary KC and MIP-2. Burn injury also induces a delay in neutrophil apoptosis, and ethanol exposure before burn and infection impairs neutrophil apoptosis even further. Therefore, we suggest that a decrease in neutrophil apoptosis is mediated by the combined insult of ethanol and burn injury in mice infected with *P. aeruginosa*. Hence, it is challenging to create effective immunomodulatory therapies for the critically ill, to maintain a delicate balance of neutrophil infiltration and functional properties promoting the beneficial bactericidal function of these phagocytes without their tissue-damaging effects. Further investigation is needed to understand the mechanisms of how ethanol mediates these effects in this model.

Acknowledgments

The authors thank Patricia Simms for the technical assistance in experiments involving flow cytometry. They also thank Drs. Douglas Faunce and Phong Le for their thoughtful discussions on this project.

This work was supported by the National Institutes of Health R01 AA012034 (E.J.K.), Institutional Training Grant T32 AA013527 (E.J.K.), NIH F31 AA017032 (E.L.M.), NIH F31 AA017027 (J.K.), Illinois Excellence in Academic Medicine Grant (E.J.K.), Ralph and Marian C. Falk Research Trust, and Margaret O. Baima Endowment Fund.

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Fig. 1. Total WBC count in infected mice exposed to ethanol before burn injury

At 24 and 48 h after injury, blood was obtained from mice in all treatment groups by cardiac puncture. Absolute blood counts were performed in whole blood using a multispecies hematology analyzer. Data are expressed as mean WBC ($\times 1,000/\mu\text{L}$) $\pm$ SEM, $n = 6$ animals per group, $*P < 0.05$ from burn vehicle + *P. aeruginosa* and sham + *P. aeruginosa* regardless of ethanol exposure at 24 h. Dashed line indicates total WBC counts in unmanipulated animals.

Fig. 2. Peripheral blood neutrophils in infected mice exposed to ethanol before burn injury
 At 24 and 48 h after injury, differential blood counts were performed in whole blood using a multispecies hematology analyzer. Data are expressed as mean $\times 1,000/\mu\text{L}$ neutrophils $\pm$ SEM, $n = 5$ animals per group, $*P < 0.05$ from all treatment groups at 24 h. No significant differences were detected between groups at 48 h. Dashed line indicates peripheral blood neutrophils in unmanipulated animals.

Fig. 3. Immunostaining for neutrophils and macrophages in the lungs of infected mice exposed to ethanol before burn injury

At 48 h after injury, the lungs from all four treatment groups were collected. Lungs were sectioned and stained with the rat anti-Gr-1 (green) and the rat anti-MOMA-2 (red) antibodies. Hoechst stain was used to label nuclei. Representative sections of the four treatment groups are shown, original magnification $\times 400$.

Fig. 4. Neutrophils in the lungs of infected mice exposed to ethanol before burn injury

At 48 h after injury, lungs were obtained from all four treatment groups, sectioned, and stained with antibodies against neutrophils and macrophages. Positive fluorescent pixels were calculated using Axio Vision Rel 4.5 software (Zeiss). A, Positive green fluorescent pixels indicating neutrophils (Gr-1⁺/MOMA-2⁻ cells) and (B) positive red fluorescent pixels indicating macrophages (MOMA-2⁺/Gr-1⁻ cells) were calculated for each animal in the experiment. Data are represented as the average pixels per ten 200× fields for each group ± SEM. n = 4 animals per group. **P* < 0.05 compared with all other treatment groups.

Fig. 5. Bronchoalveolar lavage cells obtained from infected mice exposed to ethanol and burn injury

Bronchoalveolar lavage cells were harvested at 24 h after injury from all four treatment groups. Cells were stained for the F4/80 and Ly6C/6G (Gr-1) antigens and analyzed by flow cytometry. Representative panels of the four treatment groups are shown above. Cell viability was determined at 98% to 99%. Dead cells were excluded.

Fig. 6. Bronchoalveolar lavage cells obtained from infected mice exposed to ethanol before burn injury

Bronchoalveolar lavage cells were obtained at 48 h after injury from all four treatment groups. Cells were stained for the F4/80 and Ly6C/6G (Gr-1) antigens and analyzed by flow cytometry. A, Representative panels of the four treatment groups. B, Total number of neutrophils in the BAL. Data are represented as mean of total neutrophils per group $\pm$ SEM. $n = 5-6$ animals per group. # $P < 0.05$ compared with sham injured and infected animals regardless of ethanol exposure; * $P < 0.05$ compared with all other treatment groups.

Fig. 7. Pulmonary chemokines in infected mice exposed to ethanol and burn injury

Levels of (A) KC and (B) MIP-2 were measured in lung homogenates from mice in all four groups at 24 and 48 h. Data are expressed as the mean in picograms per milligram of protein $\pm$ SEM. $n = 5$ animals per group, $*P < 0.05$ compared with sham animals regardless of ethanol exposure at 24 h; $\#P < 0.05$ compared with burn vehicle and sham animals regardless of ethanol exposure at 48 h.

Fig. 8. Pulmonary proinflammatory cytokines in infected mice exposed to ethanol before burn injury

Levels of (A) IL-1 β and (B) TNF- α were measured in lung homogenates obtained from mice in all four groups at 24 and 48 h. Data are expressed as the mean in picograms per milligram of protein $\pm$ SEM. $n = 5$ animals per group, * $P < 0.01$ vs. infected sham animals regardless of ethanol exposure at 24 h; # $P < 0.05$ vs. all other treatment groups at 48 h.

Fig. 9. Apoptosis in neutrophils isolated by BAL from infected mice exposed to ethanol before burn injury

At 24 h after injury, neutrophils were isolated by BAL from all treatment groups and analyzed by flow cytometry. A, Cells stained and incubated with FLICA 8 and PI. Data are represented as the percent caspase 8–positive cells $\pm$ SEM, $n = 6$ (sham) and 8 (burn) animals per group. $*P < 0.02$ compared with burn vehicle + *P. aeruginosa* and sham ethanol + *P. aeruginosa*. B, Cells stained and incubated with FLICA 9 and PI. Data are represented as the percent caspase 9–positive cells $\pm$ SEM, $n = 6$ (sham) and 8 (burn) animals per group. $*P < 0.05$ compared with all other groups.