Neutrophil chemokines and their role in IL-18-mediated increase in neutrophil O$_2^-$ production and intestinal edema following alcohol intoxication and burn injury

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Burn injury. Male rats (250 g) were gavaged with EtOH to achieve...disease associated with EtOH exposure, major trauma, and burn injury (5, 17, 24, 43, 48, 49).

Additional findings from our laboratory have shown that the increase in intestinal permeability following a combined insult of EtOH and burn injury was accompanied with an increase in interleukin (IL)-18 production (26, 28, 42). IL-18, like IL-12, was discovered initially to be a cytokine that drives the T cell toward T helper-1 cell subtype and thus was referred as interferon (IFN)-γ-inducing factor (37, 38). However, subsequent studies found that IL-18 is pleiotropic in nature and may cause tissue damage in various inflammatory and disease conditions (7, 20–22, 36, 46, 50, 53). Although many of these studies indicated IL-18-induced IFN-γ to be the cause of tissue damage, we found that IL-18 promotes recruitment of neutrophils to lungs and intestine and thus causes tissue damage in those organs (26, 28, 42).

Studies have shown that neutrophils migrate through the endothelium of blood vessels to extravascular inflammatory sites to destroy pathogens by releasing toxic oxygen radical species and proteolytic enzymes. However, excess release of these agents may cause tissue damage in various inflammatory conditions, such as shock, trauma, and burn injury (10, 15, 39, 43, 49). In a recent study, we observed that acute EtOH intoxication potentiates neutrophil release of superoxide anions (O$_2^-$) (29). Thus an increase in neutrophil accumulation and the release of O$_2^-$ and proteolytic enzymes (e.g., elastase) may result in intestinal epithelial damage, capillary leakage, alteration of intestinal permeability, and increase in translocation of bacteria to extraintestinal sites (8, 9, 12, 24, 43). Treatment of animals with antineutrophil antiserum to deplete neutrophils prevented neutrophil-mediated intestinal injury (28). These findings strongly suggest that neutrophils play a critical role in organ damage following EtOH intoxication and burn injury.

Although the mechanism by which EtOH combined with burn injury upregulates neutrophil tissue-damaging actions remains unknown, our recent findings indicate that IL-18 upregulates cytokine-induced neutrophil chemokines (CINC)-1 and CINC-3 and intercellular adhesion molecule 1 in the intestine and lungs following EtOH and burn injury (25, 26, 28). Because neutrophils are known to have receptors for IL-18, the present study investigated the role of IL-18 in increased neutrophil O$_2^-$ and elastase release. We also examined the role of neutrophil chemokines CINC-1 and CINC-3 in increased neutrophil recruitment to the intestine following EtOH and burn injury. Moreover, to determine whether or not the neutrophil chemokines influence neutrophil activation, we further examin...
ined the role of CINC-1 and CINC-3 in IL-18-mediated increase in neutrophil O$_2^-$ and elastase release following EtOH and burn injury.

MATERIALS AND METHODS

Animals and reagents. Male Sprague-Dawley rats (225–250 g) were obtained from Charles River Laboratories (Wilmington, MA). Anti-rat IL-18 antibody, recombinant (r) rat CINC-1, and recombinant rat CINC-3 were purchased from R&D Systems (Minneapolis, MN). Rat model of acute EtOH and burn injury. Rats were divided into two major groups, saline + sham and EtOH + burn. In the EtOH group, the level of blood EtOH equivalent to 90–100 mg/dl was achieved by gavage feeding of 5 ml 20% EtOH in saline. In the saline group, animals were gavaged with 5 ml of saline. Four hours after gavage, all animals were anesthetized and transferred into a template, which was fabricated to expose ~12.5% of the total body surface area. For burn injury, rats were immersed in boiling water (~97°C) for 10–12 s (25, 26, 28, 29). Sham rats were subjected to identical anesthesia and immersed in lukewarm water. Animals were resuscitated intraperitoneally with 10 ml physiological saline, and were administered anti-rat IL-18 antibody (80 μg/kg) or isotype IgG (Santa Cruz Biotechnology, Santa Cruz, CA) intraperitoneally. After 20 min, anti-IL-18 antibody–treated rats were given either recombinant rat CINC-1 (12 μg/kg) or CINC-3 (20 μg/kg). On day 1 after injury, rats were euthanized.

All the experiments were carried out in adherence to the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and were approved by the University of Alabama at Birmingham and Loyola University Medical Center, Maywood, Animal Institutional Care and Use Committees.

Isolation of neutrophils. As described in our previous study (29), blood was drawn via cardiac puncture into a heparinized tube. Heparinized whole blood was diluted 1:2 with PBS. The blood was then added slowly to Ficoll Paque (GE Healthcare, Uppsala, Sweden) from the side of the tube and centrifuged at 300 g for 40 min. The pellet containing red blood cells (RBCs) and neutrophil was suspended in PBS and mixed with 3% dextran (Fisher Scientific, Pittsburgh, PA). The cell suspension was left on a plane surface for 1 h at room temperature. Neutrophil-rich supernatant was collected and centrifuged at 300 g for 20 min at 10°C. The RBCs were lysed by the addition of sterile distilled water followed by the addition of 10× HBSS and centrifuged at 300 g for 30 min at 10°C. The purified neutrophils settled at the bottom were resuspended in HBSS and used for subsequent studies.

Measurement of neutrophil O$_2^-$ anions. As we have described previously (29), neutrophil superoxide anion release was determined by cytochrome c reduction assay. Briefly, 0.1 ml of neutrophil (5 × 10$^6$ cells/ml in HBSS) was incubated with cytochrome c or cytochrome c plus superoxide dismutase for 5 min at 37°C in a 96-well plate. Neutrophil O$_2^-$ production was initiated by adding phorbol esters (PMA) at a dose of 500 ng/ml. Although we have used lower doses of PMA (50 and 100 ng/ml), a maximum response was obtained with a dose of 500 ng/ml. The absorbance of reduced cytochrome c was measured continuously for 1 h at 550 nm. The peak O$_2^-$ concentration was achieved ~20–25 min after neutrophil stimulation with PMA. These peak values were recorded, pooled, and are expressed as means ± SE in RESULTS.

Measurement of neutrophil elastase. Elastase production was measured by activating the neutrophils (2.5 × 10$^6$ cells/ml with PMA (500 ng/ml) at 37°C for 1 h. Cells were washed and lysed, and the supernatant was collected for estimation of elastase activity (54). The lysates (25 μl) were incubated in a 96-well plate at room temperature for 60 min with 1 mM methoxy-tyrosine-tyrosyl-tyrosine-tyrosyl-p-nitroanilide (Sigma Chemical), 0.1 M HEPES, and 0.5 M NaCl (pH 7.5) in a total volume of 150 μl. Absorbance was measured at 405 nm.

Measurement of intestinal tissue edema. A piece of small intestine was removed, weighed, and dried for 24 h at 80°C. Water content (%) of intestinal tissue was calculated as (wet weight − dry weight)/wet weight × 100 and was used as a measure of tissue edema (25, 26, 28, 29).

Preparation of intestinal homogenates. Immediately after the rats were anesthetized, intestine was exposed. Leaving approximately the first 15 cm proximal segment of intestine, a 3-cm length of intestine was removed, cleaned, and snap frozen. Equal weights (100 mg wet weight) of intestine from various groups were suspended in 1 ml PBS and sonicated at 30 cycles, twice, for 30 s on ice. Homogenates were cleared by centrifuging at 10,000 revolution/min (or 10,600 g) at 4°C, and the supernatants were stored at −70°C (25, 26, 28, 29). Protein levels in the homogenates were determined using the Bio-Rad (Hercules, CA) assay kit.

Measurement of intestinal tissue myeloperoxidase levels. Myeloperoxidase (MPO) activity was measured by incubating intestinal tissue homogenates (10 μl) in a 96-well plate with 290 μl of 50 mM potassium phosphate buffer, 3 μl of substrate solution containing 200 mg/ml o-dianisidine hydrochloride, and 3 μl of 20 mM H$_2$O$_2$. The reaction was stopped by adding 3 μl of 30% sodium azide. Plates were read at 460 nm (25, 26, 28, 29).

Measurement of intestinal tissue IL-18, CINC-1, and CINC-3. IL-18, CINC-1, and CINC-3 levels in the intestinal tissue homogenates were measured using the ELISA kits (R&D Systems) following the manufacturer’s instructions. The concentrations in the samples were determined using the standard curve and by normalizing with protein (25, 26, 28, 29).

Immunohistochemical detection of neutrophils. Approximately 0.5–1.0-cm-long small intestine rings were fixed in formaldehyde, embedded in paraffin, and cut into ~5-μm-thick sections using a microtome. After dewaxing and rehydrating, the antigenic site retrieval of the sections was accomplished by boiling each slide for 20 min in 0.01 M citric acid buffer (pH 6.0). Nonspecific staining was blocked with 5% goat serum in PBS buffer. Thereafter, sections were incubated with anti-neutrophil antibody (Accurate Chemical and Scientific, Westbury, NY) for 2 h at room temperature. After being washed in PBS, endogenous peroxidase in intestine sections was blocked using 0.5% hydrogen peroxide solution in PBS. Sections were further incubated with biotinylated goat anti-rabbit secondary antibody. After being washed in PBS buffer, sections were incubated with diaminobenzidine substrate solution with peroxidase enzyme and counterstained with hematoxylin solution (48). Representative sections were selected for the presentation. Some sections were also stained with hematoxylin-eosin for histological analysis (42, 48). Representative sections were selected for the presentation.

Statistical analysis. These data are presented as means ± SE and were analyzed using the ANOVA statistical program. A P value <0.05 between groups was considered statistically significant.

RESULTS

Intestinal IL-18, CINC-1, and CINC-3 levels. We have shown earlier that on day 1 after EtOH intoxication or burn injury there was no significant change in the intestine tissue levels of IL-18, CINC-1, and CINC-3 compared with sham gavaged with saline (26, 28). However, a significant increase in IL-18, CINC-1, and CINC-3 was observed in the intestine of rats subjected to a combined insult of EtOH intoxication and burn injury compared with shams (Fig. 1). To determine whether the increase in IL-18 is responsible for the increase in CINC-1 and CINC-3, a group of EtOH plus burn-injured rats was treated with anti–IL-18 antibodies, and the effect of this treatment was determined on intestinal levels of IL-18, CINC-1, and CINC-3. The results shown in Fig. 1 clearly indicate that administration of IL-18-neutralizing antibodies...
have significantly higher $O_2^-$ production compared with the neutrophils obtained from sham rats. Treatment with anti-IL-18 antibody significantly reduced the level of $O_2^-$ production. When IL-18 antibody-treated animals were administered with rCINC-1, the level of neutrophil $O_2^-$ production was restored to the vehicle-treated EtOH plus burn group levels. In contrast, administration of anti-IL-18 antibody-treated animals with rCINC-3 did not alter the level of neutrophil $O_2^-$ production.

Neutrophil elastase release. Neutrophils from the vehicle-treated EtOH plus burn group showed significant increase in the intracellular elastase levels compared with the sham group (Fig. 3). Treatment with anti-IL-18 antibody reduced the level of elastase to the sham level. The restitution of CINC-1 or CINC-3 in anti-IL-18 antibody-treated rats restored neutrophil elastase release to the levels observed in vehicle-treated EtOH plus burn group.

Small intestinal neutrophil infiltration. As shown in Fig. 4, MPO activity in the intestine increased significantly in the vehicle-treated EtOH plus burn group compared with shams. Administration of anti-IL-18 antibodies prevented the increase in intestinal MPO activity following EtOH plus burn injury. The restitution of CINC-1 in anti-IL-18-treated animals resulted in a significant increase in the MPO activity compared with shams or anti-IL-18 antibody-treated group. However, there was no increase in the intestinal MPO activity after restitution of CINC-3.

The neutrophil presence was further confirmed by immunohistochemical analysis of intestine sections using anti-neutrophil antibody. The results as shown in Fig. 5 indicate a large number of neutrophils in the intestine sections prepared from rats receiving EtOH plus burn injury compared with shams (Fig. 5). Administration of IL-18 antibody in rats following EtOH and burn injury reduced the number of neutrophils in the intestinal tissue. The restitution of CINC-1 in anti-IL-18-

![Fig. 1. Intestinal IL-18, cytokine-induced neutrophil chemokine (CINC)-1, and CINC-3 levels.](image1)

(80 µg/kg) decreased the levels of IL-18 (Fig. 1A) as well as CINC-1 (Fig. 1B) and CINC-3 (Fig. 1C) to the levels observed in sham animals. The restitution of CINC-1 or CINC-3 in anti-IL-18 antibody-treated rats did not affect the intestinal IL-18 levels; however, the levels of CINC-1 and CINC-3 were respectively restored to the levels observed following EtOH plus burn injury. Although administration of recombinant CINC-3 also enhanced the levels of CINC-1 in anti-IL-18 antibody-treated rats following EtOH plus burn injury, those levels were not significantly different from sham or the IL-18 antibody-treated group. Similarly, restitution of CINC-1 slightly increased the CINC-3 level, but it was not significantly different from sham or the anti-IL-18 antibody-treated group.

Neutrophil superoxide anion production. Figure 2 shows that neutrophils from the vehicle-treated EtOH plus burn group
treated animals increased the neutrophil infiltration in the intestine following EtOH and burn injury. Administration of rCINC-3 in anti-IL-18 antibody-treated animals, however, did not affect the neutrophil number in the intestine following EtOH and burn injury.

Intestinal villus area and tissue edema. There was a significant increase in the intestinal villus area of rats receiving EtOH plus burn injury compared with the shams (Fig. 6). Treatment of rats with anti-IL-18 antibody reduced the villus area to the sham level. However, the restitution of CINC-1 and not CINC-3 in anti-IL-18 antibody-treated animals increased the villus area to levels similar to those observed following EtOH and burn injury.

Intestinal tissue edema as determined by water content was significantly increased in rats receiving EtOH plus burn injury (Fig. 7). The treatment of rats with anti-IL-18 antibodies significantly reduced the edema formation following EtOH and burn injury. However, the restitution of CINC-1 in anti-IL-18 antibody-treated animals significantly increased the intestinal tissue water content. In contrast, CINC-3 restitution did not influence the intestinal water content in anti-IL-18 antibody-treated animals following EtOH and burn injury.

DISCUSSION

In our previous studies, we have shown that on day 1 after EtOH intoxication or burn injury alone there was no significant change in the intestine tissue levels of IL-18, CINC-1, and CINC-3 compared with shams gavaged with saline (26, 28). Furthermore, neutrophil O$_2^-$ and its infiltration into the intestine was not found to be significantly different following EtOH intoxication or burn injury alone compared with sham rats gavaged with saline (29). Additionally, unpublished findings from our laboratory indicate that EtOH or burn injury alone did not significantly affect the neutrophil elastase levels (X. Li and M. A. Choudhry). However, a combined insult of EtOH intoxication and burn injury resulted in increased intestinal levels of IL-18, neutrophil chemokines (CINC-1 and CINC-3), neutrophil superoxide anion and elastase, neutrophil accumulation, MPO activity, and intestinal edema. Treatment of rats with anti-IL-18 antibody immediately after injury prevented the increase in the above parameters following EtOH intoxication and burn injury. The restitution of CINC-1 in anti-IL-18 antibody-treated rats markedly increased intestinal levels of CINC-1, neutrophil superoxide anion and elastase, neutrophil accumulation, MPO activity, and intestinal edema. The restitution of CINC-3 in IL-18 antibody-treated animals, on the other hand, did not influence the above parameters except the neutrophil elastase, which was restored to the levels observed after EtOH and burn injury. These findings indicate that, similar to IL-18, CINC-1 may also help in the neutrophil recruitment to the intestine and in the production of superoxide anion and elastase. Nevertheless, the finding that treatment of rats with anti-IL-18 antibodies inhibits CINC-1 and CINC-3 further supports the notion that IL-18 plays a critical role in increased neutrophil tissue-damaging action following a combined insult of EtOH intoxication and burn injury.

IL-18 is a member of the IL-1 cytokine superfamily. It was initially characterized as IFN-γ-inducing factor and thus was recognized as an important player in host defense (37, 38). IL-18 is synthesized as a precursor protein (pro-IL-18), and, in the presence of IL-1β-converting enzyme (or caspase-1), it matures into 18-kDa active protein (6, 13, 35). IL-18 is produced from macrophage-like cells and from epithelial cells, including intestinal epithelial cells (6, 13, 35). In our previous studies, we have reported that EtOH intoxication combined with burn injury increases the level of IL-18 in intestine and lungs (25, 26, 28). We further found that IL-18 helps in the recruitment of neutrophils (25, 26, 28, 29). Jordan et al. (20) have shown that intratracheal administration of IL-18 resulted in increased infiltration of neutrophils and caused significant increase in lung vascular permeability. Conversely, intratracheal instillation of anti-IL-18 antibodies in inflamed lung...
greatly reduced the recruitment of these cells and prevented increase in vascular permeability (20). Studies have also shown that intratracheal administration of IL-18 bp resulted in suppressed lung vascular permeability and decreased bronchoalveolar lavage content of neutrophils, cytokines, and chemokines (20). Moreover, our recent findings showed that the depletion of neutrophils prevents intestinal and lung tissue damage (25, 28). Altogether, these findings strongly support the suggestion that neutrophils are critical in IL-18-dependent intestinal tissue damage (25, 28). Alternatively, it is also possible that IL-18 induces the production of neutrophil chemotactic factors such as CINC, which in turn may play a role in neutrophil recruitment to intestine and other organs in conditions such as EtOH intoxication and burn injury.

Neutrophil infiltration and subsequent edema are the early markers of tissue damage. Earlier studies have shown that EtOH intoxication with or without burn injury activates neutrophils to release O$_2$ and proteases (8, 16). Although such release of O$_2$ and proteases by neutrophils is important for the host defense and the killing of pathogens, excessive release of these products may cause tissue damage. It is widely believed that, after performing their functions, neutrophils undergo apoptosis and are cleared by macrophages. However, studies
have shown that following major injury the production of inflammatory mediators not only activates the neutrophils but also enhances their survival. Consistent with these findings, we have recently shown that EtOH intoxication combined with burn injury delays neutrophil apoptosis (29) and thus may further add to the neutrophil-dependent pathology associated with EtOH and burn injury.

Our results indicate that IL-18 plays a role in the increased release of neutrophil $O_2^-$ and elastase levels after the combined insult of EtOH intoxication and burn injury. We further found that CINC-1 may also independently enhance neutrophil ability to produce $O_2^-$ and elastase. In contrast, CINC-3, another potent neutrophil chemokine, did not influence the neutrophil effector responses following EtOH and burn injury. Both CINC-1 and CINC-3 are members of the IL-8 family and are potent chemotactic factors for neutrophils (14, 41). Chemotaxis of neutrophils is an important, functional response to chemoattractants and is a key event in the recruitment of neutrophils in inflammation. CINC is a member of the subfamily of chemokines and is classified as CINC-1, CINC-2α, CINC-2β, and CINC-3. Although all three CINCs play roles in neutrophil recruitment, many studies have suggested that CINC-1 and CINC-3 play a predominant role in neutrophil recruitment (14, 25, 26, 28, 41). With the use of CINC antibodies, it was demonstrated that CINC-1 and CINC-3 contribute significantly to the influx of neutrophils in rat inflammation models, including lung injury and lipopolysaccharide-induced inflammation (18, 44). In our studies, we observed that the levels of both CINC-1 and CINC-3 are elevated after a combined insult of EtOH and burn injury. However, the administration of anti-IL-18 antibody prevented the increase in CINC-1 and CINC-3, suggesting a role of IL-18 in their upregulation.

The mechanism by which IL-18 and neutrophil chemokines modulate the neutrophil $O_2^-$ production and elastase release remains to be investigated. Studies have shown that neutrophils constitutively express IL-18 receptor. Findings from multiple studies have shown that a membrane-bound multicomponent NADPH oxidase consisting of six subunits is responsible for the production of oxygen radicals (1, 45). In resting neutrophils, four of the six subunits, p47$^{phox}$, p67$^{phox}$, p40$^{phox}$, and the small GTPase and Rac2, are localized in the cytoplasm, whereas the remaining two subunits, gp91$^{phox}$, and gp22$^{phox}$, form a heterodimeric membrane-bound flavocytochrome known as cytochrome b$_{558}$ (1, 45). Upon activation, the cytosolic components p40$^{phox}$, p47$^{phox}$, p67$^{phox}$, and Rac-2 translocate to the membrane where they associate with flavocytochrome b$_{558}$ to form the active oxidase complex. Previous studies have shown that neutrophil exposure to low levels of cytokines and chemotactic factors (e.g., tumor necrosis factor-α, platelet-activating factor, and lipopolysaccharide) does not fully activate the NADPH oxidase system but results in 10- to 20-fold increase in $O_2^-$ production, a stage referred to as “neutrophil priming” (45). When these primed neutrophils encounter another signal, they become fully activated to produce reactive oxygen species and elastase. Furthermore, it has also been suggested that at high concentrations the same priming agents may lead to the activation of these cells (2, 43, 54). In our recent study, we found that EtOH combined with burn injury activated the p47$^{phox}$ and p67$^{phox}$ (2, 43, 54). Our findings further indicate that the increase in neutrophil $O_2^-$ or p47$^{phox}$ and p67$^{phox}$ could result from a decrease in heme oxygenase (HO)-1 expression. HO-1 has an antioxidant role in the cell, and a decrease in HO-1 may hamper the ability of cells to scavenge the $O_2^-$ and thus contribute to increased neutrophil $O_2^-$ levels. It is likely that IL-18 directly modulates NADPH assembly and thereby enhances neutrophil $O_2^-$ production. Recent findings indicate that IL-18 downregulates HO-1 expression in endothelial cells (52). Thus it is also possible that an increase in IL-18 following EtOH and burn injury may cause a decrease in HO-1 and thereby cause an increase in neutrophil $O_2^-$ under those conditions. However, both these
possibilities remain to be investigated. In addition to IL-18, our findings also indicate that restitution of CINC-1 but not CINC-3 activates the neutrophils to produce O$_2^-$ in contrast to restitution of both CINC-1 and CINC-3 caused an increase in neutrophil elastase release. The precise reason for the observed differences in CINC-1 and CINC-3 action on neutrophils remains to be investigated. Nonetheless, studies have indicated that CINC-1 and CINC-3 may exert different biological activities through distinct G proteins. Several other mediators including leukotrienes, vasoactive peptides, cytokines, and chemokines may activate neutrophils (43).

The present study has utilized a relatively small total body surface area burn injury, which by itself did not cause any change in the neutrophil effector responses nor has produced any deleterious effects on the intestine on day 1 after injury. There is evidence that burn injury size is a critical factor in postburn complications (28). However, other factors such as age, sex, and preclinical manifestation can also influence the outcome of burn patients especially patients with small burn injury. Similarly, EtOH consumption before burn injury has been shown to further confound postburn pathogenesis (5, 19, 23, 32–34, 47). Thus a smaller burn or single dose of EtOH by itself may not have any significant effect on neutrophil effector responses measured in this study. However, when the two insults are combined, they become severe and detrimental.

In summary, results presented in this study suggest a role of IL-18 and neutrophil chemokines in the activation of neutrophils to produce more O$_2^-$ and elastase. Furthermore, like IL-18, CINC-1 may also modulate neutrophil effector responses following EtOH and burn injury. CINC-3, in contrast, did not appear to influence neutrophil responses. Regardless of the mechanism of neutrophil activation, the resulting release of O$_2^-$ and elastase may contribute to the intestinal edema formation and tissue damage following EtOH and burn injury.

GRANTS
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G346 IL-18 AND NEUTROPHIL ROS PRODUCTION


