Interleukin-18 Delays Neutrophil Apoptosis following Alcohol Intoxication and Burn Injury

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Studies have shown that burn patients who are intoxicated at the time of injury are more susceptible to infection and have a higher incidence of mortality. A major cause of death in burn and trauma patients regardless of their alcohol (EtOH) exposure is multiple organ dysfunction, which is driven in part by the systemic inflammatory response and activated neutrophils. Neutrophils are short lived and undergo apoptosis to maintain homeostasis and resolution of inflammation. A delay in apoptosis of neutrophils is one important mechanism which allows for their prolonged presence and the release of potentially harmful enzymes. The purpose of this study was to examine whether EtOH intoxication combined with burn injury influences neutrophil apoptosis and whether IL-18 plays any role in this setting. To accomplish this investigation, rats were gavaged with EtOH (3.2 g/kg) 4 h before being subjected to sham or burn injury of ~12.5% of the total body surface area, and then killed on d 1 after injury. Peripheral blood neutrophils were isolated and lysed. The lysates were analyzed for pro- and antiapoptotic proteins. We found that EtOH combined with burn injury prolonged neutrophil survival. This prolonged neutrophil survival was accompanied by a decrease in the levels of the neutrophil proapoptotic protein Bax, and an increase in antiapoptotic proteins Mcl-1 and Bcl-xl. Administration of IL-18 antibody following burn injury normalized the levels of Bax, Mcl-1 and Bcl-xl. The decrease in caspase-3 and DNA fragmentation observed following EtOH and burn injury was also normalized in rats treated with anti–IL-18 antibody. These findings suggest that IL-18 delays neutrophil apoptosis following EtOH and burn injury by modulating the pro- and antiapoptotic proteins.

INTRODUCTION

Major trauma remains a leading cause of death in humans of all ages. Approximately one million burn injuries are reported every year within the United States, and nearly half of them occur in individuals who are under the influence of alcohol/ethanol (EtOH) (1–3). Studies have shown that patients who are intoxicated at the time of injury are more susceptible to infection and have a higher incidence of mortality compared with burn patients who have not consumed EtOH at the time of injury (2,4,5). Similarly, findings from experimental studies have also shown that EtOH intoxication before burn injury exacerbates the sup-

pression of immunity, impairs intestinal barrier function, and increases bacterial translocation relative to either EtOH intoxication or burn injury alone (6–11).

EtOH is widely known to cause hepatocyte apoptosis and alcoholic liver disease (12–14). Chronic EtOH exposure sensitizes Kupffer cells, the resident macrophages in liver, to activation by lipopolysaccharide (LPS). This sensitization increases the production of proinflammatory mediators, such as tumor necrosis factor-α (TNF-α) and reactive oxygen species, that contribute to hepatocyte dysfunction and induction of apoptosis (14). In a recent study, we found that EtOH intoxication combined with

burn injury delays neutrophil apoptosis (11). This effect was accompanied by marked neutrophil accumulation in intestinal tissue (15). Neutrophil apoptosis occurs both in the bloodstream and in tissue (16,17). The delay in cellular apoptosis could be the result of interference with either the intrinsic pathway (mitochondrial, stress induced) or the extrinsic pathway (death receptor dependent), or both (18). The intrinsic apoptotic pathway involves mitochondria, which release cytochrome *c* into the cytoplasm following the activation of proapoptotic proteins, such as Bax and Bad, belonging to the Bcl-2 family. Cytochrome *c* then associates with Apaf-1 (apoptotic protease-activating factor 1) and procaspase-9 to form the apoptosome. Caspase 9 is activated on the apoptosome and subsequently activates caspase-3, which is a critical step in cell apoptosis (19). Antiapoptotic proteins Bcl-2 and Bcl-xl, also from the Bcl-2 family, inhibit the release of cytochrome *c* from the mitochondria

into the cytoplasm, thereby preventing the cellular apoptosis (20).

We have shown previously that IL-18 plays a key role in increased neutrophil recruitment to the intestine and the lung following EtOH intoxication and burn injury (10,15,21). IL-18, a proinflammatory cytokine, belongs to the IL-1 cytokine superfamily and is synthesized as a precursor protein (pro-IL-18). In the presence of IL-1β–converting enzyme (ICE, or caspase-1), the precursor protein matures into an 18-kDa active protein (22), which is produced by macrophages, dendritic cells, neutrophils and epithelial cells (22,23). Neutrophils constitutively produce both IL-18 and its antagonist, IL-18 BP (24). Neutrophils were also found to constitutively express IL-18 receptors (α and β) (25), and thus an increase in IL-18 levels following EtOH and burn injury may modulate neutrophil effector functions, including their survival. In the present study we investigated whether acute EtOH exposure before burn injury modulates the expression of pro- and antiapoptotic proteins of neutrophils and whether IL-18 has any role in the modulation of these proteins.

MATERIAL AND METHODS

Animals and Reagents

Male Sprague-Dawley rats (250–275 g) were obtained from Harlan (Indianapolis, IN, USA). Anti–rat IL-18 antibody was purchased from R&D Systems (Minneapolis, MN, USA). Antibody for Mcl-1 was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All the other antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA).

Rat Model of Acute EtOH and Burn Injury

As in our previous studies (6–8,10,11), male rats (250–275 g body weight) were randomly divided into four groups: sham vehicle, sham EtOH, burn vehicle and burn EtOH. In EtOH-treated groups, a blood EtOH level equivalent to 90–100 mg/dL was achieved by gav-

age feeding of 5 mL of 20% EtOH in water, which was ~3.2 g/kg body weight. In the vehicle groups, rats were gavaged with 5 mL of water. Four hours after gavage, all animals were anesthetized and transferred into a template, which was fabricated to expose 12–15% of the total body surface area. For burn injury, rats were immersed into a boiling water bath (~97°C) for 10–12 s. Immediately after injury, animals were resuscitated intraperitoneally with 10 mL of saline. A group of sham vehicle and burn EtOH animals was administered 100 μg/kg anti–rat IL-18 antibody (R&D Systems, Minneapolis, MN, USA) intraperitoneally, as in our previously described study (6). On d 1 following injury, rats were killed and blood was collected by cardiac puncture. 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 Chicago Medical Center institutional animal care and use committees.

Isolation of Neutrophils

As described previously (6,11), heparinized whole blood was diluted 1:2 with phosphate-buffered saline. The blood was then added slowly into Ficoll Paque (GE Healthcare, Uppsala, Sweden) from the side of the tube and centrifuged at $300g$ for 40 min. The pellet was suspended in 3% Dextran solution and left on plane surface for 1 h. Supernatant was separated and centrifuged to get the pellet. The remaining red blood cells were lysed by the addition of sterile-distilled water, and the neutrophils were collected after centrifugation and used in the subsequent experiments. This procedure gives ~98% viable neutrophils and a purity of ~95% as reported in our earlier studies (26).

Immunoblotting

Neutrophils were lysed in lysis buffer (50 mmol/L HEPES, 150 mmol/L NaCl, 1 mmol/L EDTA, 100 mmol/L NaF,

1 mmol/L $MgCl_2$, 10 mmol/L $Na_4P_2O_7$, 200 μmol/L Na_3VO_4, 10% glycerol and 0.5% Triton X-100) and centrifuged at $9000g$ for 30 min at 4°C as described in a report of our previous studies (11). The supernatant was collected and equal amounts of protein from neutrophil lysates were separated on sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to immobilon membrane by using a semidry Trans-Blot system (Bio-Rad, Hercules, CA, USA). The membranes were saturated with blocking buffer (10 mmol/L Tris, 150 mmol/L NaCl and 0.05% Tween 20, supplemented with 5% dry milk) for 2 h at room temperature and incubated with the desired primary antibody at 4°C overnight. The membranes were washed with Tris-buffered saline supplemented with 0.05% Tween 20 (TBST). The membranes were incubated with appropriate secondary antibody conjugated with horseradish peroxidase for 1 h at room temperature. The membranes were washed with TBST and probed using ECL (enhanced chemiluminescence) dye (PerkinElmer, Waltham, MA, USA). Membranes were stripped by Western blot stripping buffer (Pierce, Rockford, IL, USA) and reblotted with anti–β-actin antibody to confirm equal protein loading.

Neutrophil Apoptosis

As we described in reports of our previous studies (11), neutrophil apoptosis was measured by determining neutrophil caspase-3 activity and cell death by using assay kits available from Invitrogen (Carlsbad, CA, USA) and Roche Applied Science (Palo Alto, CA, USA), respectively.

Statistical Analysis

Results are presented as mean ± SEM and were analyzed by using ANOVA. The significance between the groups was determined by using Tukey's and Fisher's least significant difference test (GB-Stat School Pak). A *P* value < 0.05 between groups was considered statistically significant.
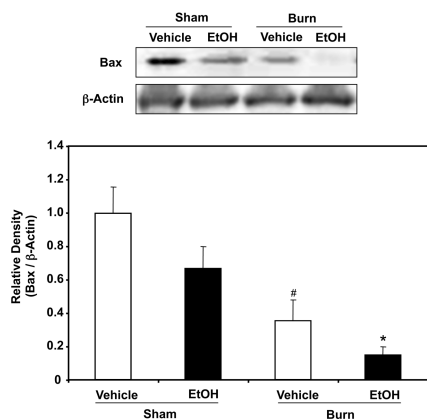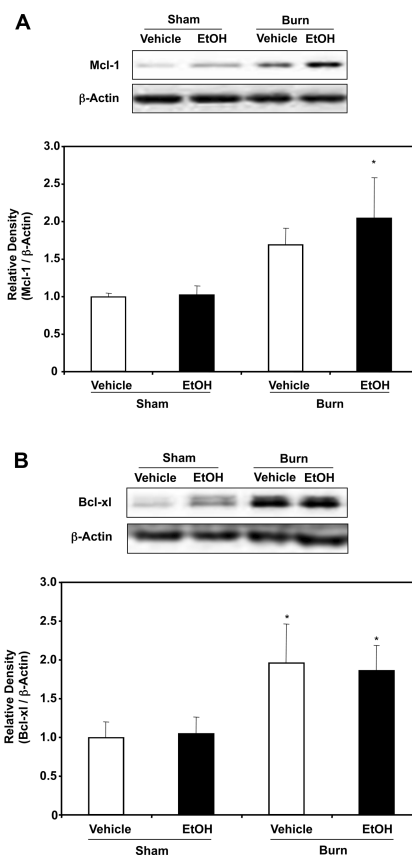
Figure 1. Representative blot showing neutrophil expression of Bax on d 1 following EtOH intoxication and burn injury. The amount of Bax was quantitated by use of densitometry. The densitometric values were normalized to the β-actin and are shown as mean ± SEM from at least six animals in each group. *$P < 0.05$ versus sham, #$P < 0.05$ versus vehicle + sham.

RESULTS

After its activation, Bax, a proapoptotic protein belonging to the Bcl-2 family, translocates to the mitochondria for the subsequent cytochrome *c* release from the mitochondria into the cytosol. This process leads to additional activation of the signaling cascades, eventually leading to cellular apoptosis. We examined Bax expression in neutrophil lysates and the results indicate a slight but not significant decrease in Bax expression in neutrophils isolated from rats that received EtOH compared with those that received vehicle (Figure 1). An ~50% decrease in Bax levels was observed in neutrophils from rats that received vehicle plus burn injury compared with sham animals gavaged with water. Although there was a tendency toward additional decrease in Bax levels in the neutrophils harvested from the burn-EtOH group, this decrease was not found to be significantly different from that observed in burn animals gavaged with water (Figure 1).

Mcl-1 and Bcl-xl are the members of the Bcl-2 family that promote cell sur-

Figure 2. Representative blot showing neutrophil expression of Mcl-1 (A) and Bcl-xl (B) on d 1 following EtOH intoxication and burn injury. The amount of Mcl-1 and Bcl-xl was quantitated by use of densitometry. The densitometric values were normalized to the β-actin and are shown as mean ± SEM from at least six animals in each group. *$P < 0.05$ versus sham.

vival. There was no difference in Mcl-1 and Bcl-xl in neutrophils from rats receiving EtOH compared with vehicle. However, as compared with shams, there was a significant increase in the level of Mcl-1 and Bcl-xl in neutrophils from burn-EtOH groups (Figure 2). Bcl-xl also showed a significant increase in neutrophils from burn vehicle group compared with shams. No significant difference in Mcl-1 and Bcl-xl was found in neutrophils from the burn-vehicle or burn-EtOH group.

To determine the role of IL-18 in the modulation of pro- and antiapoptotic
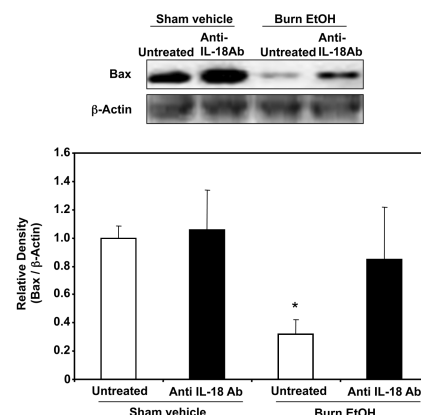
Figure 3. The effect of anti–IL-18 antibody (Ab) on neutrophil expression of Bax on d 1 following EtOH intoxication and burn injury. The amount of Bax was quantitated by use of densitometry. The densitometric values were normalized to the β-actin and are shown as mean ± SEM from 3 to 6 animals in each group. *$P < 0.05$ versus other groups.

signals, rats receiving sham and burn EtOH were divided into two subgroups to receive anti–IL-18 antibody or be left untreated. The results summarized in Figures 3 and 4 clearly indicate that the administration of anti–IL-18 antibody in the burn-EtOH group of rats normalized neutrophil Bax (Figure 3), Mcl-1 and Bcl-xl levels similar to those observed in sham animals (Figure 4). No difference in the expression of pro- and antiapoptotic proteins in neutrophils was observed between sham animals untreated and those treated with anti–IL-18 antibody.

Caspase-3 is one of the key components of executioners of apoptosis, because it is either partially or totally responsible for the proteolytic cleavage of many key proteins such as the nuclear enzyme poly (ADP-ribose) polymerase. In a recent study, we have shown a significant decrease in cleaved caspase-3 expression in neutrophils from rats receiving a combined insult of EtOH intoxication and burn injury compared with rats receiving either EtOH exposure or burn injury alone (11). We found that the administration of anti–IL-18 antibody

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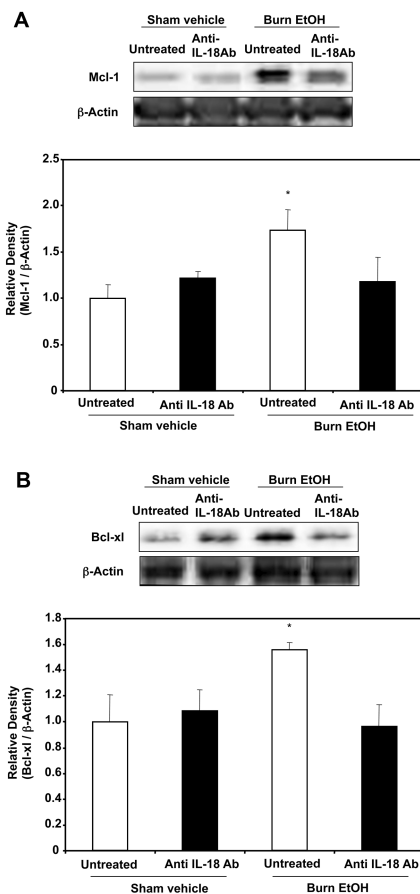
Figure 4. The effect of anti–IL-18 antibody (Ab) on neutrophil expression of Mcl-1 (A) and Bcl-xl (B) on d 1 following EtOH intoxication and burn injury. The amount of Mcl-1 and Bcl-xl was quantitated by use of densitometry. The densitometric values were normalized to the β-actin and are shown as mean ± SEM from 3 to 6 animals in each group. *$P < 0.05$ versus vehicle treated saline plus sham and anti–IL-18 Ab–treated EtOH and burn groups.

normalized caspase-3 activity to the sham levels (Figure 5). However, administration of anti–IL-18 antibody in sham animals did not influence the caspase-3 activity.

In addition to caspase-3 activity, neutrophil apoptosis was further confirmed by measuring cytoplasmic histone-associated DNA fragments in neutrophil lysates. As has been shown in our previous study (11), EtOH intoxication combined with burn injury in rats resulted in ~50% decrease in apoptosis in freshly
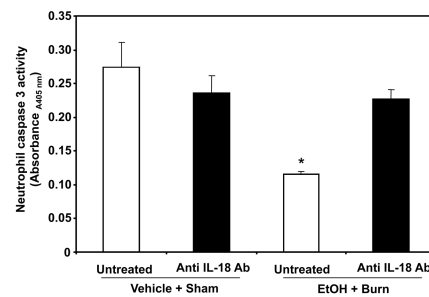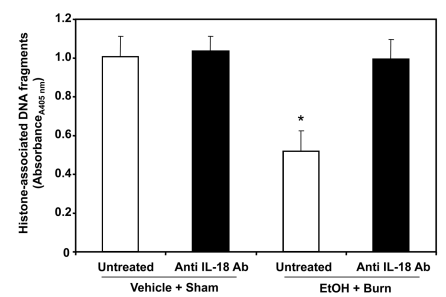

Figure 5. The effect of anti–IL-18 antibody (Ab) on neutrophil Caspase-3 activity on d 1 following EtOH intoxication and burn injury. Neutrophils were lysed and an equal amount of the protein (25 μg/well) was used for the determination of caspase-3 activity. Values are mean ± SEM from 3 to 4 animals in each group. *$P < 0.05$ versus other groups.

isolated neutrophils compared with sham animals. However, treatment of animals with anti–IL-18 antibody restored neutrophil apoptosis to levels similar to those observed in sham animals (Figure 6).

DISCUSSION

In the present study, we have shown that EtOH intoxication combined with burn injury decreases neutrophil apoptosis by modulating pro- and antiapoptotic proteins of the neutrophils. Furthermore, we observed that IL-18 plays a major role in the modulation of pro- and antiapoptotic proteins of the neutrophils following EtOH and burn injury. Studies have shown that under normal physiological conditions, neutrophils are short-lived leukocytes; however, under pathological conditions their life span is modulated (27–30). Several lines of evidence indicate that as a result of major trauma or burn injury, a large amount of proinflammatory cytokines, such as IL-6, TNF-α and IL-1β, are released. Many of these inflammatory cytokines have been shown to cause a delay in neutrophil apoptosis (29–31). In addition, the presence of granulocyte macrophage–colony-stimulating factor (GM-CSF) was also shown to inhibit neutrophil apoptosis following burn injury (32). We have shown previously

Figure 6. The effect of anti–IL-18 antibody (Ab) on neutrophil apoptosis on d 1 following EtOH intoxication and burn injury. Neutrophils were lysed and an equal amount of the protein (25 μg/well) was used for the determination of apoptosis. Values are mean ± SEM from 3 to 6 animals in each group. *$P < 0.05$ versus other groups.

that EtOH intoxication combined with burn injury results in an increase in IL-18 levels in the intestine and lung tissues (10,15,33). Furthermore, we found that IL-18 helps the recruitment of neutrophils to intestine and lungs following EtOH intoxication and burn injury.

The findings reported here further confirm that IL-18 inhibits neutrophil apoptosis by modulating various pro- and antiapoptotic proteins following EtOH and burn injury. Although neutrophils do not express Bcl-2, they express Bcl-xl, Mcl-1 and the proapoptotic protein Bax (34,35). In the present study, we found that in animals with EtOH intoxication combined with burn injury, the levels of the antiapoptotic proteins Mcl-1 and Bcl-xl were significantly increased compared with the levels in sham animals. The level of proapoptotic protein, Bax, was significantly downregulated following EtOH and burn injury compared with the level in sham animals. The upregulation of Mcl-1 and Bcl-xl, and downregulation of Bax, may subsequently decrease caspase-3 activity, resulting in a decrease in neutrophil apoptosis following EtOH and burn injury. In a previous study we have shown an increase in the neutrophil release of proteases (for example, elastase) and reactive oxygen species (for example, O_2^-)

following EtOH intoxication and burn injury (11). The delay in neutrophil apoptosis following EtOH intoxication and burn injury may prolong the ability of neutrophils to produce and accumulate O_2^- and proteases in excess. Such an increase in neutrophil proteases and O_2^- may accelerate tissue damage and organ dysfunction (15,36,37).

Knowledge about the signaling mechanisms involved in the regulation of neutrophil apoptosis is still fairly limited because of methodologic limitations such as the short lifespan of neutrophils. There has been much interest in the role of p38 mitogen-activated protein kinase (MAPK) in the regulation of neutrophil apoptosis and survival (38,39). Reported findings suggest that the inactivation of the intrinsic p38 MAPK activity increases neutrophil apoptosis. Phosphorylation of p38 MAPK induces the phosphorylation of the serine 150 residue of caspase-3 and the serine 364 residue of caspase-8, which impairs the activities of these caspases, thereby favoring survival of the neutrophils (39). It has been suggested that the activation of caspase-3 is initiated during the period of inhibition of p38 MAPK activity and that the survival-signaling cytokine GM-CSF counteracted the transient inhibition of p38 MAPK in isolated neutrophils. These findings suggest that p38 MAPK contributes to neutrophil survival (38). In the present study, we observed a decrease in caspase-3 activity in neutrophils after the combined insult of EtOH and burn injury, and normalization of the caspase-3 activity following the administration of anti–IL-18 antibody. IL-18 has been shown to activate the p38 MAPK pathway in neutrophils (24,40). IL-18 activated p38 MAPK in a Ca^{2+}-dependent manner that was directly influenced by the IL-18 concentration and the incubation time (40). The observation that pretreatment with an anti–IL-18 antibody protects against LPS-induced liver injury, and the report of a similar kind of protection observed in IL-18–knockout mice, further support our hypothesis (41,42). The molecular mechanisms whereby IL-18 modifies the

activity of p38 MAPK in neutrophils undergoing apoptosis are currently unknown.

Apart from activating the p38 MAPK pathway, IL-18 has also been shown to activate the phosphatidylinositol-3 kinase (PI3K)/Akt pathway (24). PI3K is another key signal transducer for neutrophil survival (43,44). Induction of neutrophil survival by various antiapoptotic factors such as GM-CSF, IL-8, interferon (IFN)-β and LPS has been reported to occur through the PI3K pathway (45–47). PI3K-mediated Akt activity can lead to prosurvival phosphorylation of Bax (48). Furthermore, glucocorticoids have been shown to inhibit neutrophil apoptosis, and this effect of glucocorticoids was found to be mediated in part by signaling through PI3K (43). PI3K activation following burn injury results in a downstream activation of nuclear factor–κB and Bad phosphorylation in neutrophils. This PI3K activation also results in the upregulation of antiapoptotic factor Bcl-xl, which subsequently decreases neutrophil apoptosis (44).

Results of other studies have suggested that IL-18 plays a role in the production of IFN-γ and TNF-α (49,50). One of the findings has demonstrated that IL-18 activates and attracts neutrophils by inducing the production of TNF-α, which, in turn, induces the synthesis of leukotriene B_4, a well-known chemoattractant of neutrophils (51). In a previous study we found that IL-18 induces neutrophil chemoattractants cytokine-induced neutrophil chemoattractant (CINC)-1 and CINC-3 following EtOH and burn injury (6). Thus multiple mechanisms may exist by which IL-18 may modulate the neutrophil effector responses, and more studies are needed to delineate these mechanisms.

In the present study we assessed apoptosis in neutrophils isolated from the peripheral blood. However, it remains unclear whether these cells reflect the apoptosis in neutrophils after their infiltration into the tissue. Nevertheless, blood continuously circulates through the organs; as a result neutrophils travel

through the organs and return to the circulation under normal conditions. In contrast, in inflammatory conditions, some neutrophils are retained in the organs each time they pass through them. Moreover, the neutrophils may get activated or their effector functions may change each time they pass through the injured/inflamed tissue or organs. Thus, neutrophils from the peripheral blood obtained from animals receiving EtOH and burn injury may represent a mixed population that has both naïve neutrophils as well as the neutrophils that have been exposed to the injured tissue. In our present study, we did not distinguish between naive neutrophils and neutrophils recruited to the injured tissue; thus, this is a limitation of this study. Furthermore, concern may also be raised because we did not identify the site of anti–IL-18 antibody action in the present study. Our previous studies have shown an elevation in IL-18 levels in most of the organs on day 1 following EtOH and burn injury (10,15,33). However, IL-18 levels in circulation were not found to be significantly different at this time point following EtOH and burn-injury exposure compared with sham exposure. Because we observed a significant elevation in IL-18 levels in the tissue and not in circulation on day 1 after injury, we suspect that anti–IL-18 antibody is primarily neutralizing the release from the tissues. Alternatively the possibility of an increase in IL-18 levels in circulation at an earlier time point and its neutralization by the anti–IL-18 antibody was not ruled out in this study.

In summary, in the present study we demonstrated that a suppression of the mitochondrial (intrinsic) pathway contributes to the delay in circulating neutrophil apoptosis following EtOH intoxication and burn injury. The data also demonstrate that the modulation of anti- and proapoptotic proteins of neutrophils following EtOH intoxication and burn injury is mediated by IL-18, which subsequently leads to prolonged neutrophil survival and possibly causes host tissue damage under those conditions.

ACKNOWLEDGMENTS

DISCLOSURE

The authors declare that they have no competing interests as defined by *Molecular Medicine,* or other interests that might be perceived to influence the results and discussion reported in this paper.

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