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PULMONARY INFLAMMATION AFTER ETHANOL EXPOSURE AND BURN INJURY IS ATTENUATED IN THE ABSENCE OF IL-6

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Abstract

Alcohol consumption leads to an exaggerated inflammatory response after burn injury. Elevated levels of interleukin-6 (IL-6) in patients are associated with increased morbidity and mortality after injury, and high systemic and pulmonary levels of IL-6 have been observed after the combined insult of ethanol exposure and burn injury. To further investigate the role of IL-6 in the pulmonary inflammatory response, we examined leukocyte infiltration and cytokine and chemokine production in the lungs of wild-type and IL-6 knockout mice given vehicle or ethanol (1.12 g/kg) and subjected to a sham or 15% total body surface area burn injury. Levels of neutrophil infiltration and neutrophil chemoattractants were increased to a similar extent in wild-type and IL-6 knockout mice 24 hours after burn injury. When ethanol exposure preceded the burn injury however, a further increase of these inflammatory markers was seen only in the wild-type mice. Additionally, signal transducer and activator of transcription-3 (STAT3) phosphorylation did not increase in response to ethanol exposure in the IL-6 knockout mice, in contrast to their wild-type counterparts. Visual and imaging analysis of alveolar wall thickness supported these findings and similar results were obtained by blocking IL-6 with antibody. Taken together, our data suggest a causal relationship between IL-6 and the excessive pulmonary inflammation observed after the combined insult of ethanol and burn injury.

Keywords

lung; neutrophils; cytokines; alcohol; trauma; burn

Introduction

Alcohol exposure prior to injury greatly exaggerates immune dysfunction resulting in increased morbidity and mortality (Howland and Hingson, 1987; Kowal-Vern et al., 1994; McGill et al., 1995; Silver, 2008). In the US, nearly 50% of patients who present with burn injuries have ethanol in their system at the time of admission (Silver, 2008). Additionally, a blood alcohol content of greater than 0.1g/100mL prior to burn injury has been shown to increase the risk for developing nosocomial pneumonia (Griffin, 2009). In animal models,

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the combined insult of ethanol and burn injury also results in increased distal organ inflammation and susceptibility to infection (Bird and Kovacs, 2008). This dysregulated immune response in humans and mice can be characterized by higher levels of pro-inflammatory cytokines, including interleukin-6 (IL-6), which has been linked with decreases in survival not only after burn, but following other injuries as well (Biffl et al., 1996). However, as discussed below, the role of IL-6 in the lung can vary substantially depending on the etiology of the insult and the involvement of IL-6 in the clinically relevant setting of burn injury with prior ethanol exposure is yet to be elucidated.

Depending on the tissue or cell type in which it is expressed, IL-6 can exert numerous different biological activities, which include fever induction, acute phase protein synthesis, lymphocyte differentiation and activation, and regulation of cytokine production. Following binding of its receptor, IL-6 activates the JAK-STAT pathway leading to transcription of many different genes. Signal transducer and activator of transcription-3 (STAT3) was shown to be important for neutrophil accumulation following bacterial pneumonia (Jones et al., 2006). However, there exists a dichotomy in the literature on the role of IL-6 in pulmonary inflammation and injury.

Several studies demonstrated decreased pulmonary inflammation as measured by neutrophil infiltration and chemokine production in response to cigarette smoke (Yu, 2002) or after acute kidney injury (Klein et al., 2008) in mice genetically deficient in IL-6. In contrast, IL-6 was not found to be required for immune complex-mediated vascular injury (McClintock et al., 2005) and even deemed protective in experimental ventilator-induced lung injury (Wolters, 2009). The role of IL-6 in lung injury induced by sepsis is also complicated in the literature with reports of IL-6 being both beneficial (Quinton, 2008) and detrimental (Riedemann, 2003) depending on the circumstances. In addition to the cellular source and setting, the amount of IL-6 is crucial to its action. In patients, high levels of IL-6 were linked with poor outcome (i.e. mortality) after acute respiratory distress syndrome (ARDS), regardless of the underlying etiology for pulmonary failure (Meduri et al., 1995). ARDS is characterized by an increase in capillary permeability, neutrophil infiltration, and often a medical emergency. The subsequent edema can impair gas exchange and neutrophil degranulation can damage the delicate architecture of the lung parenchyma causing diffuse alveolar damage. Our laboratory has previously demonstrated an increase in both circulating and pulmonary levels of IL-6 after ethanol and burn injury relative to either insult alone (Bird et al., 2010a; Bird et al., 2010b; Faunce et al., 1997, 1998; Fontanilla et al., 2000; Messingham et al., 2000), which coincided with increased chemokine production and neutrophil infiltration (Bird et al., 2010a; Bird et al., 2010b). We propose that IL-6 has an essential function in the heightened congestion and neutrophil infiltration seen in our animal model of burn and ethanol as well as in the worsened outcomes seen clinically in patients who ingest alcohol before they are thermally injured.

The studies described herein examine this requirement for IL-6 signaling in the aberrant pulmonary inflammation following the combined insult of ethanol exposure and burn injury. To accomplish this we used IL-6 knockout mice to evaluate lung inflammation by visual assessment, computational measurements of congestion, pulmonary chemoattractant and activated STAT3 levels, and neutrophil infiltration. Our studies reveal that in the absence of IL-6, the exaggerated pulmonary inflammation observed in the setting of ethanol exposure prior to burn is attenuated to the levels of a burn injury alone. Furthermore, these results were substantiated by the use of a monoclonal antibody against IL-6 in wild-type animals. Taken together our data suggest a causal relationship between IL-6 and the excessive pulmonary inflammation found when ethanol exposure precedes a burn injury.

Materials and Methods

Mice

Male wild-type (C57BL/6) and homozygous IL-6 knockout (B6.129S2-IL6(tm1Kopf)/J) mice were purchased from Jackson Laboratories (Bar Harbor, ME) and sacrificed when between 8–10 weeks old. Mice were housed in sterile micro-isolator cages under specific pathogen-free conditions in the Loyola University Medical Center Comparative Medicine facility. All experiments were conducted in accordance with the Institutional Animal Care and Use Committee.

Murine Model of Ethanol and Burn Injury

A murine model of a single (acute) ethanol exposure and burn injury was employed as described previously (Faunce et al., 1997). Briefly, mice were given a single intraperitoneal dose of 150 μ l of 20% (v/v) ethanol solution (1.12 g/kg) resulting in a blood ethanol concentration of 150 mg/dl at 30 minutes. The mice were then anesthetized (100 mg/kg ketamine and 10 mg/kg xylazine), their dorsum shaved, and placed in a plastic template exposing 15% of the total body surface area and subjected to a scald injury in a 92–95° C water bath or a sham injury in room-temperature water. The scald injury resulted in an insensate, full-thickness burn (Faunce et al., 1999). The mice were then resuscitated with 1.0 ml saline and allowed to recover on warming pads. In separate studies, wild-type mice were given either rat anti-mouse IL-6 antibody or isotype control (IgG; 5 μ g i.p., eBioscience) at 30 minutes post-injury as described previously (Fontanilla et al., 2000). As no differences were found between IgG or anti-IL-6 treatment in the sham groups and burn vehicle group, only mice given ethanol and burn injury plus anti-IL-6 are shown in the results section. All other groups are treatment plus IgG.

Histopathologic Examination of the Lungs

At 24 hours post-injury mice were euthanized, blood was collected via cardiac puncture and lungs were harvested. The upper right lobe was inflated with 10% formalin and fixed overnight as described previously (Patel et al., 1999). The lung was then embedded in paraffin, sectioned at 5 μ m, and stained with hematoxylin and eosin (H&E). The sections were analyzed microscopically in a blinded fashion for pathologic changes, and numbers of neutrophils in 10 high power fields were counted as a marker of inflammation (Patel et al., 1999).

Photographs were taken in a blinded fashion of 10 high power fields (400 $\times$) per animal and analyzed using the Java-based imaging program ImageJ (National Institutes of Health, Bethesda, MD). The images were converted to binary to differentiate lung tissue from air space and then analyzed for the percent area covered by lung tissue in each field of view as described by Papadopoulos et al.

Cytokine Analysis of Lung Homogenates

In the same animals, the right middle lung lobe was snap-frozen in liquid nitrogen. The tissues were then homogenized in 1 ml of BioPlex cell lysis buffer according to manufacturer's instructions (BioRad, Hercules, CA). The homogenates were filtered and analyzed for cytokine production using BioRad multiplex assay or ELISA. The results were normalized to total protein present in the homogenate using the BioRad protein assay (BioRad, Hercules, CA).

Measurement of Phosphoprotein Levels in the Lung

Lung homogenates were also analyzed for levels of phosphorylated proteins using BioRad Phosphoprotein multiplex assay according to manufacturer's instructions (BioRad, Hercules, CA). The mean fluorescent intensity above background of phosphorylated STAT3 was measured in each sample.

Statistical Analysis

Statistical comparisons were made between wild-type and knockout animals in the sham vehicle, sham ethanol, burn vehicle, and burn ethanol treatment groups, resulting in 8 total groups analyzed. One-way analysis of variance was used to determine differences between treatment responses, and Tukey's post-hoc test once significance was achieved ($p < 0.05$). Data are reported as mean values $\pm$ the standard error of the mean.

Results

Verification of IL-6 knockout and monoclonal IL-6 antibody treatment

To verify the absence or decrease of IL-6 in the knockout groups or after antibody administration, IL-6 was measured in lung homogenates following all treatments. No detectable IL-6 was found in the knockout animals following any of the four treatments (Figure 1). This is in contrast to wild-type animals, which had significantly higher concentrations of pulmonary IL-6 after burn compared to sham groups with further increase when ethanol preceded the burn ($p < 0.05$). Additionally, in vivo treatment of wild-type mice with anti-IL-6 antibody reduced lung IL-6 levels by over 90% after the combined insult compared to IgG-treated controls (Figure 1). No differences were found between IgG-treated mice and those not given antibody, or between IgG-treated mice and those given anti-IL-6 in the Sham and Burn Vehicle groups (data not shown).

Increase in Alveolar Wall Thickness After Ethanol and Burn is Reduced in the Absence of IL-6

At 24 hours after ethanol exposure and burn injury there is a marked increase in the thickness of the alveolar wall, which is more pronounced than after burn alone (Figure 2). When ethanol and a burn injury are given to mice deficient in IL-6, this amplified thickness is no longer apparent (Figure 2E, F).

The alveolar wall thickness and cellularity was quantified by using imaging software to measure the area of lung tissue in 10 high power fields per animal which is reported as a percentage of the entire field of view. A significant increase in tissue area, corresponding to a relative decrease in air space, was found after burn injury in both wild-type and IL-6 KO mice (Figure 3A). Similar to what was seen visually, however, only in wild-type mice did ethanol exposure prior to burn expand the tissue area further ($p < 0.05$), indicating increased congestion. These findings were repeated with wild-type animals given either anti-IL-6 antibody or control IgG antibody after ethanol and burn (Figure 3B).

Neutrophil Infiltration in the Lung is Mitigated in the Absence of IL-6

To determine if IL-6 is important in the neutrophilic infiltrate observed after the combined insult of ethanol and burn, lungs from wild-type and IL-6 KO mice were examined microscopically as previously described (Bird et al., 2010a; Bird et al., 2010b; Patel et al., 1999). In wild-type mice, a ten-fold increase (Figure 4A) in neutrophils were found in the lungs after burn compared to sham groups ($p < 0.05$). Ethanol exposure prior to burn injury resulted in a two-fold greater number of neutrophils than burn alone in wild-type mice. IL-6 KO mice also had neutrophil infiltration into the lungs after burn injury but in contrast to

their wild-type counterparts, ethanol exposure prior to burn did not augment neutrophil numbers. Consistent with these results, neutralization of IL-6 by monoclonal antibody diminished the level of neutrophil infiltration by 33% after ethanol and burn injury compared to control IgG-treated animals (Figure 4B). Taken together these data may suggest a causal relationship between IL-6 and the amplified pulmonary neutrophil infiltration seen after the combined insult of ethanol and burn.

Pulmonary KC levels in the Absence of IL-6

Similar to previous studies (Bird et al., 2010a; Bird et al., 2010b; Patel et al., 1999), following the combined insult of ethanol and burn, there was a 20-fold increase ($p < .05$) in KC compared to sham animals and a 2-fold elevation ($p < .05$) compared to burn alone (Figure 5A). This increase was also observed in the IL-6 KO animals after burn alone but in the absence of IL-6, KC levels did not significantly raise above burn alone when given the combined injury though an increase was observed. Additionally, the KC response to combined injury in wild-type animals was abrogated by 70 percent ($p < .05$) by treatment with anti-IL-6 antibody when compared to IgG-treated controls (Figure 5B).

STAT3 Signaling after Ethanol and Burn Injury

Levels of STAT3 activation in the lung after ethanol and burn injury in the presence and absence of IL-6 were measured by multiplex assay. As shown in Figure 6A, lungs from animals receiving burn injury had 4-fold higher phosphorylated STAT3 (pSTAT3) compared to sham mice ($p < .05$) with ethanol exposure prior to burn raising pSTAT3 levels above burn injury alone ($p < .05$). Interestingly, pSTAT3 was also elevated 4-fold in lungs from IL-6 KO mice after burn ($p < 0.05$) but without IL-6, prior ethanol exposure no longer raised pSTAT3 levels further above burn alone (Figure 6A) and remained below levels seen in wild-type counterparts ($p < 0.05$). Consistent with the data described above, neutralization of IL-6 with monoclonal antibody prevented the increase in STAT3 activation observed in the lung after ethanol and burn injury compared to burn injury alone (Figure 6B).

Discussion

Multiple organ failure and sepsis are common complications after a substantial injury and the lungs are among the first organs to fail (Solomkin, 1990). Specifically, pulmonary failure may be induced by leakage of pro-inflammatory mediators, endotoxin, pathogens from both the circulation and the airway, and subsequent neutrophil damage to the delicate architecture of the lung itself. Additionally, chronic ethanol consumption is linked to increased risk of ARDS (Moss et al., 1996). Previous studies in our laboratory have demonstrated that relative to either insult alone, the combined injury of ethanol exposure and burn results in elevated levels of neutrophil infiltration and pro-inflammatory cytokine production in the lung (Bird et al., 2010a; Bird et al., 2010b; Patel et al., 1999), though the mechanism for this harmful increase is unknown. This inflammation was blunted in the absence of either ICAM-1 or TLR4 (Bird et al., 2010a; Bird et al., 2010b), which coincided with decreased amounts of systemic and pulmonary IL-6. We therefore sought to determine directly, the importance of IL-6 in the exaggerated pulmonary inflammation seen when ethanol exposure precedes burn injury.

We show in Figure 2 a visual representation of the state of the lungs 24 hours after a burn injury. The congestion and alveolar wall thickening that characterizes ARDS is present after burn (Figure 2C), appears exacerbated by ethanol (Figure 2D), and dependent on IL-6 when ethanol precedes a burn (Figure 2E,F). When the alveolar wall thickness and cellularity was quantified, the superfluous expansion of lung tissue after the combined injury compared to burn alone was again found to be dependent on IL-6 (Figure 3). Taken together, the visual

representation and imaging analysis may suggest a direct relationship between IL-6 and the increased congestion seen in the lungs when burn is preceded by ethanol exposure. However, this amelioration seems to apply specifically to the excessive inflammation of the combined injury as throughout our studies we see a restoration of inflammatory markers in the absence of IL-6 but only to the degree seen in burn injury alone. Indeed, this held true when evaluating neutrophil infiltration (Figure 4) as well as neutrophil chemotactic factors (Figure 5).

Neutrophils are a common measure of acute inflammation and migrate from the blood into tissues along a density gradient of chemoattractants, which in the mouse include KC. In response to a burn injury in mice, pulmonary KC increases and ethanol has been shown to amplify this accumulation (Bird et al., 2010a; Bird et al., 2010b). In IL-6 KO mice an apparent decrease in comparison to wild-type was seen after burn injury and ethanol exposure though it no longer reached significance when compared to any other burn group. This lack of significance may be a factor of the sample size (n=10–15 per group) or a possible early IL-1 or tumor necrosis factor (TNF) compensatory response in the absence of IL-6. This trend did reach significance when wild-type mice were given anti-IL-6 antibody after the combined injury where pulmonary KC diminished to levels seen in burn alone (Figure 5B).

As mentioned above, IL-6 was linked with poor outcomes in patients with ARDS, which is characterized by inflammation and edema in the lung parenchyma leading to impaired gas exchange. In a study conducted in patients with ARDS, high circulating levels of IL-6 correlated with increased mortality, where elevated IL-6 in the serum on day one of ARDS significantly increased the risk of mortality. Moreover, levels of IL-6 greater than 400 pg/ml during the first week of ARDS was linked to poor survival (Meduri et al., 1995). Consistent with above observations in ARDS patients, we have found that IL-6 is elevated in the circulation and in the lung at 6, 12, and 24 hours following the combined insult of ethanol and burn injury [Figure 1, (Bird and Kovacs, 2008; Bird et al., 2010a; Bird et al., 2010b)]. The role of IL-6 in pulmonary inflammation appears to be dependent on the amount, specific tissue, and type of insult or injury. In hypoxia-induced acute lung injury (ALI), IL-6 was shown to be protective as animals overexpressing IL-6 had decreased mortality, cell death, and membrane injury and leakiness (Ward et al., 2000). Additionally, the acute phase response was diminished in mice genetically deficient in IL-6 following tissue injury (Kopf et al., 1994). Several other studies showed decreased inflammation in the lungs in response to irritants, like ozone, or acute kidney injury in IL-6 KO mice (Johnston, 2004; Klein et al., 2008). In contrast, IL-6 is not required for immune complex-mediated vascular injury, as IL-6 KO mice had similar levels of vascular permeability and leukocyte migration into the lungs compared to wild-type mice (McClintock et al., 2005). In burn patients, ethanol exposure prior to burn results in increased susceptibility to pneumonia (Griffin, 2009). With nearly half of admitted burn patients having positive blood alcohol content, this immune dysfunction can have substantial impact on morbidity, mortality and socioeconomic cost. Our study demonstrates that aberrant pulmonary inflammation, as measured by alveolar wall thickness, KC levels, and neutrophil infiltration, in the setting of ethanol exposure and burn is dependent on IL-6.

IL-6 was shown to be involved in neutrophil chemotaxis to the lungs during pneumonia, through mechanisms involving STAT1 and STAT3 (Jones et al., 2006). Along these lines, alveolar epithelial cell production of STAT3 is critical in protection against *E. coli*-induced pneumonia (Quinton et al., 2008) though, like IL-6, the role of STAT3 in lung inflammation is dependent on the etiology of the injury. As shown in Figure 6A, Tyr705 phosphorylation of STAT3 was significantly increased after the combined insult of ethanol and burn in wild-type but not IL-6 KO mice. Therefore the increased neutrophils present in the lung may

result from increased STAT3 signaling. Though more experiments are needed to establish the function of STAT3 in our burn and ethanol model, the correlation between IL-6, STAT3, and neutrophil levels is supported by our use of anti-IL-6 antibodies. While other cytokines such as IL-11 and G-CSF are known to activate pulmonary STAT3 in response to insults (Gao et al., 2004) our studies indicate a role for IL-6 activation of STAT3 in the setting of burn and ethanol exposure.

In summary, increased alveolar wall thickening, neutrophil infiltration, chemokine production, and phosphorylated STAT3 were observed in the lungs of burn injured mice. These markers of inflammation were further increased when ethanol exposure preceded the burn in wild-type but not IL-6 depleted animals. This reduction of aberrant inflammation to the levels of burn injury alone was consistently achieved by both genotypic knockout of IL-6 and by neutralization of IL-6 by monoclonal antibody. The functional role of IL-6 is highly dependent on the circumstances and its part in the clinically relevant setting of burn injury preceded by ethanol exposure has not been described. With clinical data linking raised levels of IL-6 with increased mortality in ARDS patients (Meduri et al., 1995) and ethanol exposure to morbidity and mortality in burn patients (Silver, 2008), IL-6 may play a crucial role in this setting. Indeed, the data presented herein illustrate a direct and causal relationship between IL-6 and the deviant pulmonary inflammation seen after ethanol and burn injury.

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*p<0.05 compared to all groups

#p<0.05 compared to Sham and IL6KO groups

Figure 1. Pulmonary IL-6 in experimental treatment groups

Levels of IL-6 in lung homogenates were quantified by multiplex and concentrations were normalized to total protein in the sample. Data are presented as mean values $\pm$ the standard error of the mean. N = 12–20 animals per group.

Figure 2. Lung histology at 24 hours after burn

H&E stained lung sections 24 hours after ethanol and burn injury. Top panels are wild-type mice given sham injuries without (A) and with (B) prior ethanol exposure. Middle panels are wild-type mice given burn injuries without (C) and with (D) prior ethanol exposure. Bottom panels were given ethanol and burn injury but are either IL-6 KO (E) or wild-type mice given anti-IL-6 antibody (F). Images were obtained at 1000 $\times$.

Figure 3. Quantification of lung congestion

The area of 10 high power fields (400×) per animal covered by alveolar tissue on H&E-stained sections was measured using ImageJ software. The alveolar thickness is reported as a percentage of the total field of view. Data are shown in wild-type and IL-6 KO mice (A) or in wild-type mice given control IgG antibody or anti-IL-6 antibody (B) and presented as mean values ± the standard error of the mean. N = 6–8 animals per group.

Figure 4. Neutrophil infiltration in the lungs of mice after ethanol and burn injury

Neutrophils were counted by light microscopy in H&E stained lung sections 24 hours after ethanol and burn injury. Data are shown as the total number of neutrophils in 10 high power fields (400×) in wild-type and IL-6 KO mice (A) or in wild-type mice given control IgG antibody or anti-IL-6 (B). Data are presented as mean values $\pm$ the standard error of the mean. N = 10–18 animals per group.

*p<0.05 compared to Sham and Burn Vehicle groups

#p<0.05 compared to Sham groups

\$p<0.05 compared to all groups

Figure 5. Pulmonary KC following ethanol and burn injury in the absence of IL-6

Levels of KC in the lung were quantified by BioRad multiplex bead array in lung homogenates from wild-type and IL-6 KO mice (A) or wild-type mice given control IgG antibody or anti-IL-6 (B). Cytokine concentrations were normalized to total protein in the sample as determined by BioRad protein assay. Data are presented as mean values $\pm$ the standard error of the mean. N = 10–15 animals per group

Figure 6. STAT3 signaling in the lung after ethanol and burn injury

Levels of phosphorylated STAT3 in lung homogenates were determined by BioRad phosphoprotein detection multiplex bead array. Samples were obtained from wild-type and IL-6 KO mice (A) or from wild-type mice receiving control IgG or anti-IL-6 antibody (B). Data are presented as mean values $\pm$ the standard error of the mean. N = 6–16 animals per group.