GM-CSF receptor expression and signaling is decreased in lungs of ethanol-fed rats

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Joshi, Pratibha C., Lisa Applewhite, Patrick O. Mitchell, Khaled Fernainy, Jesse Roman, Douglas C. Eaton, and David M. Guidot. GM-CSF receptor expression and signaling is decreased in lungs of ethanol-fed rats. Am J Physiol Lung Cell Mol Physiol 291: L1150–L1158, 2006. First published July 28, 2006; doi:10.1152/ajplung.00150.2006.—Alcohol abuse dramatically increases the risk of acute lung injury. In an experimental rat model of ethanol-mediated susceptibility to lung injury, recombinant granulocyte/macrophage colony-stimulating factor (GM-CSF) restored alveolar epithelial barrier function both in vitro and in vivo, even during acute endotoxemia. These findings suggested that the alveolar epithelium, which secretes GM-CSF into the airway where it is required for alveolar macrophage maturation, likewise responds to GM-CSF priming in a receptor-mediated manner. In this study we determined that both the GM-CSF receptor α- and β-subunits (GM-CSFRα and GM-CSFRβ) are expressed throughout the rat airway epithelium and that this expression was significantly decreased in the alveolar epithelium following chronic ethanol ingestion (6 wk). In parallel, PU.1, the master transcription factor for GM-CSF signaling in hematopoietic cells, is also expressed in alveolar epithelial cells, and ethanol ingestion likewise decreased PU.1 protein expression and nuclear binding in the alveolar epithelium. Finally, GM-CSF signaling as reflected by PU.1 expression and nuclear binding was restored with recombinant GM-CSF treatment in vitro. We conclude that chronic ethanol ingestion decreases GM-CSF receptor expression and signaling in the lung epithelium. Consequently, we speculate that dampening of GM-CSF stimulation of the alveolar epithelium is responsible at least in part for the diverse functional defects that characterize the alcoholic lung and could be a therapeutic target in acute lung injury.

acute respiratory distress syndrome; signal transduction; type II cells; transcription factor; alcohol abuse; granulocyte/macrophage colony-stimulating factor

CHRONIC ALCOHOL ABUSE SIGNIFICANTLY increases the incidence and severity of the acute respiratory distress syndrome (ARDS), a severe form of acute lung injury, with an overall mortality of 40–60%, and to date is the only comorbid variable identified that independently increases the risk of developing ARDS in critically ill patients (14, 16). In fact, based on these epidemiological studies, it has been speculated that ~50% of patients with ARDS have a significant history of alcohol abuse that contributes to the pathophysiology of acute lung injury in those individuals. Therefore, it is important to investigate the mechanisms by which chronic alcohol abuse renders the lung susceptible to acute injury and identify effective therapies, particularly since ARDS has such a grim prognosis, and at present the treatment is limited to supportive care.

To study potential mechanisms that could explain the association between alcohol abuse and the increased risk for ARDS, we developed a rat model of ethanol-mediated susceptibility to acute lung injury. Although chronic (6 wk) ethanol ingestion alone does not cause acute lung injury, it produces multiple defects in alveolar epithelial function and renders the lung intrinsically susceptible to acute edematous injury. These defects include decreased alveolar liquid clearance, increased permeability to proteins, decreased surfactant secretion, and increased susceptibility to oxidant-mediated apoptosis and necrosis due to depletion of glutathione (2, 3, 6, 8). All told, the experimental “alcoholic lung” shows significant defects in alveolar epithelial, endothelial, and macrophage functions, along with aberrant tissue remodeling during sepsis (12, 15). Therefore, on the basis of controlled experimental studies, it is now apparent that chronic ethanol ingestion, even in the absence of concomitant smoking, malnutrition, or other factors, produces previously unrecognized defects in lung cellular function that render it vulnerable to inflammatory damage.

Our group has sought to identify novel therapeutic strategies that could rapidly correct the alcoholic lung phenotype and therefore decrease the incidence, or at least the severity, of ARDS in at-risk individuals such as alcoholics. One attractive candidate is granulocyte/macrophage colony-stimulating factor (GM-CSF). GM-CSF is a 23-kDa protein that was first identified in mouse lung extracts where it is secreted primarily by alveolar epithelial type II cells (20). Its best-known function is priming the terminal differentiation of circulating monocytes into functional alveolar macrophages. This function was elucidated when a GM-CSF knockout mouse was developed and was found quite unexpectedly to have normal bone marrow maturation but defective alveolar macrophage maturation and a phenotype essentially identical to the human disease pulmonary alveolar proteinosis (4). In contrast, mice that overexpress GM-CSF in the lung have alveolar epithelial type II cell hyperplasia and increased lung size (9), a feature of the GM-CSF transgenic mouse models that has received less attention. This was intriguing to us and is consistent with a trophic effect of GM-CSF that has not been well studied but in fact may not even be restricted to lung epithelium, as GM-CSF promotes skin wound epithelialization in experimental models (5, 7). Interestingly, ARDS patients with relatively higher levels of endogenous GM-CSF in their lung lavage fluids were...
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reduced expression of the GM-CSF receptor (GM-CSFR) in the 

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factor for GM-CSF signaling, PU.1, was decreased by ethanol 

ingestion (11). Remarkably, treatment with recombinant GM-

CSF restored GM-CSFR and PU.1 expression as well as 

bacterial phagocytic capacity in the alveolar macrophage (11). 

However, at present there is virtually no information about the 

functional role of GM-CSF in maintaining the normal alveolar 

epithelium, let alone in pathological states such as alcohol 

abuse. Furthermore, whether or not the lung epithelium even 

expresses GM-CSFRs or PU.1 is unknown, and this study was undertaken to address these questions.

**MATERIALS AND METHODS**

**Ethanol feeding.** Adult male Sprague-Dawley rats (150–200 g; Charles River Laboratory, Wilmington, MA) were fed the Lieber-DeCarli liquid diet (Research Diets, New Brunswick, NJ) containing either ethanol (36% of total calories) or an isocaloric substitution with maltin-dextrin ad libitum for 6 wk as previously published (3). All work was performed with the approval of the Institutional Care and Use of Animals Committee at Atlanta Veterans Affairs Medical Center.

**Isolation of alveolar epithelial cells.** Alveolar epithelial cells from control and ethanol-fed rats were isolated using an established protocol (17). Briefly, rats were anesthetized, and lungs were removed en bloc following tracheostomy. Lungs were lavaged with 40 ml of PBS (pH 7.4) to remove alveolar macrophages. Lungs were perfused with buffer to remove blood cells and then filled with elastase solution to dissociate cells from lungs. The lung tissue was minced in a solution containing DNase I and newborn calf serum. The finely minced lung tissue was successively filtered through 100- and 20-

µm nylon mesh. The cell suspension was plated on bacteriological plastic plates coated with IgG to remove remaining alveolar macrophages. After 1 h of incubation at 37°C, the nonadherent type II epithelial cells were carefully removed. Cells obtained by this method were always >95% viable by trypsin blue exclusion. Flow cytometric analyses showed the following phenotype: >90% keratin positive, ~90% surfactant protein (SP)-C positive, <1% CD14 (macrophage marker) positive, <0.2% CD32 (alveolar macrophage marker) positive, and <1% vimentin (fibroblast marker) positive. Cultured cells at 48 h continued to show a typical epithelial-like morphology and were uniformly positive for SP-C by immunochemistry.

**Epithelial cell line.** To confirm that our findings in the primary alveolar epithelial cells were not attributable to low-level contamination by alveolar macrophages or fibroblasts (<1% by flow cytometry analyses as noted), we also analyzed a commercially available rat lung epithelial cell line [American Type Culture Collection (ATCC) CCL-149]. These cells were maintained in F-12K complete media with 10% fetal bovine serum and subcultured using trypsin-EDTA solution. This cell line was used to confirm the expression of GM-CSFR subunits as well as PU.1 in lung epithelial (i.e., non-hematopoietic) cells. As a positive control for these studies, we also analyzed the rat alveolar macrophage cell line (ATCC NR8383) for PU.1 protein expression.

**Primary alveolar epithelial cell culture.** Alveolar type II cells were cultured with or without recombinant rat GM-CSF (PeproTech, 10 ng/ml) for either 2 or 18 h. After the treatment, cells were stored for PCR or Western blots as described below.

**RNA extractions and reverse transcriptase PCR.** Total RNA was extracted from the isolated alveolar epithelial type II cells or from the cultured ATCC CCL-149 epithelial cells using the Qiagen RNA extraction kit. RNA from each sample was reverse transcribed by a standardized protocol (10) followed by PCR with gene-specific primers. PCR amplification was performed according to following schedule: denaturation, annealing, and elongation at 94°C, 48–60°C, and 70°C for 45, 45, and 90 s, respectively. The number of cycles was chosen from our preliminary optimization experiments for each gene product. GAPDH mRNA level was used as a control. PCR products were separated on a 2% agarose gel containing ethidium bromide. For quantification, PCR bands were scanned using an imaging system. Relative amounts of GAPDH, PU.1, and GM-CSFRα- and β-subunits (GM-CSFRα and GM-CSFRB) were quantified and expressed as PU.1 or GM-CSFR/GAPDH ratios. Specific primers used were as follows: PU.1 (sense) 5′ CAACAGTGTGGAAAGACTCC 3′, (antisense) 5′ GCGGCATCTTTCTGTA 3′; GM-CSFRα (sense) 5′ GCTGCAACCCAGTACATC 3′, (antisense) 5′ GAAGGCCGAAG-GCTTGTGC 3′; GM-CSFRB (sense) 5′ GAGATCCCRTGGTCTCAC 3′, (antisense) 5′ GCCGAGGCACTAGTGC 3′; GAPDH (sense) 5′ TGAAGGTCGTTGTCGAAGGATTGGC 3′, (antisense) 5′ CATGGGATTGAGGTCCACCAC 3′.

GM-CSFRα, GM-CSFRB, and PU.1 primers were designed in our laboratory and were obtained from Sigma-Genosys (Woodland, TX). GAPDH primers were purchased from Promega (Palo Alto, CA). In some experiments, these optimized primer pairs were used in the LightCycler (Roche) real-time PCR protocol. The reaction mixture (10 µl) contained PCR-grade water, 10× PCR buffer, MgCl2, BSA, dNTPs, primer pairs, Jump Start Accu Taq DNA polymerase mix (Sigma), SYBR green, and cDNA. The PCR conditions used for GAPDH, GM-CSFRα and GM-CSFRB, and PU.1 were as follows: at 94°C for 30 s followed by 45 cycles of 48–60°C for 10 s and 72°C for 16–39 s. Melting curve analysis showed one species of amplicon for each primer set.

**Flow cytometric detection of membrane and intracellular receptor expression.** Membrane and intracellular expression of GM-CSFRs on alveolar epithelial cells were measured by an established protocol (10). Briefly, cells were incubated for 30 min at room temperature with rabbit polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) to either the rat GM-CSFRα- or β-subunit, or to an isotype-matched control antibody. Cells were washed to remove unbound antibody followed by a 30-min incubation at room temperature with secondary anti-rabbit antibody conjugated to FITC. For intracellular staining of the receptors, cells were first made permeable with 0.1% saponin in PBS followed by staining with the antibody. Cells were washed with PBS-saponin before adding FITC-conjugated secondary antibody (Santa Cruz Biotechnology). Cells were washed with PBS and kept in the dark at 4°C until analyzed. The labeled cells were analyzed by FACScan flow cytometer (Becton Dickinson, San Jose, CA). Data are expressed both as % cells positive for the α-subunit or the β-subunit as well as the mean channel fluorescence for positive cells in each group.

**PU.1 electromobility shift assay.** Cells were washed with cold PBS, and nuclear binding proteins were extracted. Protein concentration was determined by the Bradford method using BioRad protein assay reagent. Double-stranded PU.1 consensus oligonucleotide (5′ TGA AAG AGG AAC TTG GT) is radiolabeled with [γ-32P]ATP using T4 polynucleotide kinase enzyme. Nuclear protein (10 µg) was incubated with radiolabeled PU.1 for 30 min at room temperature. For competition reactions, non-radiolabeled consensus and mutated PU.1 dou-
ble-stranded oligonucleotides (5’ TGA AAG AGC TAC TTG GT) were added to the reaction mixture at 50× molar concentration as a control to confirm the identity of the PU.1-DNA complexes. DNA-protein complexes were separated on 6% native polyacrylamide gel (20:1 acrylamide/bis ratio) for 2–3 h. Gels were fixed in a 10% acetic acid/10% methanol solution for 10 min, dried under vacuum, and exposed to phosphoscreen.

Western blots. Cell lysates were prepared by adding lysing reagent to alveolar epithelial cells. Fifty micrograms of protein from each sample was loaded onto a 12% acrylamide gel and electrophoresed at

Fig. 1. Granulocyte/macrophage colony-stimulating factor (GM-CSF) receptor α- and β-subunits (GM-CSFRα and GM-CSFRβ) protein expression is shown in the lung epithelium. A: representative immunohistochemistry images of GM-CSFRα (left), GM-CSFRβ (middle), and control IgG (right) in trachea (A; ×4 magnification), bronchi and alveoli (B; ×20 magnification), and in alveoli alone (C; ×40 magnification). B shows representative immunocytochemistry images of GM-CSFRα, GM-CSFRβ, and control IgG, and C shows a representative immunofluorescence image using an antibody for surfactant protein C (a type II cell-specific marker) in freshly isolated alveolar epithelial cells after 48 h in culture.
150 V for 75 min. The separated proteins were transferred to a 0.45 μM polyvinylidine difluoride membrane at 15 V for 75 min. Membranes were blocked at room temperature for 1 h in Tris-buffered saline with 0.2% Tween 20 (TBS-T) containing 5% non-fat dry milk in TBS-T. Primary antibody for PU.1 (Santa Cruz Biotechnology) at 1:50 in 5% milk in TBS-T was added to the membranes and kept at 4°C overnight. After several washing steps to remove unbound primary antibody, membrane was incubated at room temperature with horseradish peroxidase-labeled anti-rabbit IgG secondary antibody in 5% milk in TBS-T for 2 h. After ECL chemiluminescence reagent (Amersham, Arlington Heights, IL) was added to the membranes, bands were detected using a BioRad Imaging System.

Statistics. Data are presented as means ± SE. Data analysis was done either by Student’s t-test or by ANOVA with Student-Newman-Keuls test for group comparison, and differences were considered statistically significant at P < 0.05.

RESULTS

GM-CSFR expression throughout the lung epithelium. We first examined the rat airway for expression of the GM-CSFR by immunohistochemistry staining of lung tissue using monoclonal antibodies that recognize the GM-CSFRα- and GM-CSFRβ-subunits. Representative images of tracheal, bronchial, and alveolar epithelium are shown in Fig. 1A. As is evident in these immunohistochemistry images, both the GM-CSFRα- and GM-CSFRβ-subunits are expressed throughout the entire airway epithelium. Also shown in the right column are the control stains performed with an isotype-matched antibody. Figure 1B shows immunocytochemistry on isolated alveolar epithelial type II cells after 48 h in culture using the same antibodies as in the immunohistochemistry images in Fig. 1A. Figure 1C shows immunofluorescent staining for the type II cell-specific marker, SP-C, performed on comparable cultured alveolar epithelial cells, confirming that these cells are indeed alveolar epithelial type II cells. To our knowledge, this is the first reported evidence that both of the GM-CSFR subunits are expressed in lung epithelium.

The effects of chronic ethanol ingestion on GM-CSFRα and GM-CSFRβ gene and protein expression in isolated alveolar epithelial cells. Although the results shown in Fig. 1 indicate that the GM-CSFR is expressed throughout the airway epithelium, we focused the rest of this study on the alveolar epithelial cell since our previous studies on the effects of chronic ethanol ingestion have to date centered on alveolar epithelial and macrophage dysfunction. We chose to examine the GM-CSFR and its downstream transcription factor, PU.1, as we previously determined that chronic ethanol ingestion has no effect on alveolar fluid levels of GM-CSF protein (11). However, in that study we determined that chronic ethanol ingestion significantly decreased alveolar macrophage expression of both the GM-CSFR and PU.1. It is known that GM-CSF plays a major role in the lung innate immune response by activating alveolar macrophages via this specific signaling pathway. However, to our knowledge, GM-CSF expression in alveolar epithelial cells has not been reported, and we speculated that chronic ethanol-mediated alveolar epithelial dysfunction could parallel the observed defects in the alveolar macrophage. Furthermore, we had also previously determined that recombinant GM-CSF delivered via the airway rapidly restores alveolar epithelial barrier function in ethanol-fed rats (17). Therefore, it seemed likely that the alveolar epithelium not only expresses the GM-CSFR and PU.1, but that their expression might be impaired in the alcoholic lung.

To test these speculations, we isolated alveolar epithelial type II cells from control-fed and ethanol-fed rats and analyzed their expression of GM-CSFRα and GM-CSFRβ. As shown in Fig. 2, GM-CSFRα and GM-CSFRβ are expressed at the mRNA level in alveolar epithelial cells, but no significant differences were found between control- and ethanol-fed animals. Figure 2A shows representative gels, and Fig. 2B shows the summary data for GM-CSFRα and GM-CSFRβ mRNA expression normalized to mRNA expression of the housekeeping gene GAPDH. Although chronic ethanol ingestion had no apparent effect on GM-CSFRα and GM-CSFRβ mRNA levels, it nevertheless had a significant impact on GM-CSFRα and GM-CSFRβ protein expression in the alveolar epithelium as shown in Fig. 3. Specifically, we quantitated intracellular and membrane expression of GM-CSFRα and GM-CSFRβ by flow cytometry. The results for GM-CSFRα expression are shown...
in Fig. 3A, and the results for GM-CSFRβ expression are shown in Fig. 3B. We determined that although alveolar epithelial cells expressed both GM-CSFRα and GM-CSFRβ on their cell membranes, the percentage of cells that expressed either subunit was surprisingly low (<5%), and chronic ethanol ingestion had no significant effect on membrane expression of either subunit. However, a much higher percentage of cells was positive for intracellular expression of both GM-CSFRα and GM-CSFRβ, and chronic ethanol ingestion significantly \((P < 0.05)\) decreased the percentage of cells that expressed these subunits (Fig. 3, A and B). Representative histograms for the intracellular expression of GM-CSFRα and GM-CSFRβ are shown above the summary data in Fig. 3, A and B. Furthermore, ethanol ingestion also decreased \((P < 0.05)\) the relative intensity of GM-CSFRα and GM-CSFRβ expression (as reflected by mean channel fluorescence) in those cells that were positive for these subunits (Fig. 3, A and B). Interestingly, we consistently identified what appeared to be a very small subpopulation of epithelial cells from the control-fed rats that was intensely positive for GM-CSFRβ expression (small second peak in the histogram). This small peak was not seen in the cells from ethanol-fed rats. Whether this represents a unique (albeit very small) subpopulation of epithelial cells that is preferentially lost during chronic ethanol ingestion or simply an artifact of the flow cytometric analyses is unknown and was not pursued in this study. Together, these studies show that alveolar epithelial cells express the GM-CSFR and that the intracellular expression and pool size of both GM-CSFRα and GM-CSFRβ are decreased by chronic ethanol ingestion.

The effects of chronic ethanol ingestion on PU.1 gene and protein expression in isolated alveolar epithelial cells. Previously, PU.1 expression was thought to be restricted to hematopoietic cells. However, our finding that the lung epithelium expresses the GM-CSFR suggested that the master transcription factor for GM-CSF, namely PU.1, might be expressed in this cell type as well. Otherwise, an alternative signaling pathway would need to be present to explain the ability of GM-CSF to induce a phenotypic change in isolated alveolar epithelial cells (17). As shown in Fig. 4, PU.1 mRNA was present in freshly isolated alveolar epithelial cells. However, chronic ethanol ingestion had no effect \((P > 0.05)\) on this expression from both control-fed and ethanol-fed rats. Figure 4A shows representative PCR gels, and Fig. 4B shows the summary data for all of the determinations normalized to mRNA expression of the housekeeping gene GAPDH. Although mRNA levels for PU.1 were not apparently affected by chronic ethanol ingestion, PU.1 protein levels were significantly decreased \((P < 0.05)\) in alveolar epithelial cells from ethanol-fed rats (Fig. 5). Overall, these studies provide novel evidence that the GM-CSF master transcription factor, PU.1, is expressed in a non-hematopoietic cell type (i.e., the alveolar epithelium) and that PU.1 protein levels are decreased by chronic ethanol ingestion.

Expression of GM-CSFRα- and GM-CSFRβ-subunits and PU.1 in the rat lung epithelial cell line. To gather additional evidence that PU.1 is indeed expressed in the lung epithelium and that our findings in Figs. 4 and 5 were not explained by low-level contamination of the cell isolates with alveolar mac-

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**Fig. 3.** Flow cytometric expression of membrane and intracellular GM-CSFRα and GM-CSFRβ protein is shown in alveolar epithelial cells of control-fed and ethanol-fed rats. Shown are the relative numbers of cells that were positive for the GM-CSFR subunits in the membrane and the intracellular compartments as well as the mean channel fluorescence in each compartment. **A:** the data for the GM-CSFRα-subunit. **B:** the data for the GM-CSFRβ-subunit. Representative histograms for the intracellular pools are shown above the summary data for these determinations in **A** and **B**. Each value shown in the summary data for percentage positive and mean channel fluorescence represents the mean ± SE of 5 or more determinations. \(* P < 0.05\) compared with control.
rophages (known to express PU.1), we screened for PU.1 expression (using Western blot analyses and PCR) in a rat lung epithelial cell line (ATCC CCL-149). As shown in Fig. 6, these pure epithelial cells not only express both GM-CSFR subunits (protein expression by flow cytometry in Fig. 6A and mRNA expression by real-time PCR in Fig. 6B), they also express PU.1 at both the gene level (mRNA expression by real-time PCR in Fig. 6B) and at the protein level (Western blot analyses in Fig. 6C). As shown in Fig. 6C, the rat alveolar macrophage cell line (ATCC NR8383), which we used as a positive control for these studies, strongly expresses PU.1 protein as expected. Together, the results in Figs. 4–6 provide strong evidence that the GM-CSF master transcription factor, PU.1, is expressed by lung epithelial cells.

**GM-CSF treatment in vitro increases PU.1 protein expression and nuclear binding in alveolar epithelial cells isolated from ethanol-fed rats.** As discussed, we previously determined that recombinant GM-CSF treatment restored alveolar epithelial barrier function in ethanol-fed rats (17). In a related study, we determined that in alveolar macrophages from ethanol-fed rats, GM-CSF treatment in vitro restored bacterial phagocytic function (11), and this was associated with a GM-CSF-dependent increase in PU.1 protein expression and nuclear binding (11). Therefore, in this current study, we likewise determined whether or not GM-CSF treatment increased its master transcription factor in the alveolar epithelium of ethanol-fed rats as a potential mechanism by which it stimulates epithelial function. We examined PU.1 expression as well as nuclear binding in freshly isolated alveolar epithelial cells from control-fed and ethanol-fed rats ± treatment with recombinant GM-CSF (10 ng/ml) for 18 h in vitro. For these experiments, PU.1 protein expression was quantitated and expressed relative to the housekeeping protein GAPDH. This was done to verify that any GM-CSF-mediated increases in PU.1 protein expression were not due solely to generalized growth factor effects of GM-CSF on the alveolar epithelium. As shown in Fig. 7A, recombinant GM-CSF treatment in vitro significantly ($P < 0.05$) increased cellular PU.1 protein expression in alveolar epithelial cells from ethanol-fed rats by ~48% after in vitro incubation. However, this increase in PU.1 protein did not appear to be secondary to an increase in gene transcription, since the levels of PU.1 mRNA (as determined by RT-PCR) were not affected by GM-CSF treatment (not shown). By comparison, GM-CSF treatment had no significant effect ($P > 0.05$) on PU.1 protein expression in alveolar epithelial cells from control-fed rats. In parallel, recombinant GM-CSF treatment increased nuclear binding of PU.1 in alveolar epithelial cells from ethanol-fed rats. As shown in Fig. 7B, GM-CSF treatment in vitro increased PU.1 nuclear binding in alveolar epithelial cells from both control-fed and ethanol-fed rats, although in general this effect was more dramatic in epithelial cells from ethanol-fed rats. Also evident in this representative gel is that chronic ethanol ingestion decreased PU.1 nuclear binding in parallel to the decrease in cellular PU.1 protein expression shown in Fig. 5. Together, the results in Fig. 7 suggest that recombinant GM-CSF treatment in vitro restores PU.1 protein expression and nuclear binding in alveolar epithelial cells from ethanol-fed rats. (A) Western blot analyses of PU.1 and GAPDH expression in control and ethanol-fed rats. *$P < 0.05$ compared with control-fed rats. (B) Summary data for the relative amount of PU.1 protein compared with the housekeeping protein GAPDH in whole cell lysates of alveolar epithelial cells from control- and ethanol-fed rats. Each value represents the mean ± SE from 4 rats in each group.
fed rats, and this increased PU.1 expression corresponds to restoration of alveolar epithelial function that we have reported previously in this same experimental model (17).

**DISCUSSION**

This study provides four novel observations regarding the role of GM-CSF signaling and function in the lung. First, GM-CSFRs are expressed ubiquitously throughout the airway epithelium from the trachea to the alveolar space. Second, the master transcription factor for GM-CSF signaling, PU.1 (which was thought to be restricted to hematopoietic cells), is expressed by a non-hematopoietic cell (i.e., lung epithelial cells). Third, chronic ethanol ingestion, known to impair alveolar epithelial function and predispose to acute lung injury, decreases expression of both the GM-CSFR and PU.1 in the alveolar epithelium. Fourth and finally, recombinant GM-CSF treatment, which we have shown can rapidly restore alveolar epithelial barrier function in the alcoholic rat lung, increases GM-CSFR and PU.1 expression in alveolar epithelial cells from ethanol-fed rats. Importantly, we also determined that a rat alveolar epithelial cell line also expressed the GM-CSFR as well as the GM-CSF master transcription factor, PU.1. These findings argue strongly that our findings in the primary alveolar epithelial cell preparations could not be attributed to low-level contamination by alveolar macrophages or fibroblasts.

Our findings in this current study provide a plausible and in fact predictable explanation for functional studies we had previously performed. Specifically, we determined that GM-CSF treatment restored alveolar epithelial barrier function, both in vitro and in vivo, in this same model of chronic ethanol ingestion (17). Furthermore, this study complements our recent parallel studies in which GM-CSF treatment restored both GM-CSF signaling as well as innate immune function (as reflected by bacterial phagocytosis) to alveolar macrophages from ethanol-fed rats (11). Overall, this study provides compelling evidence that the lung epithelium expresses the cellular machinery to respond to GM-CSF signaling. Therefore, our findings provide potentially novel insights into the role of GM-CSF-mediated alveolar epithelial barrier function as well as alveolar macrophage function in the normal lung and insight into how alcohol abuse appears to interfere with this critical signaling. Although one can only speculate at present, these findings could explain at least in part why otherwise healthy alcoholic subjects are more susceptible to ARDS.

GM-CSF is a 23-kDa glycosylated monomeric peptide that is secreted by many cell types, including the alveolar epithelium. It was initially named for its ability to stimulate the growth of granulocytes and macrophages from cultured hematopoietic progenitor cells. However, as noted, mice that overexpress GM-CSF in the lung also have alveolar epithelial type II cell hyperplasia and increased lung size (9), suggesting that GM-CSF has important autocrine effects on the alveolar epithelium in addition to paracrine effects on the alveolar macrophages. In that study (9), the authors also determined that the GM-CSFRα-subunit was present in the mouse lung epithelium by immunohistochemistry. Moreover, we recently reported that GM-CSF treatment improved lung liquid clearance and decreased epithelial protein leak in ethanol-fed rats (17). Therefore, these findings argue strongly that our findings in the primary alveolar epithelial cell preparations could not be attributed to low-level contamination by alveolar macrophages or fibroblasts.

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GM-CSF is a 23-kDa glycosylated monomeric peptide that is secreted by many cell types, including the alveolar epithelium. It was initially named for its ability to stimulate the growth of granulocytes and macrophages from cultured hematopoietic progenitor cells. However, as noted, mice that overexpress GM-CSF in the lung also have alveolar epithelial type II cell hyperplasia and increased lung size (9), suggesting that GM-CSF has important autocrine effects on the alveolar epithelium in addition to paracrine effects on the alveolar macrophages. In that study (9), the authors also determined that the GM-CSFRα-subunit was present in the mouse lung epithelium by immunohistochemistry. Moreover, we recently reported that GM-CSF treatment improved lung liquid clearance and decreased epithelial protein leak in ethanol-fed rats (17).
Therefore, the alveolar epithelium must have specific mechanisms to transduce GM-CSF signaling, and the primary goal of this study was to determine whether the alveolar epithium expressed receptors for GM-CSF as well as its master transcription factor, PU.1, and whether or not ethanol ingestion affected this expression. We report here for the first time that both GM-CSFRα and GM-CSFRβ subunits are expressed throughout the rat airway epithelium. Specifically, the GM-CSFRα- and GM-CSFRβ-subunits were visualized in the tracheal, bronchial, and alveolar epithelia by immunohistochemistry. Moreover, in the isolated alveolar epithelial cells, both the GM-CSFRα- and GM-CSFRβ-subunits were present at the membrane and intracellular levels as shown by flow cytometric analysis. Although GM-CSFRα are known to be present on the alveolar macrophage, to our knowledge this is the first comprehensive evidence that lung epithelial cells also express these receptors. Furthermore, although chronic ethanol ingestion had no apparent effect on GM-CSFRα or GM-CSFRβ gene expression, the total protein expression (membrane + intracellular) of these receptors was significantly decreased in the epithelial cells from ethanol-fed animals. This parallels our recent findings in alveolar macrophages in this same model, in which GM-CSFRα or CSFRβ protein expression, but not gene expression, was significantly decreased by chronic ethanol ingestion (11). Because we already determined that GM-CSF protein levels in the alveolar space are not affected by ethanol ingestion (11), it seems unlikely that the observed decrease in GM-CSFR membrane expression in either the alveolar macrophage or the alveolar epithelium is due to internalization of the ligand-receptor complex, as might be seen if GM-CSF levels were somehow increased significantly in the alcoholic lung. It is important to note that GM-CSFR protein expression in the membrane of the alveolar epithelium was relatively low compared with the alveolar macrophage. In our previous study, we determined that 25–35% of the alveolar macrophages were positive for GM-CSFRα and GM-CSFRβ membrane expression by flow cytometry in the normal rat lung (11), whereas in this study we determined that only 5% or fewer freshly isolated alveolar epithelial type II cells showed membrane expression. One potential explanation for these differences is that the alveolar macrophage may be relatively more dependent on GM-CSF signaling, and indeed it is well known that in the absence of GM-CSF priming, the alveolar macrophage fails to mature (19). Alternatively, the isolation of the alveolar epithelial type II cells, which requires elastase digestion (see materials and methods), could damage and/or induce internalization of some of the receptors. However, approximately one-third of the freshly isolated epithelial cells were positive for GM-CSFRα and GM-CSFRβ expression by flow cytometry, and the entire airway epithelium was qualitatively positive for all GM-CSFRα and GM-CSFRβ expression by immunohistochemistry. Therefore, when one combines these new findings with previous studies by our group and others on the physiological effects of GM-CSF (9, 17), it is not at all surprising that the airway epithelium expresses functional GM-CSFRs and that these receptors play a crucial role in maintaining normal barrier function. Furthermore, this expression and function is significantly perturbed by chronic ethanol ingestion, and this is a plausible explanation for the barrier defects that characterize the alcoholic lung (3, 6, 8). The recognition that GM-CSF appears to have an important role in regulating the lung epithelium raises important new questions as to which functions are under its control. Based on our previous work, one could speculate that GM-CSF influences epithelial barrier formation, perhaps through effects on junctional proteins.

Fig. 7. Shown are the effects of recombinant GM-CSF treatment on PU.1 protein expression (A) and nuclear binding (B) in alveolar epithelial cells. Freshly isolated alveolar epithelial type II cells from control-fed and ethanol-fed rats were cultured for 18 h ± GM-CSF (10 ng/ml) for 18 h and then analyzed for PU.1 protein expression as well as PU.1 nuclear binding. A: the cellular PU.1 protein expression relative to the housekeeping protein GAPDH in each experimental group, with each value representing the mean ± SE of 6 determinations. *P < 0.05 compared with untreated, ethanol-fed group. The inset shows a representative Western blot for cells from 2 ethanol-fed animals ± GM-CSF probed with the polyclonal antibody for PU.1. The band at 40 kDa is consistent with the known size of PU.1, and as shown in the right-hand side of the gel, this band is eliminated in the presence of a ×20 concentration of the control peptide. B: representative electrophoretic shift assay for nuclear extracts from alveolar epithelial cells in each experimental group (C, control diet; E, ethanol diet). Lane 0 is free probe without nuclear extract. Lanes 1–4 were probed with a 32P-labeled PU.1 consensus oligonucleotide. Lanes 1 and 2 are extracts from untreated controls, and lanes 3 and 4 are extracts from GM-CSF-treated cells. Lanes 5–8 show the results of probing nuclear extracts from untreated cells from control-fed and ethanol-fed rats with the 32P-labeled PU.1 consensus oligonucleotide and either a ×50 concentration of unlabeled PU.1 consensus nucleotide (lanes 5 and 6) or a ×50 concentration of a 32P-labeled mutated form of the PU.1 consensus nucleotide (lanes 7 and 8) to confirm that the observed nuclear binding is PU.1 specific.
parallel, it could influence surfactant synthesis and secretion in parallel to its well-studied role in regulating surfactant recycling by the alveolar macrophage. The mechanism(s) by which ethanol ingestion impairs GM-CSFR expression, and consequently GM-CSF-dependent functions within the lung epithelium, are at present unknown but clearly merit investigation now that this phenomenon has been recognized.

In parallel, or perhaps as a consequence of decreased GM-CSF expression, chronic ethanol ingestion also impaired downstream GM-CSF signaling by decreasing PU.1 expression and nuclear binding. PU.1 is the master transcription factor for GM-CSF signaling, and its expression is lost in alveolar macrophages of patients with pulmonary alveolar proteinosis as well as in GM-CSF knockout mice (1, 20). Again, to our knowledge this is the first report of PU.1 expression in a non-hematopoietic cell, and this expression was significantly decreased at the protein level by chronic ethanol ingestion. Overall, these findings are also not surprising in that we had recently shown that ethanol ingestion similarly interferes with GM-CSF priming of alveolar macrophages (11). Therefore, it appears that dampening of GM-CSF responsiveness within the alveolar space (and perhaps elsewhere in the airway) is a relatively proximal mechanism by which chronic alcohol abuse renders the lung susceptible to both acute lung injury and to pulmonary infections.

In summary, we report here for the first time that pulmonary epithelial cells express receptors for GM-CSF and for their downstream transcription factor, PU.1, which ultimately transduces GM-CSF signaling. Therefore, this study provides novel evidence that GM-CSF is a critical regulator of both alveolar macrophage and epithelial function within the alveolar space and it may well regulate epithelial function throughout the airway. Importantly, chronic ethanol ingestion decreases protein expression of the GM-CSFRα- and GM-CSFRβ-subunits as well as the transcription factor, PU.1, findings that may well explain how alcohol abuse renders individuals so susceptible to acute lung injury. Furthermore, as we had previously shown in the alveolar macrophages of ethanol-fed rats, recombinant GM-CSFR treatment restored GM-CSFR expression in the alveolar epithelium and increased PU.1 protein expression and nuclear binding, all of which parallel correction of alveolar epithelial barrier function by GM-CSF treatment in ethanol-fed rats. Together, our studies raise the exciting possibility that recombinant GM-CSF therapy, which is already used clinically in other settings, could significantly improve alveolar epithelial and macrophage function in critically ill patients, particularly those with alcohol abuse who are at an excessive risk of developing acute lung injury and serious pulmonary infections.

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