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ALCOHOL PRIMES THE AIRWAY FOR INCREASED INTERLEUKIN-13 SIGNALING

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Abstract

Background—Using an experimental model of airway fibrosis following lung transplantation, we recently showed that chronic alcohol ingestion by donor rats amplifies airway fibrosis in the recipient. Associated with alcohol-mediated amplification of airway fibrosis is increased transforming growth factor beta-1(TGFβ₁) and alpha-smooth muscle actin (α-SMA) expression. Other studies have shown that interleukin-13 (IL-13) modulates TGFβ₁ signaling during experimentally-induced airway fibrosis. Therefore, we hypothesized that IL-13 is a component of alcohol-mediated amplification of pro-fibrotic mediators in the alcoholic lung.

Methods—To test this hypothesis, we analyzed tracheal epithelial cells and type II alveolar cells from control- or alcohol-fed rats, alcohol-treated mouse lung fibroblasts and human bronchial epithelial cells in vitro for expression of various components of the IL-13 signaling pathway. Signaling via the IL-13 pathway was assessed by measuring levels of phosphorylated signal transducers and activators of transcription-6 (STAT6). In addition, we performed heterotopic tracheal transplantation using control-fed and alcohol-fed donor rats and analyzed tracheal allografts for expression of components of the IL-13 signaling pathway by RT-PCR and immunocytochemical analyses.

Results—IL-13 expression was detected in type II alveolar epithelial cells and human bronchial epithelial cells, but not in lung fibroblasts. IL-13 expression was decreased in whole lung and type II cells in response to alcohol exposure. In all cell types analyzed, expression of IL-13 signaling receptor (IL-13Rα₁) mRNA was markedly increased. In contrast, mRNA and protein expression of the IL-13 decoy receptor (IL-13Rα₂) were decreased in all cells analyzed. Exposure to alcohol also increased STAT6 phosphorylation in response to IL-13 and lipopolysaccharide.

Conclusions—Data from multiple cell types in the pulmonary system suggest that IL-13 and its receptors play a role in alcohol-mediated activation of pro-fibrotic pathways. Taken together, these data suggest that alcohol primes the airway for increased IL-13 signaling and subsequent tissue remodeling upon injury such as transplantation.

Keywords

alcohol; airway fibrosis; obliterative airway disease; IL-13; IL-13 receptor

INTRODUCTION

Obliterative bronchiolitis (OB), and its clinical manifestation bronchiolitis obliterans syndrome, is a fibrotic airway obstruction disease that develops in response to many different stimuli, including toxicant exposure (Konichezky et al., 1993; Matarese and Matthews, 1986; Tasaka et al., 1995) and respiratory infections (Coultras et al., 1986; Prabhu et al., 1991; Sung et al., 1992; Wiebicke and Seidenberg, 1990). OB is most prevalent in recipients following lung transplantation (Boehler et al., 1998) and is the leading cause of long-term graft failure, affecting 50-60% of lung transplant recipients that survive 5 years post-transplant (Boehler et al., 1998; Estenne and Hertz, 2002). Unfortunately, predicting which lung transplant recipients develop OB pathology has been unsuccessful to date due to a lack of discernible risk factors. Because OB is progressive and unresponsive to available therapies (Boehler and Estenne, 2003; Boehler et al., 1998) there is a need to identify risk factors for the development of these post-transplant complications.

IL-13 is a pleiotropic cytokine that plays a key role in pulmonary pathophysiological conditions (Joshi et al., 2006a) and has recently been shown to mediate post-transplant airway fibrosis (Joshi et al., 2006a; Keane et al., 2007; Lama et al., 2006). IL-13 acts through its remodeling effects on airway epithelial cells (Laoukili et al., 2001; Relova et al., 2001; Richter et al., 2001; Zhu et al., 1999). IL-13 binds with high affinity to the IL-13 receptor $\alpha 1$ (IL-13R α_1) and IL-4 receptor α_1 . A second IL-13-binding protein, IL-13 receptor $\alpha 2$ (IL-13R α_2) binds IL-13 with high affinity but is non-signaling, thereby serving as a decoy receptor (Mentink-Kane and Wynn, 2004). IL-13 signaling through IL-13R α_1 increases phosphorylation of STAT6 which in turn increases expression of chemokines such as eotaxin (Matsukura et al., 2001; Zhu et al., 1999). Using the HTT model of post-transplant airway fibrosis, IL-13 acts upstream of, and increases expression of TGF β_1 (Lama et al., 2006). TGF β_1 is a critical pro-fibrotic factor known to induce the transformation of fibroblasts into myofibroblasts and extracellular matrix deposition that occurs during airway fibrosis (Hu et al., 2003; Ramirez et al., 2006). Thus, IL-13 is an important cytokine in promoting tissue remodeling associated with airway inflammation and fibrosis.

Recent studies have shown that chronic alcohol ingestion primes the lung for injury (Holguin et al., 1998; Lois et al., 1999; Velasquez et al., 2002) and increases levels of TGF β_1 in the lung (Bechara et al., 2004; Bechara et al., 2005). Clinically, alcoholics have increased risk of acute respiratory distress syndrome following stresses such as sepsis or trauma (Moss et al., 1996; Moss et al., 2003). Young and otherwise healthy alcoholics have impaired alveolar epithelial barrier function despite having normal airway physiology (Moss et al., 2000). Chronic alcohol ingestion modulates a complex network of signaling pathways that eventually lead to dysfunction of alveolar type II (AT2) cells within the respiratory epithelium (Bechara et al., 2003; Bechara et al., 2005; Brown et al., 2001; Guidot et al., 2000; Joshi et al., 2006b). Although this alcohol-mediated pathophysiology is clinically silent in otherwise healthy individuals, it nevertheless renders the lung susceptible to injury. Overall, these studies suggest that clinically silent alcohol-mediated lung dysfunction could promote tissue remodeling and fibrosis following injuries such as transplantation. In support of this contention, we recently demonstrated that chronic alcohol ingestion by donor rats amplifies airway fibrosis in recipients using the heterotopic tracheal transplant (HTT) model of airway fibrosis (Mitchell and Guidot, 2007). Associated with this alcohol-mediated amplification in airway fibrosis was an increase in TGF β_1 expression (Mitchell and Guidot, 2007). The mechanism by which prior alcohol ingestion by donor rats amplifies airway fibrosis in the transplanted airway is unclear; however, modulation of the interleukin-13 (IL-13) signaling pathway represents a plausible pathway by which alcohol 'primes' the airway for pro-fibrotic signaling upon injury.

Therefore, we hypothesized that IL-13 is a component of alcohol-mediated amplification of pro-fibrotic mediators in the alcoholic lung. To test this hypothesis, we analyzed the expression profile of components of the IL-13 signaling pathway in a variety of cells of the pulmonary system from disparate species that were exposed to alcohol and subsequent injury.

MATERIALS AND METHODS

Animals, alcohol feeding, and heterotopic tracheal transplantation (HTT)

All procedures involving animals were performed with approval by the VA Medical Center institutional animal care and use committee (IACUC), and in accordance with the NIH (Publication 85-23) Guide for the Care and use of Laboratory Animals, the Animal Welfare Act, and applicable VA Medical Center policies. Male Sprague Dawley (SD; Charles River) rats (3 – 5 months of age) were fed the Lieber-DeCarli isocaloric liquid diet (Research Diets, New Brunswick, NJ), containing either alcohol (36% total calories) or an isocaloric substitution with Maltin-Dextrin (control diet) for 8 weeks as described previously (Mitchell and Guidot, 2007). HTT was performed as described previously (Ramirez et al., 2004). Briefly, allografts were obtained by transplanting tracheal segments from control- or alcohol-fed donor SD rats subcutaneously into a suprascapular pouch in Fischer 344 recipient rats. Tracheas were harvested prior to transplant (un-transplanted) or at 21 days post-transplant (upon complete occlusion of the allograft lumen by a fibrotic lesion) and either cut into 6 μ m-thick sections for histological and immune-histological analyses, or homogenized for RT-PCR analyses. All recipient rats were fed normal rodent chow and water. Male C57BL/6 mice (10-12 weeks of age; Charles River Laboratories) were used to obtain primary lung fibroblasts.

Isolation of Alveolar type II epithelial (AT2) cells

AT2 cells from control- and alcohol-fed rats were isolated using an established protocol in our laboratory (Pelaez et al., 2004). Briefly, after lungs were removed en bloc following tracheostomy, alveolar macrophages were removed by lavaging the lungs with 40 ml phosphate-buffered saline (PBS). The lungs were then filled with an elastase solution to dissociate cells, the lung tissue was minced in a solution containing DNase I, and newborn calf serum and successively filtered through 100- and 20- μ m nylon meshes. The cell suspension was plated on sterile bacteriological plastic plates coated with IgG to remove remaining alveolar macrophages for 1 hr at 37°C. The nonadherent AT2 cells were collected for experiments and cells obtained by this method were always >95% viable by trypan blue exclusion. For RT-PCR analyses, collected AT2 cells were immediately centrifuged and resuspended in Trizol reagent (Life technologies). For immuno-cytochemical staining, AT2 cells were cultured for 12 hours on glass coverslips at 37°C in 90% air/10% CO₂ to facilitate adhesion.

Isolation of mouse primary lung fibroblasts (PLF)

PLF were harvested from normal C57BL/6 male mice as described by Roman et al. (Roman et al., 2005). Briefly, the outer 3 mm of lung periphery was discarded and cutting the remaining lung parenchyma tissue into 1-mm sections. Tissue sections were washed twice in sterile PBS, resuspended in DMEM with 4.5 g/l glucose supplemented with 10% FBS and 1% antibiotic-antimycotic solution (100 U/ml penicillin G sodium, 100 U/ml streptomycin, 0.25 μ g/ml amphotericin B), transferred to a tissue culture dish, and incubated in a humidified 5% CO₂ incubator at 37°C for 1–3 wk to allow fibroblasts to migrate out of tissue sections. PLF were between three and five passages when used for all experiments.

Cell culture and treatments

All cells were incubated in a humidified 5% CO₂ incubator at 37°C. PLF were maintained in DMEM supplemented with 10% FBS until the time of treatment. Beas2B cells (ATCC) were maintained below 50% confluency on plates that were pre-coated with 0.01 mg/ml fibronectin, 0.03 mg/ml bovine collagen type I, and 0.01 mg/ml bovine serum albumin dissolved in BEBM serum-free media (Clonetics Corporation). For all experiments, Beas2B cells were cultured in BEBM supplemented with 0.5 ng/ml recombinant epidermal growth factor, 500 ng/ml hydrocortisone, 0.005 mg/ml insulin, 0.035 mg/ml bovine pituitary extract, 500 nM ethanolamine, 500 nM phosphoethanolamine, 0.01 mg/ml transferrin, 6.5 ng/ml 3,3',5-triiodothyronine, 500 ng/ml epinephrine, and 0.1 ng/ml retinoic acid (Clonetics). Prior to incubation with alcohol (60 mM) or nicotine (50 µg/ml), PLF or Beas2B cells were plated at 2×10^6 cells (for 48 hr treatments) or 0.5×10^6 cells (for 5 day treatments) per well of 6-well plates. For alcohol and nicotine treatments, PLF were incubated in media containing 2% FBS. Media containing alcohol was replaced daily. For Western blot analyses of STAT6 phosphorylation, Beas2B cells were cultured in 150 mm dishes for 5 days in the presence / absence of ethanol (60 mM). On day 4, cells were incubated in the presence / absence of lipopolysaccharide (1 µg/ml), and human recombinant IL-13 (10 ng/ml) was added for the last 30 minutes. Cells were harvested using the NE-PER nuclear / cytoplasmic extraction kit (Pierce Biotechnology) according to the manufacturer's protocol.

Immunostaining

Anti-IL-13R α_2 and anti- α -SMA antibodies were obtained from R&D Systems and LabVision Corp, respectively. We were unable to obtain a suitable commercially-available anti-IL-13R α_1 antibody suitable for immuno-staining in mouse or rat cells. Biotinylated donkey anti-mouse and donkey anti-rabbit secondary antibodies were obtained from Jackson ImmunoResearch. Immuno-staining was performed as described previously (Mitchell and Pavlath, 2004). Briefly, sections (6 µm) from untransplanted tracheae or allografts, and cells were fixed in 4% formaldehyde for 10 minutes. Endogenous peroxidase activity was quenched with 3% H₂O₂ for 10 minutes. Sections were permeabilized with 0.05% Tween-20 (PBS-T), blocked with 5% donkey serum, incubated overnight at 4°C with primary antibodies. After washing with PBS-T, sections were incubated with biotinylated secondary antibodies followed by horseradish peroxidase-streptavidin (Vectastain kit; Vector Laboratories). Color was developed with 3,3'-diaminobenzidine tetrahydro-chloride substrate (Vector), counterstained with hematoxylin, and coverslipped. Appropriate isotype IgGs were used to control for nonspecific antibody binding. Multiple low- and high-power photomicrographs were obtained using a Leica DM4000B microscope. Relative positive immunostaining between experimental groups was determined by quantifying staining of multiple sections (for tissue) or multiple high power fields (for cells). Briefly, multiple photo-micrographs were imported into Photoshop 7.0 (Adobe Systems Inc.). A color sampler tool was used to gate representative shades of brown DAB staining. To determine the stain density of each field, the area of the field containing the selected color stain was selected, the remaining background was removed, and the resulting image was imported into Scion Image (Scion Corporation). The density of staining was averaged and calculated for each section or field of cells.

Detection of STAT6/phospho-STAT6 by Western blot

Protein concentrations for cytoplasmic and nuclear extracts Beas2B cells were determined using the BCA protein assay. 20 µg of extract were resolved by 4-20% gradient polyacrylamide gel electrophoresis (NuSep). After transfer to PDVF membranes, extracts were blocked in 5% non-fat dry milk and incubated overnight in primary antibody (5 µg/ml anti-STAT6 or anti-phospho-STAT6; BD Biosciences). Primary antibody binding was detected using horseradish-peroxidase conjugated donkey anti-mouse secondary antibody

(1:5,000; Jackson ImmunoResearch) followed by enhanced chemiluminescence (ECL; Amersham). Immuno-reactive bands at ~100 kD were considered specific for STAT6 / phospho-STAT6. Bands were quantitated using Scion Image and values obtained for nuclear phospho-STAT6 were normalized to the corresponding cytoplasmic STAT6 levels.

RNA isolation and reverse transcriptase PCR

Total RNA isolation and reverse-transcriptase PCR were performed as previously described (Mitchell et al., 2005). Total RNA was isolated from whole lung tissue, untransplanted tracheae, transplanted tracheal allografts, or cells using Trizol Reagent following the manufacturer's protocol. Total RNA (2.5 µg) was reverse transcribed in a 20 µl final reaction volume using random hexamer primers and M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA). The reverse transcription reaction was incubated at 42°C for 60 min. 1 µl (for IL-13R α_1 and 18S rRNA) or 2 µl (for IL-13 and IL-13R α_2) of cDNA were amplified using gene-specific primers in a 50 µl reaction (see Table 1 for primer sequences). Samples were incubated at 95°C for 15 min, followed by 29-35 cycles of 95°C denaturation, primer pair-specific annealing (See Table 1), and 72°C extension. For all amplicons, reactions were optimized by first determining the linear range of amplification for the relevant 'control' sample for each group. All reactions were performed in triplicate and normalized to the corresponding 18S rRNA amplicon for each sample. Amplicon pixel intensity for each gene-specific primer pair was quantitated using Scion Image software, normalized to the corresponding 18S rRNA amplicon for each sample, and then presented as a fold change relative to the appropriate 'control' group for each gene.

Statistical analyses

To determine significance between two groups, comparisons were made using Student's *t*-test. Analyses of multiple groups were performed using one-way ANOVA with Bonferroni's post-test using GraphPad Prism version 4.0a (GraphPad Software). For all statistical tests, $p < 0.05$ was accepted for statistical significance. All graphical data are displayed as mean $\pm$ SEM.

RESULTS

Chronic alcohol exposure in rats increases expression of IL-13R α_1 and decreases IL-13R α_2 expression in the whole lung

In order to determine the effects of chronic alcohol ingestion on IL-13 pathway expression in the lung, whole lung sections from control- or alcohol-fed rats were analyzed for expression of IL-13R α_2 protein expression by immuno-histochemical staining. Chronic alcohol exposure decreased IL-13R α_2 protein expression 55% in cells lining the alveolar wall (Figure 1A; panel *ii*) when compared to lungs from control-fed rats (panel *i*). In parallel, RT-PCR was performed on whole lung to determine the expression profile of components of the IL-13 signaling pathway (Figure 1B and 1C). Although IL-13 mRNA expression was decreased by 49% in the lungs of alcohol-fed rats, IL-13R α_1 mRNA expression increased ~3.5-fold. In agreement with the immuno-histochemical data shown in Figure 1A, IL-13R α_2 mRNA decreased by 46%.

Chronic alcohol exposure in rats increases components of pro-IL-13 signaling in type II alveolar epithelial (AT2) cells and primary lung fibroblasts

In an attempt to identify cell types within the lung that are affected by alcohol, we first isolated AT2 cells from the lungs of control- and alcohol-fed rats and immune-stained for IL-13R α_2 expression. IL-13R α_2 expression was decreased by 50% in AT2 cells from chronic alcohol-fed animals (Figure 2A; panels *i* and *ii*). Interestingly, IL-13R α_2 protein

expression was localized mostly intracellularly and specifically in a perinuclear / cytoplasmic granular pattern. These data are consistent with studies that have described a granular cytoplasmic localization pattern in bronchial epithelial cells and fibroblasts (Konstantinidis et al., 2008; Yoshikawa et al., 2003). AT2 cells were also processed for RT-PCR analyses of mRNA expression for components of IL-13-mediated signaling (Figure 2B and 2C). IL-13 expression was consistently decreased by ~40% in AT2 cells from alcohol-fed rats. IL-13R α_1 mRNA expression was increased 2.5-fold and IL-13R α_2 expression was decreased ~50% compared to AT2 cells from the lungs of control-fed rats (Figure 2B and 2C).

In addition, we analyzed the effects of alcohol exposure on the IL-13 pathway in mouse primary lung fibroblasts (PLF) by RT-PCR analyses. PLF were cultured for 48 hr in the presence of 60 mM alcohol or 50 μ g/ml nicotine as a positive control for pro-fibrotic signaling (Roman et al., 2005). IL-13 mRNA was not detected in PLF under any treatment or PCR conditions analyzed (data not shown). These data suggest that fibroblasts respond to IL-13 secreted by other cell types. Consistent with the effects of alcohol in AT2 cells (Figure 2B), mRNA for both IL-13 receptors (IL-13R α_1 and R α_2) were expressed by PLF under normal culture conditions (Figure 2D and 2E). Upon incubation with 60 mM alcohol for 48 hr, a marked increase (4-fold) in IL-13R α_1 mRNA expression was detected with a concomitant decrease (~50%) in IL-13R α_2 mRNA expression (Figure 2D and 2E).

Alcohol exposure increases expression of pro-IL-13 signaling components in epithelial cells of the conducting airways

IL-13 has been shown to be expressed by epithelial cells (Pawankar et al., 1995) and IL-13 expression is increased in the conducting airways during pathologic conditions such as asthma (Doherty and Broide, 2007; Wills-Karp, 2004). Thus, we endeavored to determine the effects of chronic alcohol exposure on the conducting airway epithelium. Tracheal sections from control- or alcohol-fed rats were analyzed by immuno-histochemical staining for IL-13R α_2 expression. Similar to the results obtained in whole lung sections (Figure 1A), chronic alcohol exposure decreased the expression of IL-13R α_2 protein ~50% (Figure 3A; panels ii and iv). In cultured Beas2B cells (human bronchial epithelial cell line), 48 hr exposure to 60 mM alcohol modestly increased IL-13 mRNA expression by ~40% (Figure 3B and 3C). Exposure to alcohol (60 mM) for 48 hours increased expression of IL-13R α_1 3.5-fold. In contrast, IL-13R α_2 expression was decreased 55% (Figure 3B and 3C). Similar results were obtained when Beas2B cells were exposed to 50 μ g/ml nicotine, a known inducer of pro-fibrotic signaling. Since the signaling activity of IL-13 via IL-13R α_1 is also dependent upon IL-4R α , we analyzed expression of IL-4R α in cells exposed to control media or alcohol for 48 hours (Figure 3D). No differences were observed in IL-4R α expression between control and alcohol groups.

Stimulation of STAT6 phosphorylation is amplified in cells exposed to alcohol

Upon binding to IL-13R α_1 , IL-13 increases phosphorylation of STAT6 which in turn increases expression of chemokines such as eotaxin (Matsukura et al., 2001; Zhu et al., 1999). Since alcohol increases expression of the signaling receptor (IL-13R α_1) and decreases expression of the decoy receptor (IL-13R α_2), we hypothesized that alcohol would amplify phosphorylation of STAT6 in response to IL-13. To test this hypothesis, we analyzed the phosphorylation state of STAT6 in Beas2B cells exposed to alcohol (60 mM) and a subsequent stimulus sufficient to elicit STAT6 phosphorylation. In the absence of IL-13, alcohol alone did not elicit detectable levels of phospho-STAT6 (Figure 4A). Upon exposure to IL-13, STAT6 phosphorylation increased; however, no differences were observed between control- and alcohol-treated cells (Figure 4A and 4B). To simulate the in vivo scenario whereby multiple inflammatory and pro-fibrotic mediators are present upon

injury, we incubated control- or alcohol-exposed cells to IL-13 and lipopolysaccharide. In this scenario, cells that were exposed to alcohol + LPS + IL-13 demonstrated increased STAT6 phosphorylation (2-fold) compared to cells in control media + LPS + IL-13 (Figure 4A and 4B).

Chronic alcohol exposure to allograft donors modulates expression of components of pro-IL-13 signaling in the recipient

The IL-13 signaling pathway has previously been shown to play an important role in the development of airway fibrosis after transplantation (Keane et al., 2007; Lama et al., 2006). In addition, we have previously shown that airway fibrosis is amplified in allografts originating from alcohol-fed donor rats (Mitchell and Guidot, 2007). To determine if alcohol-mediated amplification of airway fibrosis is associated with increases in IL-13 expression, we analyzed expression of IL-13 signaling components in untransplanted airways and allografts originating from control- or alcohol-fed rats. Importantly, chronic alcohol ingestion by donor rats did not elicit an increase in tissue remodeling as assessed by α -SMA immune-staining in untransplanted airways (Figure 5A). Amplified airway fibrosis in allografts due to alcohol ingestion by the donor was associated with induction (~2-fold) of IL-13 mRNA expression upon transplantation (Figure 5B and 5C). When alcohol-fed donors were used as donors for airway allografts (EtOH HTT; Figure 5B), IL-13 expression appeared to be increased relative to allografts from control-fed donors (Ctrl HTT). In agreement with our data in vitro (Figures 1, 2, and 3), IL-13R α_1 mRNA expression was increased ~100% in untransplanted airways (EtOH) compared to untransplanted airways from control-fed rats (Ctrl). Upon transplantation (Ctrl HTT and EtOH HTT), mRNA expression of IL-13R α_1 remained high. In contrast, IL-13R α_2 expression was decreased in untransplanted airways from alcohol-fed rats. Upon transplantation, there was no change in the mRNA expression levels of IL-13R α_2 in allografts from control-fed donor rats. IL-13R α_2 expression remained also unchanged at a lower level in allografts from alcohol-fed donors compared to allografts from control-fed donor rats.

DISCUSSION

In this study, we determined that chronic alcohol exposure increased the expression of IL-13R α_1 (the signaling receptor) and decreased the expression of IL-13R α_2 (the decoy receptor) in the pulmonary system. Importantly, we demonstrated these effects in multiple cell types within the pulmonary system that have been implicated in airway fibrosis and remodeling. Although this pattern of differential expression suggests the potential for increased IL-13 signaling, alcohol exposure alone did not increase expression of IL-13 cytokine (and in fact decreased IL-13 mRNA expression in AT2 epithelial cells). Taken together, these new findings suggest that alcohol alters the relative balance between the IL-13 signaling and decoy receptors and thereby “primes” the airway for fibrosis in response to a second insult that acutely increases IL-13 levels in the airway. In the context of lung transplantation, the lung allograft from an alcoholic donor would be hypersensitive to the effects of IL-13 produced during the acute stresses of ischemia-reperfusion and alloimmune inflammation. This is consistent with our recent report that chronic alcohol ingestion alone does not cause airway injury, but rather increases TGF β_1 and α -SMA expression and amplifies airway fibrosis following transplantation (Mitchell and Guidot, 2007).

Several studies have shown that chronic alcohol ingestion generally decreases immune function in a variety of cells in the lung (for reviews, see (Happel and Nelson, 2005; Nelson and Kolls, 2002)). We previously showed that chronic alcohol ingestion by donor rats amplifies the degree of airway fibrosis in transplanted allografts (Mitchell and Guidot, 2007). In that study, we demonstrated that alcohol produced its effects primarily via immune-independent mechanisms that likely involve the TGF β_1 -signaling pathway. Since

alcohol does not increase alloimmune infiltration into allografts that originated from alcohol-fed donors, nor does it 'prime' the untransplanted airway for increased alloimmune reactivity, we hypothesized that alcohol mediates its effects by amplifying pro-fibrotic TGF β ₁-dependent pathways. Several published studies have demonstrated that IL-13-mediated signaling may act upstream, and in so doing, regulates certain aspects of TGF β ₁-mediated signaling (Fichtner-Feigl et al., 2007; Fichtner-Feigl et al., 2006). We therefore hypothesized that alcohol may elicit pro-fibrotic effects at least in part via the IL-13 signaling pathway.

Previous studies from our group have demonstrated that although chronic alcohol ingestion produces long-lasting perturbations in a variety of cells of the pulmonary system, alcohol serves to 'prime' cellular activity in favor of a pro-fibrotic phenotype (Bechara et al., 2004; Guidot and Roman, 2002; Roman et al., 2005). Nevertheless, alcohol alone does not appear to cause tissue remodeling and fibrosis. Instead, an additional stimulus such as injury to the pulmonary system is required to observe the deleterious effects of alcohol. Thus, chronic alcohol ingestion produces 'sub-clinical' effects in the pulmonary system that are not detected until further insult to the cells exposed to alcohol occurs.

The complex nature of the IL-13 signaling pathway provides a plausible mechanism by which alcohol is capable of 'priming' cells for pro-fibrotic activity. IL-13 activity and subsequent downstream intracellular signaling via STAT6 is dependent on IL-13 itself, and may also be dependent on the ratio of signaling receptor (IL-13R α ₁) to decoy receptor (IL-13R α ₂). In addition, signaling of IL-13 through IL-13R α ₁ is dependent on heterodimerization of IL-13R α ₁ with IL-4R α . In this study, we show that although IL-4R α is expressed, alcohol does not modulate expression of IL-4R α . Since exposure to IL-13 stimulates STAT6-phosphorylation, sufficient IL-4R α is present to elicit IL-13-mediated signaling. The minimal stoichiometry of IL-13:IL-13R α ₁:IL-4R α required for signaling is unknown; however, in our in vitro experimental system, an adequate level of IL-4R α expression is present.

The data presented here demonstrate that alcohol, depending on the cell type analyzed, has either no effect or decreases IL-13 mRNA expression. In contrast, alcohol increases expression of IL-13R α ₁ (pro-IL-13 signaling) and decreases IL-13R α ₂ (anti-IL-13-signaling). We interpret these data to suggest that alcohol increases expression of components that favor IL-13-mediated signaling, yet IL-13 signaling per se is not increased. This contention is supported by the fact that alcohol alone does not increase STAT6 phosphorylation. We further speculate that upon injury such as transplantation (as demonstrated here using the HTT model in rats), IL-13 activity and subsequent pro-fibrotic activity is amplified due to an alcohol-mediated increase in IL-13R α ₁ and concomitant decrease in IL-13R α ₂. In support of this, cells exposed to alcohol elicited increased levels of STAT6 phosphorylation upon incubation with IL-13 and LPS.

This study has some important limitations, as the exact mechanism by which exposure to alcohol modulates the IL-13 signaling pathway is as yet unknown. First, it is unclear if alcohol increases IL-13R α ₁ mRNA expression as part of a feedback mechanism to offset the apparent decrease in IL-13 expression that we observed in some cell types. Alternatively, alcohol may first increase IL-13R α ₁ mRNA expression and as a result, IL-13 expression is decreased as part of a negative feedback mechanism. Second, the exact mechanism by which alcohol modulates the IL-13 pathway is unknown; however, we speculate the involvement of an indirect mechanism whereby chronic alcohol increases the presence of reactive oxidative species that are capable of multiple downstream effects. In support of this contention, a previous study showed that chronic alcohol ingestion in rats elicits oxidant-mediated amplification of the renin-angiotensin system (Bechara et al., 2005). In that study,

inhibition of alcohol-mediated amplification of the rennin-angiotensin system with angiotensin converting enzyme inhibitors or angiotensin II receptor blockers were cable of attenuating the deleterious effects of chronic alcohol in the lung (Bechara et al., 2003; Bechara et al., 2005).

In summary, these data support the contention that chronic alcohol ingestion renders the pulmonary system susceptible to injury, at least in part, by ‘priming’ the airway for increased IL-13 pro-fibrotic signaling upon exposure to an additional insult. Upon injury (in this study, by using the HTT model of airway fibrosis in vivo or IL-13 + LPS in vitro), subsequent amplification of TGF β ₁-dependent signaling occurs leading to increased fibrogenic activity and tissue remodeling. Future studies are planned to abrogate IL-13 signaling in an attempt to determine the exact contribution of IL-13-mediated signaling in alcohol-mediated pathophysiology of the pulmonary system.

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Figure 1. Chronic alcohol exposure in rats increases expression of IL-13R α_1 and decreases IL-13R α_2 expression in the whole lung

(A) Immuno-staining for IL-13R α_2 in whole lung sections from control- (panel *i*) or alcohol-fed (panel *ii*) rats. Chronic alcohol exposure decreases IL-13R α_2 protein expression in cells lining the alveolar walls (panels *i* and *ii*). (B) RT-PCR analyses of mRNA expression in whole lung for components of IL-13-mediated signaling. (C) Quantitation of RT-PCR results. IL-13R α_1 expression is increased 2.5-fold whereas IL-13 and IL-13R α_2 expression is decreased 49% and 46%, respectively in lungs from alcohol-fed rats. 18S rRNA was used as a control for RNA input. Immuno-staining and RT-PCR images are representative of lungs from 3 animals for each group. Bar = 100 μ m. * p < 0.05 compared to Ctrl for each gene.

Figure 2. Chronic alcohol exposure in rats increases components of pro-IL-13 signaling in type II alveolar epithelial (AT2) cells and primary lung fibroblasts
 (A) Immuno-staining for IL-13R α_2 in AT2 cells from control- (panel *i*) or alcohol-fed (panel *ii*) rats. Chronic alcohol exposure decreases IL-13R α_2 protein expression. (B) RT-PCR analyses of mRNA expression in AT2 cells for components of IL-13-mediated signaling. (C) Quantitation of RT-PCR analyses in AT2 cells. AT2 cells express IL-13 and expression is decreased 40% in cells from alcohol-fed rats. IL-13R α_1 expression is increased 2.5-fold and IL-13R α_2 expression is decreased 49% compared to AT2 cells from control-fed rats. (D) Expression of components of IL-13-mediated signaling in primary lung fibroblasts after 48 hr incubation in the presence of 60 mM or 50 μ g/ml nicotine. (E) Quantitation of RT-PCR

analyses in primary lung fibroblasts. IL-13R α_1 expression is increased 4.3-fold and IL-13R α_2 expression is decreased 57% in response to alcohol. 18S rRNA was used as a control for RNA input. RT-PCR images are representative of 3 independent experiments. Immunostaining and RT-PCR images are representative of 3 independent experiments. Bar = 50 μ m. *, ** $p < 0.05$ compared to Ctrl for each gene.

Figure 3. Alcohol exposure increases expression of pro-IL-13 signaling components in epithelial cells of the conducting airways

(A) Immuno-staining for IL-13R α_2 in tracheal sections from control- (Ctrl) or alcohol- (EtOH) fed rats. Chronic alcohol exposure decreases expression of IL-13R α_2 protein in the tracheal epithelium. (B) RT-PCR analyses of mRNA expression in cultured Beas2B cells for components of IL-13-mediated signaling. (C) Quantitation of RT-PCR results in Beas2B cells. Exposure to 60 mM alcohol for 48 hr increases IL-13 expression by 43%. In contrast, expression of IL-13R α_1 and IL-13R α_2 is increased 3.5-fold and decreased 55%, respectively. (D) RT-PCR analyses of IL-4R α mRNA expression in Beas2B cells exposed to control or alcohol (60 mM). No significant differences were observed between groups. 18S rRNA was used as a control for RNA input. Images are representative of 3 animals per group (immuno-histochemistry) or 3 independent experiments (RT-PCR). Bar = 250 μ m (panels i and ii) and 50 μ m (panels iii and iv). *, ** $p < 0.05$ compared to Ctrl for each group.

Figure 4. Stimulation of STAT6 phosphorylation is amplified in cells exposed to alcohol
(A) Western blot analyses of STAT6 phosphorylation in Beas2B cells exposed to control (Ctrl) or alcohol (EtOH; 60 mM) for 5 days. With 12 hours incubation remaining, some cells were exposed to LPS (+ LPS; 1 μ g/ml). For the last 30 minutes, IL-13 (+ IL13; 10 ng/ml) was added to some cells to induce STAT6 phosphorylation. Top panels represent phospho-STAT6 blots of nuclear extracts (~100 kD band is marked by the arrow) and bottom panels represent STAT6 blots of cytoplasmic extracts. Alcohol alone does not lead to phosphorylation of STAT6; however, STAT6 phosphorylation is induced upon addition of IL-13. **(B)** Quantitation of phospho-STAT6 Western blots. STAT6 phosphorylation is amplified 2-fold in cells exposed to alcohol + LPS + IL-13 compared to controls + LPS + IL-13. * $p < 0.05$.

Figure 5. Chronic alcohol exposure to allograft donors modulates expression of components of pro-IL-13 signaling in the recipient

(A) Immuno-staining for α -SMA in untransplanted airways from control- and alcohol-fed donor rats. α -SMA staining is unaltered in airways from control- (panels i and iii) vs. alcohol-fed donors (panels ii and iv). (B) RT-PCR analyses of mRNA expression in untransplanted airways and 21-day allografts originating from control- or alcohol-fed donors. (C) Quantitation of RT-PCR analyses in untransplanted airways and 21-day allografts. IL-13 expression is increased in 21-day allografts (Ctrl vs. Ctrl HTT). IL-13 expression is induced in allografts originating from alcohol-fed donors (EtOH vs. EtOH HTT). IL-13R α_1 expression is increased in untransplanted airways from alcohol-fed rats

(Ctrl vs. EtOH). Upon transplantation (Ctrl HTT and EtOH HTT), expression of IL-13R α_1 is increased. In contrast, IL-13R α_2 expression is decreased in untransplanted airways from alcohol-fed rats (Ctrl vs. EtOH). Upon transplantation, mRNA levels of IL-13R α_2 in allografts from alcohol-fed donors remained lower compared to allografts from control-fed donor rats. 18S rRNA was used as a control for RNA input. Immuno-staining and RT-PCR images are representative of 3 independent experiments. Bar = 250 μ m (panels i and ii) and 50 μ m (panels iii and iv). *****, $p < 0.05$ compared to Ctrl for each gene.

Table 1

List of primer sequences, annealing temperatures, and amplicon sizes (in base pairs) used for RT-PCR expression analyses of components of the IL-13 signaling pathway.

Primer	Sequence (F: forward; R: reverse)	Annealing temperature	Amplicon size
Human IL-13	F: 5'-GTAGGCGGGAAGGAGGGTTAGG-3' R: 5'-TAGGGCAGGGGAGGGGTGTAG-3'	59° C	191 bp
Rat IL-13	F: 5'-GCTCTCGCTTGCCTTGGTGGTCT-3' R: 5'-GGCCGGTCTCCCTCAGTGG-3'	60° C	382 bp
Mouse IL-13	F: 5'-CTCTTGCTTGCCTTGGTGGTCTCG-3' R: 5'-TAGTGGGGGCCTTGCGGTTACAG-3'	60° C	270 bp
Human IL-13R α_1	F: 5'-TCATCGTCGCAGGTGCAATCATAG-3' R: 5'-CGGTGCGCGACTCAACATAAAGAC-3'	55.5° C	384 bp
Rat IL-13R α_1	F: 5'-TGCCCCATTGTGCCTACTCC-3' R: 5'-AGCTCCACTTGCTCCCTCTCAG-3'	55° C	410 bp
Mouse IL-13R α_1	F: 5'-GGCCATCCTGCAAAATAGTGC-3' R: 5'-ATGGTGGTGTAGAAGGTGGAGTTT-3'	54° C	403 bp
Human IL-13R α_2	F: 5'-ACCTTTGCCGCCAGTCTATCTTAC-3' R: 5'-TGTCGGGTTTCATTTGTGTTTC-3'	53° C	198 bp
Rat IL-13R α_2	F: 5'-GAGGCCCATTCACCAA-3' R: 5'-CCAGCATTCCTCTTCACTCCAT-3'	52.5° C	209 bp
Mouse IL-13R α_2	F: 5'-AAAGGCATTGAAGGAAAGATACG-3' R: 5'-AAGCCCTCATACCAGAAAAACAT-3'	52° C	239 bp
18S rRNA	Classic II 18S Internal Standards (Ambion)	60° C	324 bp