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Alveolar macrophage inflammatory mediator expression is elevated in the setting of alcohol use disorders

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Abstract

Alcohol use disorders (AUDs) are associated with increased susceptibility to pulmonary diseases, including bacterial pneumonia and acute respiratory distress syndrome (ARDS). Alveolar macrophages (AMs) play a vital role in the clearance of pathogens and regulation of inflammation, but these functions may be impaired in the setting of alcohol exposure. We examined the effect of AUDs on profiles of cytokines, chemokines, and growth factors in human AMs isolated from bronchoalveolar lavage (BAL) samples from 19 AUD subjects and 20 age, sex, and smoking-matched control subjects. By multiplex bead array, the lysates of AMs from subjects with AUDs had significant elevation in the cytokine tumor necrosis factor α (TNF- α), as well as chemokine (C-X-C motif) ligand 8 (CXCL8), CXCL10, and chemokine (C-C motif) ligand 5 (CCL5) ($p < 0.05$). Additionally, a 1.8-fold increase in IL-1 β , 2.0-fold increase in IL-6, 2.3-fold increase in interferon gamma (IFN- γ), 1.4-fold increase in CCL3, and a 2.3-fold increase in CCL4 was observed in the AUD group as compared to the control group. We also observed compensatory increases in the anti-inflammatory cytokine IL-1RA ($p < 0.05$). AUD subjects had 5-fold higher levels of CXCL11 mRNA expression ($p < 0.05$) and a 2.4-fold increase in IL-6 mRNA expression by RT-PCR as well. In these investigations, alcohol use disorders were associated with functional changes in human AMs, suggesting that chronic alcohol exposure portends a chronically pro-inflammatory profile in these cells.

Keywords

AUD; pulmonary; inflammation; alveolar macrophage; cytokine

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INTRODUCTION

Alcohol use disorders (AUDs), including alcohol abuse and alcohol dependence, affect approximately 17 million adults in the United States each year (SAMHSA, 2012). The lifetime prevalence of AUDs is over 30% in the United States, with a higher prevalence in males than females (Hasin, Stinson, Ogburn, & Grant, 2007). AUDs are characterized by recidivism and are potentially lethal, shortening lifespans of affected individuals by more than a decade (SAMHSA, 2012; Schuckit, 2009). In addition, alcohol use leads to significant functional derangements in nearly every organ system and is responsible for a substantial portion of the health-care burden in almost all populations (Schuckit, 2009).

AUDs have been associated with an enhanced susceptibility to a number of pulmonary diseases, including bacterial pneumonia and acute respiratory distress syndrome (ARDS) (Moss, Bucher, Moore, Moore, & Parsons, 1996; Moss et al., 1999; SAMHSA, 2012; van der Poll & Opal, 2009). In addition to an increased risk for contracting community-acquired bacterial pneumonia, AUD patients who succumb to community-acquired pneumonia tend to have greater symptom severity, increased length of hospitalization, more frequent intensive care unit admission, increased need for mechanical ventilation, and poorer survival (de Roux et al., 2006; Fernandez-Sola et al., 1995). Individuals with AUDs also display an increased risk for the development of ARDS, and in this setting, have a more treacherous clinical course, with greater chances of developing non-pulmonary organ dysfunction and increased mortality (Moss et al., 1996; Moss et al., 1999).

Alveolar macrophages (AMs) are the primary effector of pulmonary innate immunity, equipped with a variety of processes implicating them in the pathophysiology of both pneumonia and ARDS. They are characterized by the ability to avidly phagocytose pathogens in the terminal airways, a function vital to the defense against bacterial pneumonia (Nelson & Summer, 1998). Another function, perhaps just as important in defending against pneumonia, is the ability to recruit granulocytes from the systemic circulation by generating chemotactic mediators. Additionally, AMs are the primary cells that ingest apoptotic granulocytes in ARDS to facilitate resolution of pulmonary inflammation, a process known as efferocytosis (Boé et al., 2010; Haslett, 1999).

The relationship of AUDs to impaired AM function may therefore contribute to the observed associations between bacterial pneumonia and ARDS that have been consistently described. Several studies conducted in animal models have reported that AM functions are significantly altered in the setting of alcohol consumption, including a diminished ability to phagocytose bacteria (Boé et al., 2010; Rimland & Hand, 1980; Romero et al., 2014). Human studies have shown increased intrapulmonary oxidative stress in the setting of AUDs, but knowledge of the effects of AUDs on human AM functions remains limited (Antony, Godbey, Hott, & Queener, 1993; L. A. Brown, Harris, Bechara, & Guidot, 2001; D'Souza, Nelson, Summer, & Deaciuc, 1996; Guidot & Brown, 2000).

We and others have previously investigated the cytokine composition of BAL fluid from control and AUD subjects (Burnham, Kovacs, & Davis, 2013). While examination of this

fluid offers insight into the global pulmonary environment, it does not clearly identify which lung cells are responsible for the secretion of these cytokines. Additionally, the dilutional effects of lavage make measurements of these mediators difficult to normalize and interpret. We sought to characterize the AM population in a cohort of patients with AUDs by delineating the cytokine, chemokine, and growth factor profiles of these cells, and to determine whether a pro- or anti-inflammatory state exists in these immune effectors in the absence of overt pulmonary symptoms. We hypothesized that the AM population of AUD subjects would demonstrate an altered inflammatory mediator profile in comparison to control subjects.

MATERIALS AND METHODS

Subject screening, recruitment, and enrollment

Subjects with AUDs were recruited between May 2012 and January 2014 at the Denver Comprehensive Addictions Rehabilitation and Evaluation Services (Denver CARES) center, an inpatient detoxification facility affiliated with Denver Health and Hospital System in Denver, CO. Control patients without AUDs were recruited via approved print and electronic flyer advertisements in the Denver, CO community. This study was approved by the Colorado Multiple Institutional Review Board (COMIRB). All subjects provided written informed consent before their participation in the study.

The Alcohol Use Disorders Identification Test (AUDIT) questionnaire was used for identification of AUD subjects and appropriate controls. The AUDIT questionnaire is a well verified and widely used 10-question screening tool used to identify hazardous and harmful patterns of alcohol consumption in the clinical setting. A score of eight and above in men, or five and above in women, identifies both heavy drinkers and those with AUDs with a sensitivity of 50–90% and a specificity of about 80% (Reinert & Allen, 2002; Schuckit, 2009).

Subjects with AUDs were eligible to participate if they met the following criteria for study entry: i) an Alcohol Use Disorders Identification Test (AUDIT) score of greater than or equal to 8 for men or greater than or equal to five for women; ii) alcohol use within 7 days of enrollment; and iii) age greater than or equal to 21 years. AUD subjects were not approached for enrollment until a sober time extrapolated from their presenting breathalyzer value had been reached. To be eligible for the control arm of the study, subjects were required to have an AUDIT score of less than these values based on gender, corresponding to abstinence or a low risk of alcohol-related problems. Cigarette smoking history was assessed by self-report.

In an effort to minimize potential confounding by comorbidities, subjects were ineligible to participate in the study if they met any of the following criteria: i) prior medical history of liver disease (documented history of cirrhosis, total bilirubin ≥ 2 mg/dL, or albumin < 3.0 g/dL), ii) prior medical history of gastrointestinal bleeding (because of the concern of varices), iii) prior medical history of heart disease (documentation of ejection fraction $< 50\%$, myocardial infarction, or severe valvular dysfunction), iv) prior medical history of renal disease (end-stage renal disease requiring dialysis, or a serum creatinine ≥ 2.0 mg/dL), v)

prior medical history of lung disease defined as an abnormal chest radiograph or spirometry (percent predicted forced vital capacity [FEV₁] <75%), vi) prior history of diabetes mellitus, vii) prior history of HIV infection, viii) pregnancy, ix) abnormal nutritional risk index (Detsky et al., 1984), or x) age >55 years due to the potentially high prevalence of asymptomatic comorbidities in these individuals. A chest radiograph was taken of each subject before enrollment; subjects were excluded if there was any evidence of an acute process or a lesion that might necessitate clinical bronchoscopy. Subjects who failed to provide informed consent were also excluded.

Whole blood samples were obtained from all subjects upon enrollment for complete blood count (CBC), complete metabolic panel (CMP), and prothrombin time with international normalized ratio (INR). Urine samples were also obtained upon enrollment to screen for cocaine, opioids and methamphetamines. Pulmonary function tests (PFTs) were completed prior to performance of the bronchoalveolar lavage (BAL).

Subject Matching

Control subjects were group-matched to AUD subjects on the basis of age, gender, smoking status, and self-reported race.

Bronchoscopy BAL

Bronchoscopy procedures were performed in the inpatient Clinical and Translational Research Center (CTRC) at the University of Colorado Hospital. All procedures were performed utilizing telemetry monitoring and standard conscious sedation protocols as previously described (Burnham et al., 2011; Hunninghake, Gadek, Kawanami, Ferrans, & Crystal, 1979). The bronchoscope was wedged into a subsegment of either the right middle lobe or the lingula. Three to four 50-mL aliquots of sterile, room-temperature, 0.9% NaCl solution were sequentially instilled and recovered with gentle aspiration. BAL fluid was then transported to the laboratory immediately in sterile 50-mL conical tubes for processing. The first aliquot was not utilized for the performance of assays in this investigation.

Cell Count, Differential, and Viability from BAL Samples

Using a small aliquot of the collected BAL fluid, cell count per milliliter was determined by microscopy as previously described (Burnham et al., 2011). Diff-quickTM (Andwin Scientific, Addison, IL) staining was performed. A minimum of 200 cells was evaluated to determine the differential cell types in all samples in a blinded manner. Viability of the cells was determined by Trypan blue exclusion, and was >97% for all samples analyzed.

Cell Pelleting and Sample Shipping

Samples were prepared as previously described (Burnham et al., 2011). Briefly, BAL fluid was aliquoted and pelleted by centrifugation (900 ×g for 5 minutes) and supernatant was removed. Sample cell pellets were snap frozen and shipped overnight on dry ice to Loyola University Health Sciences Campus where they were stored at −80°C until further processing to isolate cellular protein and RNA.

Determination of cytokine and chemokine levels via multiplex bead array

BAL cell pellets were re-suspended in cell lysis buffer (BioRad, Hercules, CA), sonicated for 20 seconds, and passed through a 1.2 µm filter before total protein concentration was determined by colorimetric assay (BioRad). The levels of 27 immunomodulating proteins were then assayed in BAL cell lysates using Bio-Rad Multiplex bead array following manufacturer guidelines as previously described (Bird et al., 2010). All samples were assayed in duplicate and the results were analyzed using the Bio-Plex manager software, version 5.0. All values were normalized to total protein concentration and reported as picograms of protein of interest per milligram of total protein.

RNA Extraction

RNA was extracted using QIAshredder columns and the RNeasy Mini Kit (Qiagen, Valencia, CA), following the manufacturer's instructions (Burnham et al., 2011). RNA was eluted in 30 µL of RNase-free water. RNA quantity and quality was determined using the NanoDrop Spectrophotometer (Thermo Scientific, Wilmington, DE).

Real-Time Reverse Transcription Polymerase Chain Reaction

For all samples, reverse transcription was performed (iScript™, Bio-Rad), using 1 µg of total RNA per reaction. RTPCR experiments were performed using the TaqMan Gene Expression system (Applied Biosystems). Assays were performed in duplicate under standard real-time PCR conditions using a 7300 Real Time PCR system (Applied Biosystems) with sequence detection software (Burnham et al., 2011). Chemokine C-X-C motif ligand 11 (CXCL11, also known as interferon-inducible T-cell alpha chemoattractant [I-TAC]) (TaqMan Gene Expression Assay, Hs04187682_g1) and interleukin 6 (TaqMan Gene Expression Assay, Hs00985639_m1) were target genes while beta-actin (TaqMan Gene Expression Assay, Hs99999903_m1) was used as endogenous control.

Statistical Analyses

The resultant protein quantities generated in multiplex bead array for all subjects were normalized to the total protein quantity of each individual subject. Standard error of these values was reported. Data was classified as parametrically or nonparametrically distributed based on testing of the variances. Parametrically distributed data were analyzed with an unpaired Student's *t*-test. Nonparametrically distributed data were analyzed with Mann Whitney U-test. To address possible confounders of age, sex, and smoking status, a multivariable linear regression analysis was performed. Statistical significance was considered to be a $p < 0.05$.

For the RTPCR data, relative quantitation of mRNA expression for control and AUD subjects was performed utilizing the comparative C_T method with matching as previously described (Burnham et al., 2011). First, duplicate values of each target gene and reference gene were averaged to obtain a mean value for each sample. Next, the mean amount of target gene expressed by AUD subjects was normalized to the group β -actin (the endogenous reference gene) expression to obtain the C_{T-AUD} . Target gene expression for control subjects was similarly normalized to the endogenous reference gene expression to

produce $C_{T-Control}$. The AUD cohort's normalized target gene expression was calibrated to the control cohort's normalized target gene expression via the equation $C_{T-AUD} - C_{T-Control}$, also referred to as ΔC_T . The mean and standard error for each cohort's ΔC_T was calculated and the amount of target gene expression in subjects with AUDs relative to controls was reported using the equation $2^{-\Delta C_T}$ (Pfaffl, 2001). An unpaired Student's t-test was performed on the amount of target gene expression and a p value of < 0.05 was considered significant.

RESULTS

Subjects

Nineteen subjects with AUDs and twenty controls were enrolled between May 2012 and January 2014. Demographics and clinical characteristics of the subjects were collected (Table 1). Mean ages of the control and AUD groups were not substantially different, nor were the percentage of men or the number of current smokers. The AUD group had a significantly higher AUDIT score ($p < 0.001$). Differential cell counts of the subjects' and controls' BAL samples did not differ significantly (Table 1), nor did cell viability results; viability was greater than 97% for all samples examined. Laboratory CBC, CMP, and INR values obtained from the enrolled subjects were compared. For all subjects, serum creatinine, total bilirubin, and albumin measurements were within normal limits. Mean serum creatinine values were 0.9 ± 0.2 mg/dL and 0.8 ± 0.1 mg/dL for the control and AUD cohorts, respectively. Mean serum total bilirubin levels were 0.9 ± 0.4 mg/dL and 1.0 ± 0.4 mg/dL, while albumin levels were 3.9 ± 0.4 g/dL and 3.9 ± 0.3 g/dL for the control and AUD groups, respectively.

To determine if pulmonary function was similar between cohorts, spirometric testing was performed (Table 2). Percent predicted forced vital capacity (FVC) was slightly increased in the AUD group, but FEV₁/FVC ratios were similar between the two groups. Neither AUD subjects nor controls had evidence of acute pulmonary illness on chest radiographs prior to procedure, and no mass lesions were identified among these individuals.

Alveolar macrophage cytokine and chemokine profiles are skewed in AUD patients

To determine whether the cytokine/chemokine milieu differed between cohorts, BAL cell lysates were analyzed. Numerous differences in quantities of specific cytokines, chemokines, and growth factors between AUD and control cohorts were observed (Figure 1). There was dramatic elevation tumor necrosis factor α (TNF- α) levels ($p < 0.05$). A 1.8-fold increase in interleukin 1 β (IL-1 β), a 2.0-fold increase in interleukin 6 (IL-6), a 1.9-fold increase in interleukin 12 (IL-12), and a 2.3-fold increase in interferon gamma (IFN- γ) were observed in the AUD group as compared to the control group. Additionally, there were dramatic elevations of multiple pro-inflammatory chemokines, including a 1.3-fold increase in (C-X-C motif) ligand 8 (CXCL8, formerly IL-8), 4.2-fold increase in CXCL10 (formerly interferon gamma-induced protein 10, or IP-10) ($p < 0.05$), a 2.3-fold increase in chemokine [C-C motif] ligand 4 or CCL4 (also known as macrophage inflammatory protein 1 β or MIP-1 β), and a 2.8-fold increase in chemokine [C-C motif] ligand 5 or CCL5 (formerly regulated on activation, normal T cell expressed and secreted [RANTES]) ($p < 0.05$).

Correspondingly, CCL2 (formerly monocyte chemotactic protein 1 or MCP-1) exhibited a 1.5-fold increase in AUD subjects and chemokine [C-C motif] ligand 3 (CCL3, or macrophage inflammatory protein 1 α [MIP-1 α]) was increased 1.4-fold in AUD subjects.

Key anti-inflammatory proteins were increased in AUD subjects as well, specifically interleukin 1 receptor antagonist (IL-1RA) with a 1.8-fold elevation ($p < 0.05$). Interleukin 10 (IL-10) exhibited a 2.1-fold increase and interleukin 13 (IL-13) exhibited a 1.7-fold increase. Finally, we observed significant increases in two growth factors that have been implicated in inflammatory cell migration in various disease states: granulocyte colony stimulating factor (G-CSF), which is increased 2.3-fold in AUD subjects ($p < 0.05$), and platelet derived growth factor (PDGF), which is elevated 2.7-fold in AUD subjects ($p < 0.05$).

Multivariable linear regression analysis, controlling for the potentially confounding variables of age, sex, and smoking status, was performed as well for each measured protein. The observed differences between inflammatory mediator levels the AM lysates of control and AUD subjects remain significant when accounting for these potential confounders, except for the chemokine CXCL8 (Table 3). Controlling for these independent variables did make the elevations we observed in IL-10, IL-12, and IFN- γ statistically significant, highlighting the contributions that age, sex, and smoking status may have on the intrapulmonary inflammatory state.

Alveolar macrophage pro-inflammatory phenotype marker gene expression is increased in AUD subjects

To further examine the inflammatory state of AMs in AUDs, we measured gene expression levels of CXCL11 and IL-6 by RT-PCR (Figure 2). CXCL11 is a consistent marker of human macrophages indicating a pro-inflammatory phenotype that has been reported to be elevated in stimulated human macrophages compared to both unstimulated, senescent macrophages and alternatively stimulated, anti-inflammatory macrophages (Jaguin, Houlbert, Fardel, & Lecureur, 2013). We observed a 5-fold increase in CXCL11 gene expression the AMs from the AUD cohort as compared to controls ($p < 0.05$). We also observed a 2.4- fold increase in IL-6 gene expression in the AMs of AUD cohort as compared to controls that did not reach statistical significance.

DISCUSSION

In these investigations, we measured protein levels and mRNA expression of BAL cell lysates from human subjects who were otherwise healthy except for a history of AUD. Our observations suggest that AUDs are associated with an inflammatory state in AMs, substantiated by the significant elevation we observed in various pro-inflammatory cytokines, chemokines, and growth factors.

Our observations of a predominantly pro-inflammatory milieu in BAL cell lysate is not entirely expected, as the alcohol and pulmonary communities for years have accepted that chronic alcohol ingestion leads to an anti-inflammatory state in the lung. In 1998, Omidvari, et al. investigated the effects of chronic alcohol consumption on AM release of TNF- α *in vitro* by collecting BAL fluid from 22 alcoholic subjects and 7 healthy volunteers. While

they reported that basal release of TNF- α by AMs was the same in alcoholics and controls, release of TNF- α after stimulation with lipopolysaccharide (LPS) was significantly suppressed in alcoholics compared to that of controls (Omidvari, Casey, Nelson, Olariu, & Shellito, 1998). These findings have been further substantiated in a number of animal studies that have shown decreased production of TNF- α by alveolar macrophages in animal models of chronic alcohol consumption (Antony et al., 1993; D'Souza et al., 1996; Greenberg et al., 1994; Standiford & Danforth, 1997). Standiford and Danforth assessed the effect of alcohol ingestion on cytokine expression of murine AMs cultured *ex vivo* after two weeks of ethanol feeding and saw substantial impairment in the LPS-induced expression of TNF- α , MIP2, and CCL3 mRNA and protein compared with production from AMs obtained from mice receiving an isocaloric control diet. They concluded that ethanol feeding results in diminished production of chemotactic and/or circulating cytokines from AMs *ex vivo* (Standiford & Danforth, 1997). Chronic alcohol consumption has also been shown to increase global lung expression of TGF- β , a cytokine implicated in several models of lung injury and traditionally held as an anti-inflammatory mediator (Bechara, Brown, Roman, Joshi, & Guidot, 2004; S. D. Brown & Brown, 2012; Curry-McCoy, Venado, Guidot, & Joshi, 2013; Thevenot et al., 2013).

The discrepancy we observed in the quantity of pro-inflammatory mediators present in cells with what we have previously reported to be present in BAL in a similar cohort of subjects suggests that active production of these mediators in the absence of frank pulmonary disease is preserved. The protein lysate data shown herein did corroborate the positive relationship between CCL5 and increasing severity of alcohol dependence, independent of cigarette smoking, that we have previously reported (Burnham et al., 2013). It has been shown that alcohol suppresses TNF- α secretion post-transcriptionally by inhibiting the interactions between TNF- α and its converting enzyme, TNF-alpha-converting enzyme, or TACE (Zhao et al., 2003). At the cell surface, TACE performs a proteolytic cleavage of pro-TNF- α , the biologically active precursor, before releasing the 17 kDa TNF- α cytokine (Mohan et al., 2002). Further supporting this idea have been findings that in animal models of acute alcohol intoxication, impaired pulmonary TNF- α protein production has been seen in response to LPS challenge without inhibition of TNF- α gene expression (Nelson, Bagby, & Summer, 1989; Szabo & Mandrekar, 2009). Another potential explanation for differences we observed in the composition of cell protein and BAL protein includes the dilutional effects of BAL. Certain proteins present in very low quantity in lung are difficult to detect in BAL given capabilities of the assay, but are more readily detectable in higher quantities of protein from cell pellets. These discrepancies lead to difficulties in comparing proteins from BAL and cells head-to-head. Another consideration is that cellular protein values were assessed in cells from subjects and controls who did not have overt evidence of pulmonary disease. Cells from individuals obtained in the setting of illness (e.g. during pneumonia) that have been exposed to pathogens (or other initiators of the immune response) might be expected to have different protein composition compared to what we observed.

Data supporting a pro-inflammatory state related to chronic alcohol exposure does exist, however. Bode et al. reported a frequency of endotoxemia of approximately 67% in patients with alcoholic liver disease (Bode, Kugler, & Bode, 1987), which led McClain and Cohen to

subsequently evaluate TNF- α activity in patients with alcoholic hepatitis. They determined that circulating monocytes from alcoholic hepatitis patients have significantly increased spontaneous and LPS-stimulated TNF- α release compared to monocytes from healthy volunteers, arguing for a state of chronic inflammation in chronic alcohol use (McClain & Cohen, 1989). Another contributor to the inflammatory state observed in cells of the AUD subjects could be depletion of glutathione with an excessively oxidized lung environment (L. A. Brown et al., 2001; Guidot & Brown, 2000; Yeh, Burnham, Moss, & Brown, 2007). Such chronic oxidative stress in an experimental setting of long term alcohol consumption has been associated with alterations in AM function (Liang, Yeligar, & Brown, 2012). The balance between the induction of pro-inflammatory mediators and antioxidant genes in response to oxidative stress, especially oxidative stress induced by chronic alcohol ingestion, requires further study (Moodie et al., 2004; Rahman, 1999). Other mechanisms for this prolonged pro-inflammatory state have been suggested as well, including chronic suppression of factors that restrict NF κ B activities in alveolar macrophages as described by Huang, et al (Huang, Chen, & Liu, 2011).

The concept of macrophage polarization, indicating that a macrophage can be classically activated into a pro-inflammatory state or alternatively activated into an anti-inflammatory state, has permeated immunology and inflammation research for years, but a consensus on how to truly define these two states is lacking (Murray et al., 2014). In a recent review by Martinez and Gordon, it was suggested to not categorize macrophages into defined subsets, but instead recognize that numerous pathways exist that lead to more complex, or even mixed, phenotypes of macrophages (Martinez & Gordon, 2014). In this study, we offer data to support a primed, pro-inflammatory state for the resident alveolar macrophage in AUD subjects without overt evidence of pulmonary disease. We observed relevant elevation in many classically pro-inflammatory cytokines and chemokines, including IL-1 β , IL-6, CXCL8, IL-12, IFN- γ , CXCL10, CCL2, CCL3 and CCL4, CCL5, and TNF- α . Interestingly, enhanced secretion of IL-10, which we observed in AUD subjects, has been also been associated with a more pro-inflammatory phenotype in human macrophages (Jaguin et al., 2013). Elevation of the gene expression of CXCL11 and IL-6 additionally support a pro-inflammatory state of cells from the AUD subjects, with upregulated RNA production suggesting that increased production of these pro-inflammatory mediators is an ongoing, dynamic process.

The higher levels of pro-inflammatory cytokines we observed in AMs in the setting of AUDs may argue that these cells could potentially respond more robustly to invading pathogens with associated improved clearance, and therefore result in better outcomes. Clinically, however, this is not what occurs in individuals with AUDs, where pulmonary infections and their attendant outcomes are often more morbid. It may be that AMs in the setting of AUDs do have the capability to produce abundant pro-inflammatory mediators when encountering pathogens, but lack the ability to release these factors into the extracellular environment (Zhao et al., 2003). Alternatively, it is possible that AMs adequately process and release pro-inflammatory mediators, but that prolonged up-regulation of mediator production is not sustained, or is in fact associated with down-regulation of downstream receptors or signaling pathways to sufficiently complete the task. Such prolonged release of pro-inflammatory mediators by these cells may also be expected

to contribute to enhanced neutrophil chemotaxis, or abnormalities in alveolar-capillary barrier function, with concomitant increased severity of infection, or even frank lung injury. Given the epidemiologic associations between AUDs and severe pneumonia/ARDS (Moss et al., 1996; Moss et al., 1999; van der Poll & Opal, 2009), the latter seems possible. The nature of the dysfunction of these AMs after chronic alcohol exposure remains an avid area of study in hopes to minimize susceptibility and to mitigate pulmonary infections common to people with AUDs.

Our investigations are not without limitations. First, the total number of subjects enrolled in our study was not large, limiting the power of the associations we observed. Nonetheless, we observed statistically significant differences in a number of mediators despite our small numbers, and the pro-inflammatory pattern that we observed persisted in multivariable analyses accounting for potential confounders including smoking. Secondly, self-reported cigarette and alcohol history is inherently subject to inaccuracy and reporter bias. We remain confident in our assessment of the AUD subjects' alcohol intake history because they were recruited from a detoxification center that holds long-standing treatment relationships with these individuals. Additional evaluation of the relationship between emerging alcohol use biomarkers, such as phosphatidylethanol (Walther et al., 2015), could potentially shed light on a dose-response relationship between AUDs and our outcome variables. We acknowledge that differences in cytokine, chemokine, and growth factor expression we observed are attributed primarily to AMs within the cell pellets. Nevertheless, lymphocytes are also present within these pellets and may have additionally contributed to our findings. However, we felt the benefit of quantifying mediators from minimally manipulated samples obtained from human subjects (e.g. not subjected to culture, cell sorting, etc) would be more informative regarding how alcohol may impact the immune response/milieu in lung. Future investigations would be important to clarify the role of lymphocytes, or AM-lymphocyte interactions, in the context of alcohol use. Finally, we recognize that these investigations do not prove that alcohol is directly causal to the cytokine differences we observed. Our findings do not explain the mechanisms by which subjects with AUDs are at higher risk for pulmonary disease. We hope that our data help direct further investigations to attempt to discover the mechanisms for our observations in important pulmonary immune cells in the population with AUDs.

In conclusion, we observed an association between increased intracellular levels of a number of pro-inflammatory cytokines, chemokines, and growth factors and AUDs. These abnormalities suggest a pro-inflammatory state in AMs in the setting of AUDs, which may provide insight regarding the reasons for increased pulmonary disease frequency and severity in these individuals.

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Highlights

- Alcohol use disorders (AUDs) are associated with increased pulmonary morbidity
- We compared alveolar macrophage (AM) lysate mediators in AUD and control subjects
- AUD subjects had significantly elevated TNF- α , CXCL10, and CCL5 expression
- There were significant increases in IL-6 and CXCL11 mRNA expression as well
- AUDs portend a chronically pro-inflammatory profile in human alveolar macrophages

Figure 1.

Control and AUD Subject BAL Cell Lysate Protein Concentrations. Protein concentrations were measured for each subject in picograms per milliliter, then normalized to each individual's total protein concentration in milligrams per milliliter. Horizontal bars represent the group mean. N = 20 control subjects and N = 19 AUD subjects. A) IL-1 β ; B) IL-1RA; C) IL-4; D) IL-5; E) IL-6; F) CXCL8; G) IL-10; H) IL-12; I) IL-13; J) IL-17; K) TNF- α ; L) IFN- γ ; M) CXCL10; N) CCL2; O) CCL3; P) CCL4; Q) CCL5; R) G-CSF; S) PDGF; T) VEGF. Significant differences are indicated by an asterisk (*) above the AUD bar of the graph. Mann Whitney U t-test was performed between groups for each protein of interest; p < 0.05 was considered significant.

Figure 2. Control and AUD Subject BAL Cell Lysate Gene Expression. Relative fold change in the expression of the gene of interest is shown. A) CXCL11, N = 11 controls and N = 12 AUD subjects; B) IL-6, N = 11 controls and N = 12 AUD subjects. Significant differences are indicated by an asterisk (*) above the AUD bar of the graph; $p < 0.05$ was considered significant.

Table 1

Control and AUD Subject Demographics and BAL Differential. Age, gender, smoking status, and alcohol history for calculation of AUDIT score were self-reported by enrolled subjects. BMI was measured on enrollment. BAL differential data is presented as cells counted in millions and values are represented as percentage of total cells counted $\pm$ standard deviation. Two hundred cells were counted per subject in a blinded manner. p values are reported values after unpaired Student's t-test analysis between groups. AUDIT, alcohol use disorder identification test; BMI, body mass index; BAL, bronchoalveolar lavage; mono/mac, monocytes or macrophages; lymphs, lymphocytes; PMNs, polymorphonuclear leukocytes; eos, eosinophils.

	Control Subjects N = 20	AUD Subjects N = 19	p value
Age	40.7 $\pm$ 8.5	42.9 $\pm$ 6.7	0.367
Male	16 (80%)	14 (70%)	0.650
Smoker	10 (50%)	10 (50%)	0.873
AUDIT Score	2.6 $\pm$ 2.2	29.5 $\pm$ 7.1	< 0.001
BMI	28.5 $\pm$ 6.0	26.5 $\pm$ 4.9	0.268
BAL Differential			
Cell count (millions)	25.4 $\pm$ 17.8	21.2 $\pm$ 14.9	0.429
% Mono/mac	86.3 $\pm$ 9.4	81.6 $\pm$ 14.6	0.386
% Lymphs	11.2 $\pm$ 8.5	17.1 $\pm$ 16.7	0.172
% PMNs	2.2 $\pm$ 3.8	1.1 $\pm$ 1.2	0.235
% Eos	0.3 $\pm$ 0.6	0.2 $\pm$ 0.2	0.364

Table 2

Control and AUD Subject Pulmonary Function Tests on Enrollment. Values are represented as mean $\pm$ standard deviation; p values are reported values after unpaired Student's t-test analysis between groups. FEV₁, forced expiratory volume in the first second of expiration; FVC, forced vital capacity.

	Control Subjects N = 20	AUD Subjects N = 19	p value
FEV ₁	3.6 $\pm$ 0.8	3.7 $\pm$ 0.6	0.839
FEV ₁ , percent predicted	94.5 $\pm$ 10.8	100.8 $\pm$ 11.8	0.088
FVC	4.6 $\pm$ 0.9	4.7 $\pm$ 0.8	0.792
FVC, percent predicted	96.2 $\pm$ 8.0	102.5 $\pm$ 11.0	0.047
FEV ₁ /FVC ratio (%)	78.4 $\pm$ 7.8	78.3 $\pm$ 6.0	0.977

Table 3

Comparison of p-values obtained with Multivariable Linear Regression Analyses and Mann-Whitney U Tests. Multivariate linear regression model controls for the independent variables of age, gender, and smoking status. Bold, italicized values are less than 0.05 and are considered significant. After controlling for age, gender, and smoking status, significance was lost in CXCL8 levels but was gained in IL-10, IL-12, and IFN γ levels. The Mann-Whitney U test values correlate to the protein levels marked as significant in Figure 1. Multivar. lin., multivariable linear.

	t value	Multivar. lin. regression p value	Mann-Whitney U test p value
IL-1 β	1.13	0.266	0.279
IL-1RA	3.01	<i>0.005</i>	<i>0.005</i>
IL-4	1.88	0.068	0.182
IL-5	2.81	0.008	0.095
IL-6	1.24	0.224	0.255
CXCL8	0.93	0.360	<i>0.027</i>
IL-10	2.13	<i>0.041</i>	0.119
IL-12	2.13	<i>0.040</i>	0.347
IL-13	2.93	0.006	0.133
IL-17	-2.42	0.021	0.100
TNF α	2.2	<i>0.034</i>	<i>0.003</i>
IFN γ	2.37	<i>0.023</i>	0.182
CXCL10	3.4	<i>0.002</i>	<i>0.004</i>
CCL2	1.16	0.253	0.491
CCL3	1.17	0.251	0.663
CCL4	1.77	0.086	0.100
CCL5	2.84	<i>0.008</i>	<i>0.002</i>
G-CSF	3.04	<i>0.005</i>	<i>0.003</i>
PDGF	4.28	<i><0.001</i>	<i><0.001</i>
VEGF	1.57	0.126	0.267