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Ethanol induced TGF- β_1 and ROS production are necessary for ethanol induced alveolar macrophage dysfunction and induction of alternative activation

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Abstract

Rationale—Previous studies have shown that chronic ethanol ingestion results in impaired alveolar macrophage function, increased TGF- β_1 production, and decreased antioxidant availability. Similarly, alternative activation (M2 activation) of alveolar macrophages also induces TGF- β_1 production and impairs macrophage function. However, the potential links between ethanol-induced alveolar macrophage derangements, M2 activation, TGF- β_1 production signaling, and oxidant stress has yet to be examined.

Objective—We hypothesized that ethanol-induced oxidant stress and induction of TGF- β_1 signaling results in alternative activation which subsequently impairs the phagocytic capacity of alveolar macrophages.

Methods—Primary rat alveolar macrophages and the alveolar macrophages cell line NR8383 was treated with 0.08% ethanol $\pm$ the antioxidant glutathione (GSH) or a TGF- β_1 neutralizing antibody for 5 days. Outcome measures included TGF- β_1 production, reactive oxygen species (ROS) production, phagocytic capacity, and expression of markers of M2 activation.

Results—Chronic ethanol treatment greatly decreased alveolar macrophage phagocytic function, increased ROS production, increased TGF- β_1 , and increased expression of markers of M2 activation. Glutathione supplementation and inhibition of TGF- β_1 signaling during ethanol treatment prevented these alterations.

Conclusions—Ethanol treatment increased oxidant stress, TGF- β_1 production, and alternative activation in NR8383 cells. However, GSH supplementation and ablation of TGF- β_1 signaling prevented these effects. This suggested the ethanol-induced switch to a M2 phenotype was a result of decreased antioxidant availability and increased TGF- β_1 signaling. Preventing ethanol-induced induction of alternative activation may improve alveolar macrophage function in alcoholic subjects and decrease the risk of respiratory infections.

Keywords

macrophage; TGF- β_1 ; oxidative stress; glutathione; alternative activation

INTRODUCTION

Abuse or excessive consumption of alcohol has numerous detrimental effects on physical and mental health, and causes extensive organ damage. Many of these effects contribute to increased mortality and morbidity and are irreversible (Boé et al., 2009; Cubero et al., 2009; Harper, 2009; Lands, 1998; Oscar-Berman and Marinkovic, 2003; Rehm et al., 2003; Room et al., 2005). Patients with alcohol use disorders (AUDs), including alcohol abuse and alcohol dependence, have a 2 fold increase in the incidence of ICU-related morbidity and mortality. This is commonly caused by acute respiratory distress syndrome (ARDS), which is characterized by lung inflammation that leads to impaired gas exchange and release of pro-inflammatory cytokines (Jurkovich et al., 1993; Moss et al., 1996; O'Brien et al., 2007). The outcome of AUD patients with ARDS is worse than in non-alcoholics, as seen by significantly higher in-hospital mortality rates. The increased incidence of ICU admittance is primarily related to an increased risk of sepsis in AUD subjects. The increased risk of respiratory infections is partially due to an impaired immune response of the alveolar macrophages, the resident phagocyte in the airspace (Aytacoglu et al., 2006; Brown et al., 2004; Joshi and Guidot, 2007).

Depending on the different stimuli within the microenvironment within the alveolar space, alveolar macrophages are activated via distinct pathways that produce opposing effects on macrophage receptor expression, cytokine expression, and phagocytic function (Abramson and Gallin, 1990; Gordon, 2003; Gordon and Taylor, 2005; McKenzie et al., 1998; Muller et al., 2007; Taylor et al., 2005). Upon exposure to microbial stimuli such as lipopolysaccharide or T_H1 cytokines, the alveolar macrophage is primed to become “classically activated” effector cells (Downing et al., 1999; Nathan and Hibbs, 1991; Noda and Amano, 1997). In response to T_H2 cytokines, alveolar macrophages become alternatively activated and exhibit a phenotype that mediates allergic responses and promotes angiogenesis, tissue repair, and antiparasitic functions. Alternative activation is characterized by pronounced expression of arginase-1, a dampened immune response and promotion of tissue repair (Gordon, 2002, 2003; Gordon and Martinez; Gordon and Taylor, 2005; Martinez et al., 2009; Stein et al., 1992; Taylor et al., 2005). While alternative activation decreases inflammation, chronic impairment of the immune response decreases the capacity to remove microbial pathogens. Alternative activation has also been shown to induce lung fibrosis via production of profibrotic factors such as fibronectin, matrix metalloproteinases, and TGF- β_1 (Isono et al., 2002; Leask and Abraham, 2004; Ruiz-Ortega et al., 2007; Wang et al., 2006). Alternative activation is distinguished by the upregulation of several markers including arginase-1, galectin-3, mannose receptor, Ym1/2, TGF- β_1 , and FIZZ-1 (Fichtner-Feigl et al., 2006; MacKinnon et al., 2008; Nair et al., 2003; Stein et al., 1992). Therefore, alternative activation is one mechanism by which the immune response or the pro-fibrotic response of alveolar macrophages is modulated.

In previous studies, chronic ethanol ingestion impaired alveolar macrophage binding and internalization of inactivated *S. aureus*. Over 50% of the macrophages isolated from ethanol-fed animals bound the pathogen but were unable to complete internalization (Brown et al., 2004). Additionally, chronic ethanol ingestion decreased antioxidant availability, leading to chronic ROS generation by alveolar macrophages (Bechara et al., 2004; Brown et al., 2004; Brown et al., 2007). In *in vivo* studies, ROS induces TGF- β_1 expression (Koli et al., 2008) which is also a characteristic of alternatively activated macrophages (Martinez et al., 2009; Song et al., 2000; Stein et al., 1992). Once TGF- β_1 is activated, it induces the depletion of the antioxidant glutathione (GSH) and increases intracellular ROS production. In contrast, *in vitro* treatments with GSH precursors induce proteolysis of the TGF- β_1 type II receptor and disintegration of TGF- β_1 (Koli et al., 2008). In other studies, TGF- β_1 has been shown to directly induce arginase-1 activity and inhibit generation of nitric oxide by inducible NO

synthase, a key step in the antimicrobial response during classical activation (Durante et al., 2001; Vodovotz et al., 1993). Lastly, Mitchell et al. demonstrated in conducting airways that chronic ethanol ingestion increased production of IL-13, a T_H2 cytokine that induces alternative activation, as well as its receptor IL-13R α_1 (Mitchell et al., 2009).

Given the similarities in phenotypes, we hypothesized that ethanol-induced alveolar macrophage dysfunction is due to dysregulation of TGF- β_1 signaling and increased ROS which induce alternative activation. Correspondingly, we examined whether antioxidant treatments and/or inhibition of TGF- β_1 signaling prevent these ethanol-induced alterations. Our data suggests TGF- β_1 signaling and GSH availability are an integral part of ethanol-induced alternative activation of alveolar macrophages, phagocytic dysfunction, and promotion of fibrogenesis.

METHODS

Cell Line Culture and Treatment

NR8383 cells, a rat alveolar macrophage-derived cell line (ATCC, Rockville, USA), were maintained in a humidified 5% CO₂ incubator at 37°C with FK-12 media supplemented with 15% heat-inactivated fetal bovine serum (FBS) and 1% antibiotic-antimycotic solution (100 U/ml penicillin G sodium, 100 U/ml streptomycin, and 0.25 μ g/ml amphotericin B). Cells were treated daily with fresh media containing 0.08% ethanol $\pm$ 500 μ M GSH, $\pm$ 8 μ g/ml TGF- β_1 neutralizing antibody (AB-101-NA, R&D Systems, Minneapolis, USA), or $\pm$ 5 μ g/ml IL-13 neutralizing antibody (MAB194, R&D Systems) for 5 consecutive days. As a control, some cells were treated with rat IgG for 5 consecutive days but no significant differences were observed when compared to ethanol alone (data not shown). Cells treated with ethanol were maintained in a closed chamber in order to maintain the ethanol concentration and media were changed daily. As a positive control for alternative activation, some cells were treated with 1.5 ng/ml IL-13 (1945-RL, R&D Systems) for 5 consecutive days.

Detection of Extracellular Hydrogen Peroxide (H₂O₂)

H₂O₂ release was determined using the oxidation of Amplex Red in the presence of horseradish peroxidase reagent according to the manufacturer's instructions (Invitrogen; Carlsbad, CA). Briefly, cells were treated for 45 min with 100 μ l of Amplex Red solution (50 μ M of Amplex Red reagent and 10 U/ml horseradish peroxidase in phosphate buffered saline). The H₂O₂ production was measured via a microplate reader by taking the absorbance at A₅₆₀ and interpolating from a standard curve generated with known freshly prepared H₂O₂ concentrations.

Detection of Intracellular H₂O₂ Production

Intracellular H₂O₂ production was determined using the Amplite Green Peroxidase Sensor (Invitrogen; Carlsbad, CA), a cell permeable sensor that fluoresces green upon reaction with H₂O₂. Using an excitation wavelength of 480 nm and emission wavelength of 522 nm, fluorescence from Amplite Green was determined via quantitative digital analysis of microscopic images using FluoView (Olympus; Center Valley, PA). Background autofluorescence was determined and subtracted from the mean relative fluorescent units. Values are presented as the mean relative fluorescent units per cell ($\pm$ SE) as tallied from at least 15 experimental fields per set.

Arginase Activity Measurements

Arginase activity was measured using the QuantiChrom Arginase Assay Kit (BioAssay Systems, Hayward, CA) according to the manufacturer's instructions. All values were normalized to protein concentration.

Measurement of TGF- β_1 and IL-13 by ELISA

The TGF- β_1 and IL-13 present in the cell lysates were quantitated by the commercially available ELISA kits TGF- β_1 Emax immunoassay system (Promega; Madison, WI) and the Quantikine Rat IL-13 Immunoassay for IL-13 (Invitrogen; Carlsbad, CA), respectively. Cell lysates were loaded in a 1:10 dilution to measure production of IL-13 and TGF- β_1 . All values were normalized to the corresponding protein concentration.

Phagocytosis of pHrodo-labeled *S. Aureus*

NR8383 cells were plated onto 8 well chamber slides at a density of 10^6 cell/ml and allowed to adhere for 1 hr. After washing twice with sterile phosphate buffered saline, the cells were incubated with pHrodo-labeled *S. aureus* (2 hours; 1 mg/mL). The rhodamine-based pHrodo dye is pH-sensitive such that it is non-fluorescent at neutral pH and fluoresces red in acidic environments such as the phagolysosome. Thus, cells with red fluorescence indicate internalization, sequestration, and a respiratory burst within the phagolysosome. After incubation, the media was removed and the cells washed twice with phosphate buffered saline before fixation with 4% paraformaldehyde. Phagocytosis of pHrodo labeled *S. aureus* was determined via quantitative digital analysis of microscopic images using FluoView (Olympus; Melville, NY). Background autofluorescence was determined and subtracted from the mean relative fluorescent units. Values are presented as mean relative fluorescent units (RFU)/cell $\pm$ SEM, the mean percentage of cells fluorescently positive (per field) $\pm$ SEM, and phagocytic index $\pm$ SEM (RFU x % positive) as tallied from at least 15 experimental fields/set.

Western Blot Analysis

Cell lysates were prepared by adding radioimmunoprecipitation assay (RIPA) buffer containing Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific; Rockford, IL). Protein concentration was determined using the Micro BCA Protein Assay Reagent Kit (Pierce). 30–50 μ g of total protein from each sample were electrophoresed on 4–15% gradient polyacrylamide gels at 200 V for 45 min and transferred to nitrocellulose membranes (*Bio Rad* Laboratories, Richmond, CA) at 100V for 1 hr. Membranes were blocked at room temperature for 1 hr in Tris buffered saline (TBS) with 0.1% Tween 20 (TBS-T) containing 5% nonfat dry milk. After washing (3 times; 10 min each), membranes were probed with primary antibodies to arginase-1 (1:100) (Santa Cruz Biotechnologies, Santa Cruz, CA) or GAPDH (1:500) (Santa Cruz Biotechnologies) diluted in 5% milk in TBS-T and kept at 4°C overnight. After 3 washes with T-TBS for 10 min, membranes were incubated at room temperature with secondary antibodies coupled to horseradish peroxidase (Santa Cruz Biotechnologies) for 1hr. After adding ECL chemiluminescence reagent (*Denville Scientific*, Metuchen, NJ) to the membranes, bands were detected and quantified via densitometry.

Capacity to Activate Fibroblasts

To determine if alternatively activated alveolar macrophages promoted fibroblast activation, NIH/3T3 fibroblasts stably transfected with a fibronectin promoter upstream of a luciferasereporter gene were used. These fibroblasts were seeded at a density of 10^6 cells/ml and allowed to adhere for 24 hrs. The 3T3 cells were then incubated for 24 hr with 300 μ l of cell-free media from the treated NR8383 cells, the 3T3 cells were detached by scraping,

washed with PBS containing 100 μ l of reporter lysis buffer (Promega; Madison, WI), and then assayed for fibronectin transcription via luciferase activity using the Promega luciferase assay system (Promega; Madison, WI). Luciferase activity was normalized to total 3T3 cellular protein.

H₂O₂ Treatment

NR8383 cells were plated onto 8 well chamber slides (5X10⁵ cells/ml) and allowed to adhere for 1 hr. After washing twice with sterile phosphate buffered saline, NR8383 cells were treated with 15 μ M H₂O₂ for 30 minutes. Where appropriate, cells were pretreated with 8 ng/ml TGF- β ₁ neutralizing antibody for 1 hour prior to H₂O₂ treatment.

TGF- β ₁ Treatment

NR8383 cells were plated onto 8 well chamber slides at a density of 5X10⁵ cells/ml and allowed to adhere for 1 hr. After washing twice with sterile phosphate buffered saline, NR8383 cells were treated with 10 ng/ml of TGF- β ₁ (T7039, Sigma-Aldrich) for 24 hours. Where appropriate, cells were co-treated with 500 μ M GSH.

Cellular Viability

To determine cellular viability following treatments, the trypan blue exclusion test was used. Cell were stained with equal volumes 0.4% trypan blue and counted using a Countess automated cell counter.

Quantitative PCR

RNA was isolated from NR8383 cells preserved in RNeasy later according to the manufacturer's instructions (RNeasy Mini; Qiagen, Valencia, Calif). Following isolation, 10 ng of total RNA per sample was reverse transcribed into cDNA using oligo (dT)-primers, according to the manufacturer's instructions (Applied Biosystems, Foster City, CA). cDNA was then used to quantitate relative levels of arginase-1, CD32, and galectin-3 gene expression, using the ABI Prism 7000 real time PCR system (Applied Biosystems). Data were normalized to glyceraldehydes 3-phosphate dehydrogenase (GAPDH). Net cycle threshold (CT) values for each gene of interest were used to calculate Δ CT values for each sample.

Alveolar Macrophage Isolation

Control male Sprague-Dawley rats (Harlan, St. Louis, MO) were used in accordance with NIH guidelines (*Guide for the Care and Use of Laboratory Animals*) as described in protocols reviewed and approved by the Emory University Institutional Animal Care Committee. Briefly, after pentobarbital anesthesia, the trachea was cannulated and the rat lung underwent 5 bronchoalveolar lavages, consisting of 10 ml of sterile phosphate-buffered saline (37°C, pH 7.4). The lavage fluid was centrifuged at 500 *g* for 8 min, and the cell pellet was resuspended in Dulbecco's modified Eagle's medium supplemented with 2% fetal bovine serum plus penicillin and streptomycin (100 U/l each). Cell count and viability was determined on the CountessTM Automated Cell Counter (Invitrogen; Carlsbad, CA). Rat primary alveolar macrophages were plated at 0.25 million cells/ml and cultured at 37°C and 5% CO₂ and allowed to adhere for 1 hr. Cells were then treated with ethanol (0.08%) $\pm$ 500 μ M GSH or 8 ng/ml TGF- β ₁ neutralizing antibody for 5 consecutive days as described above for NR8383 cells.

Statistical Analysis

Statistical analysis was performed with Sigma Stat for Windows. The data is presented as means $\pm$ SE. Results were analyzed using the Students t-test or one-way analysis of variance

where appropriate, followed by Student-Newman-Keuls test comparisons. A *p*-value of < 0.05 was considered significant.

RESULTS

Chronic Ethanol Treatment Induces Production of ROS and TGF- β_1 in NR8383 Cells

Our previous studies demonstrated that chronic alcohol alters oxidant/antioxidant balance by decreasing GSH availability and increasing ROS (Brown et al., 2007; Moss et al., 2000). In the current study, we determined the effects of chronic ethanol treatment on intracellular and extracellular production of H₂O₂. As a positive control for alternative activation, some cells were treated with IL-13. After treatment of NR8383 cells (0.08% ethanol; 5 consecutive days), there was a 6 fold increase in extracellular H₂O₂ levels (Figure 1A; ^a *p* < 0.05), and a 15-fold increase in intracellular H₂O₂ levels (Figure 1B; ^a *p* < 0.05). Similarly, IL-13 led to a 6 fold increase in extracellular H₂O₂ and a ~19-fold increase in intracellular H₂O₂ levels (Figure 1-B). Representative images for intracellular H₂O₂ production are shown in Figure 1C. In NR8383 cells, these effects of ethanol on ROS generation were time-dependent (0.08% ethanol; 1–7 days) and dose-dependent (0.02% to 0.16%; 5 days) (Supplemental Figures 1–2). TGF- β_1 was also increased ~2-fold in response to treatment with ethanol or IL-13 (Figure 1D; ^a *p* < 0.05). To determine if ethanol-induced TGF- β_1 production and ROS generation were dependant on one another, cells were treated with 0.08% ethanol plus GSH (500 μ M), or TGF- β_1 neutralizing antibody (8 ng/ml). After 5 days, GSH treatments or the TGF- β_1 neutralizing antibody normalized extracellular and intracellular H₂O₂ (Figure 1A–B), as well TGF- β_1 production (Figure 1D).

Ethanol Exposure Induces IL-13 Expression in NR8383 Cells

Since the T_H2 cytokine IL-13 induces alternative activation, we next sought to determine if ethanol induces expression of IL-13. In NR8383 cells, ethanol induced a 3.7 fold increase in IL-13 protein expression (Figure 2; ^a *p* < 0.05). GSH treatments significantly reduced IL-13 protein expression but did not normalize its protein expression (Figure 2; ^b *p* < 0.05). However, inhibition of TGF- β_1 signaling with the TGF- β_1 neutralizing antibody normalized IL-13 expression to control values (Figure 2; ^b *p* < 0.05). It is also important to note that extracellular IL-13 upregulated intracellular IL-13 ~ 70%.

Ethanol-Induces M2 Activation of NR8383 Cells and Promotes a Pro-Fibrotic Phenotype

Since ethanol increased expression of IL-13, a cytokine that induces alternative activation, we next examined whether ethanol exposure upregulated markers of alternative activation. With ethanol treatment, NR8383 cells exhibited a ~2.8 fold increase in arginase-1 expression and a ~50% increase in galectin-3 expression, similar to that observed with IL-13 alone (Figure 3A–C; ^a *p* < 0.05). Additionally, ethanol treatments caused a 2.5 fold increase in arginase-1 activity (Figure 3D; ^a *p* < 0.05). Treatment with GSH or the TGF- β_1 neutralizing antibody prevented ethanol-induced increases in arginase-1 and galectin-3 protein expression, as well as arginase-1 activity (Figure 3A–D, ^b *p* < 0.05). We next examined whether IL-13-induced alternative activation is associated with a macrophage phenotype that promotes fibroblast activation. When the cell-free supernatants from ethanol-treated NR8383 cells were overlaid onto the 3T3 fibroblasts, there was a 2.5-fold increase in fibronectin gene transcription by the 3T3 cells (Figure 3E; ^a *p* < 0.05). When the NR8383 cells were treated concomitantly with ethanol plus the TGF- β_1 neutralizing antibody, the increased fibroblast activation expected for ethanol treatment was blocked (Figure 3E; ^b *p* < 0.05). Similar results were seen when the NR8383 cells were treated with GSH during the ethanol exposure.

GSH Supplementation and Inhibition of TGF- β_1 Prevented Ethanol-Induced Loss of NR8383 Cell Phagocytic Function

Chronic ethanol exposure significantly decreased the internalization of pHrodo red labeled *S. aureus* as determined by a ~55% decrease in the phagocytic function (Figure 4A, ^a **p** <0.05; **Representative images in** Figure 4B). In NR8383 cells, ethanol-induced suppression of phagocytosis was time- and dose-dependent (Supplemental Figures 1–2). For control cells, neither GSH supplementation nor inhibition of TGF- β_1 signaling significantly altered the phagocytic capacity. However, daily supplementation with either GSH or the TGF- β_1 neutralizing antibody prevented ethanol-induced decreases in NR8383 cell phagocytic function (Figure 4A, ^b **p** <0.05).

Ethanol-Induced M2 Activation and Decreased Phagocytic Function in NR8383 Cells is Dependent on IL-13

We next examined whether ethanol promotes alternative activation and decreases phagocytic function in an IL-13 dependant manner. Similar to our previous results, ethanol treatment resulted in a ~50% decrease in phagocytic capacity, a ~3 fold increase in arginase activity, and a ~2-fold increase in TGF- β_1 (Figure 5A–C, ^a **p** <0.05). These effects of ethanol on alternative activation were time- and dose-dependent (Supplemental Figures 1–2). Treatment with the IL-13 antibody (5 μ g/ml) during ethanol treatment normalized NR8383 cell phagocytic capacity and partially blocked ethanol-induced alternative activation, as determined by decreased arginase activity and TGF- β_1 expression (Figure 5A–C, ^b **p** <0.05).

H₂O₂ and TGF- β_1 -Induced NR8383 Cell Dysregulation

To delineate the relationship between ethanol-induced intracellular ROS generation, TGF- β_1 , and alternative activation, NR8383 cells were treated with H₂O₂ or TGF- β_1 $\pm$ GSH. Where appropriate, cells were pretreated with TGF- β_1 neutralizing antibody for 1 hour prior to treatment with H₂O₂ for 30 minutes. H₂O₂ treatment resulted in a ~65% decrease in phagocytic capacity, a 32% increase in IL-13 secretion, and a 73% increase in extracellular TGF- β_1 (Figure 6; ^{a,b} **p** <0.05). Neutralization of TGF- β_1 during H₂O₂ treatment partially prevented NR8383 cell phagocytic dysfunction and normalized IL-13 secretion. Similarly, exposure to TGF- β_1 directly decreased phagocytic capacity by ~40%, increased intracellular ROS ~5.4 fold, and increased IL-13 production by ~35%. Co-treatment with 500 μ M GSH partially blocked these effects (Figure 7; ^{a,b} **p** <0.05).

Chronic Ethanol-Induced Phagocytic Dysfunction, ROS Generation, and Upregulation of TGF- β_1 and IL-13 in Rat Alveolar Macrophages

To determine physiological relevance, freshly isolated rat alveolar macrophages were treated with 0.08% ethanol for 5 consecutive days. Similar to that observed with NR8383 cells, ethanol treatment of freshly isolated rat alveolar macrophages resulted in a ~2.25 fold increases in intracellular ROS and ~41% decrease in phagocytic capacity (Figure 8A&B; ^a **p** <0.05). Treatment with either GSH or TGF- β_1 neutralizing antibody prevented these effects (Figure 8A&B; ^b **p** <0.05). Importantly, none of the treatments resulted in altered cellular viability (data not shown). In response to ethanol treatment, alveolar macrophages displayed a 5-fold increase in TGF- β_1 excretion and a 2-fold increase in extracellular IL-13 (Figure 8C&D; ^a **p** <0.05). Additionally, supplementation with either GSH or TGF- β_1 neutralizing antibody partially blocked ethanol-induced IL-13 production (Figure 8D; ^b **p** <0.05).

DISCUSSION

Numerous studies have demonstrated that patients with alcohol use disorders have an increased risk of respiratory infections, which is partially due to ethanol-induced impairment

of the immune functions of alveolar macrophages. However, mechanisms by which alcohol impairs alveolar macrophage function are poorly understood (Boé et al., 2009; Esper A., 2006; Kane and Galanes, 2004; Moss et al., 1996; Moss and Burnham, 2003). On the other hand, previous studies suggest the majority of the negative effects of alcohol on alveolar macrophage dysfunctions are due to decreased GSH availability and subsequent oxidant stress within the alveolar space (Brown et al., 2004; Brown et al., 2007). Previous studies by this laboratory group demonstrated in rodent models of alcohol abuse that ethanol-induced phagocytic dysfunction could be reversed with *ex-vivo* GSH supplementation (Brown et al., 2007). Other studies showed that *in vivo* treatments with GSH precursors prevented ethanol-induced pulmonary macrophage dysfunction.

Previous studies have shown that chronic ethanol ingestion increases TGF- β_1 production within alveolar macrophages. Our current studies demonstrated that chronic ethanol ingestion also leads to increased oxidant stress within the alveolar macrophage, as evidenced by increased intracellular and extracellular ROS. In other studies, ROS have been shown to induce TGF- β_1 activation *in vivo* (Koli et al., 2008). Similarly, alternatively activated macrophages have robust increases in TGF- β_1 production and secretion (Martinez et al., 2009; Song et al., 2000; Stein et al., 1992). Once activated, TGF- β_1 has been shown to deplete intracellular glutathione stores, and increase intracellular ROS production, thereby, creating a vicious cycle of ROS generation. TGF- β_1 also induces arginase-1 activity and inhibits generation of nitric oxide by inducible NO synthase, both markers of alternative activation. In the conducting airways, chronic ethanol ingestion increases production of IL-13, as well as its receptor IL-13R α_1 (Mitchell et al., 2009).

In the current study, chronic ethanol treatment increased expression of IL-13, a known inducer of alternative activation (Muller et al., 2007; Munder et al., 1998), as well as expression of established markers of alternative activation. Ethanol treatment also increased ROS generation and release, increased TGF- β_1 production, and severely diminished phagocytic capacity. These effects were dependent on the ethanol concentration and the time of exposure. Ablation of TGF- β_1 signaling through the use of a TGF- β_1 neutralizing antibody blocked ethanol-induced ROS generation by NR8383 cells or control alveolar macrophages, expression of alternative activation markers, impaired phagocytosis and secretion of factors that upregulate fibroblast proliferation. This suggested that ethanol induced an autocrine loop between ROS generation and TGF- β_1 expression, secretion, and signaling; thereby, making ethanol-induced increases in ROS and TGF- β_1 central to alternative activation of alveolar macrophages and impaired immune responses. These results support other studies with TGF- β_1 that demonstrate robust TGF- β_1 production by alternatively activated macrophages (Gordon, 2003; Gordon and Martinez, 2010; Martinez et al., 2009) as well as the ability of TGF- β_1 to upregulate arginase-1 activity and polyamine release, markers of alternative activation (Boutard et al., 1995; Lee et al., 2001).

In the current study, GSH treatments of NR8383 cells or primary alveolar macrophages also blocked ethanol-induced ROS generation, TGF- β_1 expression and secretion, alternative activation, impaired phagocytic capacity, and the capacity of AM to secrete factors that promote fibroblast proliferation. This further supported the concept that ethanol-induced ROS generation was central to upregulation of TGF- β_1 and subsequent switch of the alveolar macrophage to an alternatively activated phenotype with accompanying impaired immune responses. Ethanol-induced upregulation of IL-13 also plays a role in this feed forward loop as evidenced by the ability of the IL-13 neutralizing antibody to partially attenuate ethanol-induced expression of TGF- β_1 and alveolar macrophage polarization. Additionally, our studies suggest the increased expression of IL-13 can be ablated by treatment with TGF- β_1 inhibitors and attenuated with GSH. Therefore, we propose ethanol

induced production of TGF- β_1 is central to ethanol induced impairments of alveolar macrophage function and induction of alternative activation.

In conclusion, these studies with NR8383 cells and primary alveolar macrophages demonstrated that chronic ethanol exposure promotes a feed-forward loop between increased ROS generation and TGF- β_1 expression and signaling. Ethanol-induced upregulation of IL-13 was also a component of this feed-forward loop. Increased signaling through IL-13 and TGF- β_1 subsequently impaired the immune response of alveolar macrophages by inducing an alternatively activated phenotype. While the increases in galectin-3 were relatively modest compared to the other parameters, the increased activation of fibroblasts were most likely due to the combined effects of increased secretion of TGF- β_1 and IL-13 by the alternatively activated alveolar macrophage. This phenotype switch may decrease the pro-inflammatory responses of alveolar macrophages, the chronic suppression of phagocytosis decreases the capacity of alveolar macrophages to clear microbes and may explain the increased risk of respiratory infections associated with chronic alcohol use disorders. Additional studies are needed to determine if *in vivo* treatments with GSH, TGF- β_1 inhibitors or IL-13 inhibitors will reverse ethanol-induced suppression of the immune functions of alveolar macrophages and decrease the risk of respiratory infections.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

ARDS	Acute respiratory syndrome
M2 activation	Alternative activation
AUD	Alcohol use disorders
GSH	Glutathione
ROS	Reactive oxygen species
RFU	Relative fluorescent units

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Figure 1. Chronic Ethanol Treatment Increases ROS and TGF- β_1 Production

NR8383 cells were treated with 0.08% ethanol $\pm$ 500 μ M GSH or 8 ng/ml TGF- β_1 neutralizing antibody for 5 consecutive days. The extracellular level of H_2O_2 produced by NR8383 cells was determined using amplex red (A). Intracellular H_2O_2 production was quantified via amplitude green fluorescence (B). Representative confocal images are shown (C). Intracellular TGF- β_1 was also measured via ELISA (D). Bars heights represent means $\pm$ SE. ^a p 0.05 compared to controls, ^b p 0.05 compared to the ethanol treatment. n=15 or more fields per group

Figure 2. Chronic Ethanol Treatment Induces IL-13 Expression

The effects of chronic ethanol treatment on expression of IL-13 was examined in NR8383 treated with 0.08% ethanol $\pm$ 500 μ M GSH or 8 ng/ml TGF- β_1 neutralizing antibody for 5 consecutive days. Cells were also treated with IL-13 as a positive control. IL-13 protein expression was measured via ELISA (A). Bar heights represent mean $\pm$ SE. ^a p < 0.05 compared to controls, ^b p < 0.05 compared to ethanol, $n=4$ or more per group.

Figure 3. Chronic Ethanol Treatment Induces Alternative Activation

The effects of chronic ethanol treatment on alternative activation was examined in NR8383 treated with 0.08% ethanol $\pm$ 500 μ M GSH or 8 ng/ml TGF- β_1 neutralizing antibody for 5 consecutive days. Cells were also treated with IL-13 as a positive control. Expression of arginase-1 (A) and galectin-3 (B) were determined via western blot analysis. Representative immunoblots are shown in C. Assessment of arginase-1 activity in was determined and normalized to protein concentrations (D). To measure fibroblast proliferation, transfected 3T3 cells were treated with the cell-free supernatants from all NR8383 cell treatment groups for 24 hrs. The 3T3 cells were then washed and lysed before fibronectin gene transcription was measured by luminescence (E). Data are presented as average luciferase activity $\pm$ SD. Bar heights represent mean $\pm$ SE. ^a p 0.05 compared to controls, ^b p 0.05 compared to ethanol, $n=3$ or more per group.

a. Phagocytic Capacity

b. pHrodo Red Representative Images

Figure 4. Chronic Ethanol Treatment Impaired Phagocytosis of pHrodo-Labeled *S. aureus* Ethanol induced impairment of NR8383 cell phagocytosis was examined using the pH sensitive pHrodo labeled *S. aureus*. NR8383 cells were incubated with 1mg/ml pHrodo labeled *S. aureus* for 2 hr. Internalization was evaluated using quantitative digital analysis of fluorescence on the confocal microscopy. Representative photomicrographs at 20X magnification are shown in **A**. Bar heights represent the phagocytic index (**B**). Bars heights represent means $\pm$ SE. ^a p < 0.05 compared to controls, ^b p < 0.05 compared to ethanol, n=15 or more fields per group.

Figure 5. Ethanol Induced Alternative Activation and Loss of Phagocytic Function is Dependent on IL-13

The role of IL-13 in ethanol-induced NR8383 cell derangements was determined. Nr8383 cells were treated with 0.08% ethanol $\pm$ 5 μ g/ml of IL-13 neutralizing antibody for 5 consecutive days. Phagocytic capacity (A), arginase activity (B), and TGF- β_1 production (C) were determined. Bars heights represent means $\pm$ SE. ^a p 0.05 compared to controls, ^b p 0.05 compared to the ethanol treatment, $n=3$ or more per group.

a. Phagocytic Capacity

b. Extracellular TGF-β₁

c. Extracellular IL-13

Figure 6. H₂O₂ Mediated Impairment of Phagocytic Function

NR8383 cells were treated with H₂O₂ for 30 minutes ± pretreatment with 8 μg/ml TGF-β₁ neutralizing antibody for 1 hour. H₂O₂ induced impairment of NR8383 cell phagocytosis was examined using the pH sensitive pHrodo labeled *S. aureus*. NR8383 cells were incubated with 1mg/ml pHrodo labeled *S. aureus* for 2 hr. Internalization was evaluated using quantitative digital analysis of fluorescence on the confocal microscope (A). Intracellular TGF-β₁ was also measured via ELISA (B). IL-13 protein expression was measured via ELISA (C). Bars heights represent means ± SE. ^a p 0.05 compared to controls, ^b p 0.05 compared to ethanol, n=15 or more fields per group.

Figure 7. TGF- β_1 -Induced Phagocytic Dysfunction and Increased ROS

N8383 cells were treated with TGF- $\beta_1 \pm 500 \mu\text{M}$ GSH for 24 hr. Bar heights represent the phagocytic index (A). Intracellular H₂O₂ production was quantified via amplite green fluorescence (B). Extracellular TGF- β_1 was also measured via ELISA (C). IL-13 protein expression was measured via ELISA (D). Bars heights represent means $\pm$ SE. ^a p < 0.05 compared to controls, ^b p < 0.05 compared to the ethanol treatment. n=10 or more fields per group.

Figure 8. Chronic Ethanol-Induced Phagocytic Dysfunction, ROS Generation, and Upregulation of TGF- β ₁ and IL-13 in Rat Alveolar Macrophages

Freshly isolated rat alveolar macrophages were treated with 0.08% ethanol $\pm$ 500 μ M GSH or 8 ng/ml TGF- β ₁ neutralizing antibody for 5 consecutive days. Following treatment, alveolar macrophages were incubated with 1mg/ml pHrodo labeled *S. aureus* for 2 hr. Internalization was evaluated using quantitative digital analysis of fluorescence on the confocal microscopy. Bar heights represent the phagocytic index (A). Intracellular H₂O₂ production was quantified via amplite green fluorescence (B). Extracellular TGF- β ₁ was also measured via ELISA (C). IL-13 protein expression was measured via ELISA (D). Bars heights represent means $\pm$ SE. ^a p < 0.05 compared to controls, ^b p < 0.05 compared to the ethanol treatment. n=10 or more fields per group