Alcoholism causes alveolar macrophage zinc deficiency and immune dysfunction

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Author contributions: A.J.M. and D.M.G. designed and analyzed experiments, obtained samples from recruited subjects, and prepared manuscript; S.M.Y. and L.A.B. performed zinc and macrophage function assays and interpreted confocal microscopy measurements; L.E. performed statistical analysis and modeling.

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At a Glance Commentary:
Alcohol abuse causes many derangements in the lung and predisposes individuals to the development of pulmonary infections and lung injury. Animal models of chronic alcohol ingestion have implicated pulmonary zinc deficiency as an important mediator of alveolar macrophage immune dysfunction, which results in increased susceptibility and severity of pneumonia. However, human studies are lacking. Our study sheds light on the effects of chronic alcohol exposure on alveolar macrophages from human alcoholics. We show that alcoholism results in intracellular zinc deficiency and immune dysfunction of alveolar macrophages compared to non-alcoholics. Treating alveolar macrophages with zinc and/or glutathione *in vitro* reverses these defects, providing important proof-of-concept data to evaluate the role of these potential therapeutic strategies.
This article has an online data supplement, which is accessible from this issue's table of content online at www.atsjournals.org.
ABSTRACT

Rationale: Alcohol use disorders cause oxidative stress in the lower airways and increase susceptibility to pneumonia and lung injury. Currently, no therapeutic options exist to mitigate the pulmonary consequences of alcoholism.

Objectives: We recently determined in an animal model that alcohol ingestion impairs pulmonary zinc metabolism and causes alveolar macrophage immune dysfunction. The objective of this research is to determine the effects of alcoholism on zinc bioavailability and alveolar macrophage function in human subjects.

Methods: We recruited otherwise healthy alcoholics (n=17) and matched control subjects (n=17) who underwent bronchoscopy for isolation of alveolar macrophages, which were analyzed for intracellular zinc, phagocytic function, and surface expression of granulocyte-macrophage colony-stimulating factor receptor; all three of these indices are decreased in experimental models.

Measurements and Main Results: Alcoholic subjects had normal serum zinc, but significantly decreased alveolar macrophage intracellular zinc levels [adjusted means (se): 718 (41) vs. 948 (25) RFU/cell; p <0.0001], bacterial phagocytosis [adjusted means (se): 1027 (48) vs. 1509 (76) RFU/cell; p<0.0001], and expression of granulocyte-macrophage colony-stimulating factor receptor beta subunit [adjusted means (se): 1471 (42) vs. 2114 (35) RFU/cell; p<0.0001]. Treating alveolar macrophages with zinc acetate and glutathione in vitro increased intracellular zinc levels and improved their phagocytic function.

Conclusions: These novel clinical findings provide evidence that alcohol abuse is associated with significant zinc deficiency and immune dysfunction within the alveolar space and suggest
that dietary supplementation with zinc and glutathione precursors could enhance airway innate immunity and decrease the risk for pneumonia or lung injury in these vulnerable individuals.
INTRODUCTION

Alcohol use and abuse are prevalent and impose significant health burdens in our society. A recent national survey suggests that more than half of the population in the United States above the age of 12 consumes alcohol (1) and the lifetime prevalence of alcohol abuse is 18 percent (2). Tragically, alcohol abuse is the third leading cause of preventable death in the United States (3), and alcoholism is associated with numerous chronic, non-fatal health implications as well. The overall annual cost of alcohol abuse to American society has been estimated to be upwards of $200 billion dollars (4).

The consequences of alcoholism span multiple organ systems, including the heart, liver, brain, and skeletal muscle (5, 6). Among these widespread effects, alcohol abuse renders individuals susceptible to pulmonary infections and acute lung injury (7-10). We have explored potential mechanisms in experimental animal models and determined that chronic alcohol ingestion causes previously unrecognized cellular dysfunction and oxidative stress, as best reflected by significant decreases in glutathione levels, within the alveolar space (11-14). Alveolar macrophages, which are the primary immune cell in the lower airways, exhibit impaired phagocytosis and respiratory burst generation in animal models of chronic alcohol ingestion (15, 16), and treatment with anti-oxidants reverses these defects (17, 18). In addition, we established that decreased signaling of granulocyte/macrophage colony-stimulating factor (GM-CSF) mediates alcohol-induced alveolar macrophage immune deficiency (13, 19). GM-CSF is vital for the alveolar macrophage to achieve appropriate maturity, to complete terminal differentiation, and to maintain normal functioning (20).
More recently, animal models implicate zinc deficiency as a fundamental mechanism that drives the oxidative stress and impaired GM-CSF signaling in the alcoholic lung (21). Zinc is a trace metal that is an important co-factor for numerous enzymes in the body and plays a key role in the immune response (22). Zinc deficiency has been implicated in the development of pneumonia in children of third world countries (23, 24), and dietary zinc supplementation in these children decreases susceptibility to infection and improves overall health outcomes (25, 26). Importantly, recent experimental studies have shown that alcohol-fed animals have lower zinc levels in the lung compared to control-fed animals, and dietary zinc supplementation can reverse the pulmonary immune dysfunction seen with chronic alcohol ingestion (21, 27).

While these and other experimental models are important to our understanding of the alcohol lung phenotype, human studies are lacking. For this reason, we undertook this study to establish if alveolar macrophages from otherwise healthy alcoholics have significantly reduced intracellular zinc levels and phagocytic function compared to non-alcoholics, and to determine the effect of treating these alveolar macrophages in vitro with zinc and/or glutathione precursors. To answer these questions, we obtained alveolar macrophages via bronchoalveolar lavage from otherwise healthy alcoholic and non-alcoholic individuals and compared their relative intracellular levels of zinc, surface expression of the GM-CSF receptor, and phagocytic function. In parallel, we determined whether we could enhance the functional status of alveolar macrophages from alcoholics by treating them with zinc and glutathione in vitro as a pre-clinical proof-of-principle that dietary supplementation could enhance lung health in these vulnerable individuals. Some of the results of these studies have been previously reported in the form of an abstract (28).
METHODS

Study population.

The target population was otherwise healthy adults (18-55 years of age) who were diagnosed with an alcohol use disorder (AUD) at the time of enrollment. An AUD was defined as having a positive Short Michigan Alcohol Screening Test (SMAST) (29) and this was later confirmed with the Alcohol Use Disorders Identification Test (AUDIT) (30). All subjects were recruited from the Substance Abuse Treatment Program (SATP) at the Atlanta Veterans Affairs Medical Center (VAMC) in Decatur, Georgia. Non-alcoholic subjects were matched by age, gender, race, and smoking status. Greater detail regarding the study population and enrollment procedures is available in the online supplement.

Bronchoscopy procedure and bronchoalveolar lavage (BAL) fluid processing.

All study subjects underwent a bronchoscopy and BAL for collection of alveolar macrophages using standard techniques that have been previously described (31) and further procedural details are provided in the online supplement. BAL fluid was passed through sterile gauze and centrifuged at 8000 rpm for 5 min. The cell pellets contained predominantly alveolar macrophages with ~90% purity as measured by Diff-Quik (Dade Behring, Deerfield, IL) and were re-suspended at a concentration of 1 x 10^6 cells/ml in RPMI-1640 medium containing 2% FBS and 1% penicillin/streptomycin and then cultured for 2 hours as previously described (32). Some alveolar macrophages were then treated for 24 hours with 10 µM zinc acetate (Zn) (JT Baker, Phillipsburg, NJ), 500 µM glutathione (GSH) (ICN Nutritional Biochemicals, Cleveland, OH), or both Zn and GSH combined.
**Serum and alveolar macrophage studies.**

Serum zinc levels were determined by sending samples to a commercial laboratory (Quest Diagnostics, Chantilly, VA). Isolated alveolar macrophages were incubated with FluoZin-3AM dye (200 nM; Invitrogen, Carlsbad, CA) for 30 min per the manufacturer’s instructions and then fixed to chamber slides with 4% paraformaldehyde. For GM-CSF receptor measurement, we evaluated expression of the α subunit (GM-CSFRα) that mediates ligand binding and the β subunit (GM-CSFRβ) that initiates the intracellular signaling cascade that ultimately induces the expression of multiple genes responsible for alveolar macrophage function, including phagocytosis. Alveolar macrophages were fixed to chamber slides with 4% paraformaldehyde and then incubated with a primary antibody against the alpha binding and beta signaling subunit (Santa Cruz Biotechnology; 1:100), followed by incubation with a fluorescent TRITC-labeled secondary antibody. Fluorescence measurements for zinc and GM-CSF were performed using FluoView (Olympus) via quantitative digital analysis and expressed as mean relative fluorescent units (RFU) per cell. The relative phagocytic capacity of alveolar macrophages at baseline and following treatment with Zn, GSH, or Zn + GSH was assessed as previously described (32) and further details are provided in the online supplement.

**Statistical Analyses.**

For statistical comparisons between alcoholic and non-alcoholic subjects, we used general estimating equations with repeated measures to account for the matching design via SAS software’s (version 9.3) Genmod procedure (33) and 1-step robust regression estimator weighting to reduce the influence of outliers (34). Further statistical details are in the online supplement.
**Research Ethics.**

All aspects of this clinical project have been conducted according to Declaration of Helsinki principles and were approved by the institutional review board of at Emory University and the Research and Development Committee at the Atlanta VA Medical Center. Written informed consent was received from each enrolled participant.
RESULTS

Subject enrollment. A total of 375 subjects were screened for enrollment into the study; 175 with an alcohol use disorder and 200 without any history of alcohol abuse [Figure 1]. Among the screened subjects with an alcohol use disorder, 112 met at least one of the exclusion criteria and 46 declined to participate in the study. Among the non-alcoholics who were screened, 118 met at least one of the exclusion criteria, 10 declined to participate, and 55 did not have a match among the alcoholic subjects. Overall, 17 subjects with an active AUD and 17 matched non-alcoholics subjects were enrolled and underwent bronchoscopy. There was improper handling of one BAL sample from an alcoholic subject that rendered it unusable. All other samples were analyzed as described in Methods.

Subject characteristics. Alcoholic and non-alcoholic subjects were similar for gender and race (both groups were all male and 88% were Black/African-American), average age (45.2 vs. 45.8 years), and body mass index (28.0 vs. 28.6 kg/m\(^2\)). [Table 1] Eighty-eight percent of both groups smoked cigarettes; however, those in the alcoholic group smoked more heavily (medians: 360 vs. 150 cigarettes in the previous 30 days). Illegal drug use was reported by 7 (44%) of the alcoholic subjects but not by any of the control subjects. Per the inclusion criteria, all alcoholic subjects had a current alcohol use disorder and were actively drinking at the time of enrollment (mean SMAST score (se): 6.9 (3.4)), whereas the control subjects were either non-drinkers or drank socially but did not meet criteria for an AUD (mean SMAST score (se): 0.9 (0.7)). The median alcohol intake (in grams/day) for non-alcoholics was zero and 126 among alcoholic
subjects. The median time since the most recent alcohol drink was one day in the alcoholic subjects and 14 days in the control subjects.

**Alveolar macrophages isolated from subjects with an alcohol use disorder had lower intracellular zinc levels.** We previously determined that extracellular and intracellular zinc levels are decreased in an experimental animal model of chronic alcohol ingestion (27). In human subjects, we evaluated intracellular zinc status since extracellular zinc measurements in lavage fluid would be difficult to interpret given variations in sample dilution. Alveolar macrophages isolated from alcoholic subjects had lower intracellular zinc levels compared to alveolar macrophages isolated from their matched control subjects (adjusted means (se): 718 (41) vs. 948 (25) RFU/cell; p <0.0001) [see Figure 2A and Table 2].

**In contrast, serum zinc levels did not differ between alcoholic and control subjects and did not correlate with alveolar macrophage zinc levels.** There were no appreciable differences in serum zinc levels between the alcoholic subjects and the matched control subjects [Figure 3, Panel A and Table 2]. In fact, the serum zinc levels were within the normal range in all of the subjects. Further, there was no significant correlation between the serum and the alveolar macrophage zinc levels (Spearman’s $r_{\text{alcoholic}}=0.24$, $p=0.4$ v $r_{\text{non-alcoholic}}=-0.26$, $p=0.3$) [Figure 3, panel B].

**Alveolar macrophages from alcoholic subjects had decreased bacterial phagocytic capacity.** Previously, we showed that alveolar macrophages isolated from alcohol-fed animals had decreased bacterial phagocytic function compared to alveolar macrophages from control-fed animals (27). To confirm the relevance of these findings, we compared the relative bacterial
phagocytic capacities of alveolar macrophages isolated from human alcoholic and non-alcoholic subjects. The bacterial phagocytic function of alveolar macrophages was more than 30% lower in alcoholics as compared to non-alcoholics (adjusted means (se): 1027 (48) vs. 1509 (76) RFU/cell; p <0.001) [Figure 4, Panel A and Table 2]. Representative fluorescent images illustrate this decreased ability of alveolar macrophages from alcohol subjects to ingest fluorescent-labeled Staphylococcus aureus in vitro [Figure 4, Panel B].

The cell surface expression of the signaling subunit of the GM-CSF receptor was decreased in the alveolar macrophages of alcoholic subjects. The GM-CSF receptor has an α subunit (GM-CSFRα) that mediates ligand binding and a β subunit (GM-CSFRβ) that initiates the intracellular signaling cascade that ultimately induces the expression of multiple genes responsible for alveolar macrophage function, including phagocytosis. Experimental models of chronic alcohol ingestion have demonstrated a decrease in GM-CSF receptor expression and signaling in alveolar macrophages (13, 19). In this study, the relative expression of GM-CSFRα in the alveolar macrophages did not differ significantly between alcoholic and non-alcoholic control subjects [Figure 5, Panel A and Table 2]. However, the expression of the GM-CSFRβ subunit was decreased by more than 30% in the alveolar macrophages from the alcoholic subjects as compared to control subjects (adjusted means (se): 1471(42) vs. 2114 (35) RFU/cell; p <0.001) [Figure 5, panel B and Table 2].

Treating isolated alveolar macrophages with zinc acetate and/or glutathione in vitro increased intracellular zinc levels and phagocytic capacity, and these salutary effects were most pronounced in response to combination treatment. Previously we determined that treating
alveolar macrophages isolated from alcohol-fed experimental animals with either glutathione precursors (i.e., procysteine) or zinc increased their phagocytic function (17, 27). More recently, we determined that dietary zinc supplementation in alcohol-fed animals normalized alveolar redox balance and restored lung bacterial clearance (21). Taken together, these experimental findings revealed a dynamic dependence between zinc bioavailability and oxidative stress within the alveolar macrophage. However, these experimental findings have not been extended to humans. Therefore, we treated alveolar macrophages from alcoholic and control subjects with zinc acetate, glutathione, or both in vitro and compared their intracellular zinc levels and bacterial phagocytic capacities after these treatments.

Treatment with either zinc acetate or glutathione increased the intracellular zinc levels in alveolar macrophages from both alcoholic and control subjects [Figure 6, Panel A]. Interestingly, there was an even greater increase in the intracellular zinc levels when zinc and GSH were used in combination. In fact, under this treatment the intracellular zinc levels were slightly higher in the alveolar macrophages from the alcoholic subjects compared to the control subjects (adjusted means (se): 2684 (47) vs. 2512 (41) RFU/cell, p=0.0031) [Table 2].

In parallel, increased intracellular zinc levels were associated with salutary effects on bacterial phagocytic function [Figure 6, Panel B]. Treatment with either zinc acetate or glutathione increased phagocytic capacity in the alveolar macrophages from alcoholic and control subjects. Also consistent with intracellular zinc levels, the combination treatment produced the largest improvement in alveolar macrophage phagocytic function, and phagocytosis was not significantly different between the alveolar macrophages from alcoholic and control subjects.
following this treatment (adjusted means (se): 2453 (60) vs. 2560 (30) RFU/cell, p=0.1188) [Table 2].
**DISCUSSION**

In this study we determined that alveolar macrophages of otherwise healthy alcoholic subjects have significantly decreased intracellular zinc levels compared to macrophages of non-alcoholic control subjects even in the presence of normal serum zinc levels. Further, we confirmed that features of the alcoholic lung phenotype identified in the animal model, namely alveolar macrophage immune dysfunction and decreased GM-CSF receptor expression, also occur in human subjects. Finally, treatment of isolated alveolar macrophages from alcoholic subjects with either zinc or GSH in vitro increased intracellular zinc levels and alveolar macrophage phagocytic function. Taken together, these results provide new evidence that chronic alcohol abuse, even in the absence of clinically apparent zinc deficiency or end-organ damage, causes significant zinc depletion and immune dysfunction within the alveolar space. Therefore, this study extends our basic understanding of the association between alcoholism and pulmonary outcomes such as pneumonia (35, 36) and suggests that increasing zinc and glutathione bioavailability within the alveolar space with dietary supplements could mitigate these pathophysiological consequences.

Zinc deficiency has been established in alcoholic liver disease for over 50 years (37, 38). These individuals have significantly lower serum zinc levels compared to non-alcoholic controls (39), and both human studies and animal models of chronic alcohol ingestion demonstrate the presence of zinc deficiency in hepatocytes (40-42). However, much less is known about zinc balance in alcoholics without liver disease, and to our knowledge, the pulmonary zinc status in these individuals has never been examined. This is an important area for research since zinc therapy remains absent from clinical practice guidelines involving the management of alcohol-related disorders including alcohol withdrawal (43). In this study, our alcoholic and control
subjects all had normal blood zinc levels, suggesting that these serum measurements are unreliable surrogate markers for zinc metabolism at the organ level. Specifically, alveolar macrophage intracellular zinc was about 30% lower in alcoholic subjects compared to matched non-alcoholics in this study. While the clinical relevance of this finding requires additional investigation, this degree of intracellular zinc depletion has been shown to cause significant cellular and clinical derangements in other studies (44-46). Further, our clinical findings in this study are remarkably consistent with our published findings in an animal model of chronic alcohol ingestion in which we identified a similar 30% decrease in lung zinc levels, and where correction of this deficiency reversed phagocytic dysfunction in the alveolar macrophage and restored lung bacterial clearance (21, 27).

Animal models have been instrumental in characterizing what we have termed the ‘alcoholic lung phenotype’, which includes increased oxidative stress in the lower airways, immune dysfunction of the alveolar macrophage, and disruption of alveolar epithelial barrier function (7). Human studies are limited, but have confirmed the presence of oxidative stress in the alveolar space (47) and revealed alteration of gene expression in alveolar macrophages isolated from alcoholics (48). In this study, we show that isolated macrophages from human alcoholics have significantly decreased phagocytic capacity compared to matched controls. This finding is parallel to our observations in the animal model and confirms the presence of alcohol-induced immune dysfunction in the human lung. We have shown previously that decreased GM-CSF signaling secondary to alcohol exposure is at least partly responsible alveolar macrophage immune impairment in the rat lung (13, 19). Interestingly, correction of zinc deficiency improved GM-CSF signaling in this model (27), suggesting that these two pathways may be interdependent. We evaluated the role of the GM-CSF pathway in the human lung, and we
found that subjects with an AUD exhibit significantly decreased alveolar macrophage GM-CSF receptor β-subunit expression compared to matched controls and no difference in α-subunit expression. This is a similar albeit not a complete recapitulation of our findings in the animal model in which there was decreased expression of the signaling component receptor β-subunit but also of the ligand binding receptor α-subunit. The decreased expression of the β-subunit we identified in these human subjects suggests that the dampened GM-CSF signaling we discovered in the experimental model (including decreased expression and nuclear binding of its master transcription factor, PU.1) may contribute to alcohol-induced macrophage dysfunction in humans. Admittedly, we did not identify a significant decrease in expression of the α-subunit in the macrophages from alcoholic subjects as we had in the experimental model. Although an explanation for this difference is at present unknown, our findings nevertheless support the hypothesis that alcohol abuse dampens GM-CSF signaling in human alveolar macrophages as the GM-CSF receptor requires optimal expression of the β-subunit to initiate intracellular signaling.

There are no currently available treatments to mitigate the adverse effects of chronic alcohol use on the lung. Animal models have shown that dietary supplementation with zinc and thiol anti-oxidants (particularly glutathione precursors) may have a therapeutic role, but to date no human trials have been done. The use of these modalities is of particular interest as we previously showed that zinc deficiency and oxidative stress are mechanistically connected. Specifically, other investigators have described the close relationship between oxidative stress and zinc deficiency (49, 50), and we determined that correction of zinc deficiency improves redox stress in the lower airway of alcohol-fed animals (21). In this human study, treating isolated alveolar macrophages with zinc and/or GSH in vitro improved their intracellular zinc levels and phagocytic function in parallel. Understandably, this is not the same as dietary
supplementation, but these experiments provide important proof-of-concept for the development of clinical trials. Interestingly, GSH appears to improve intracellular zinc levels, strengthening the argument that zinc and oxidative stress pathways are interconnected. While these parameters also improved in alveolar macrophages isolated from control subjects, when cells are treated with both zinc and GSH there is no appreciable difference in zinc levels or phagocytic function between the two groups.

While this study furthers our knowledge of the alcoholic lung phenotype in human subjects, it has some limitations. First, this is a relatively small, single-center study, making it difficult to control for many potential confounding factors simultaneously. Due to the nature of the veteran population at our center, all enrolled subjects were male and most were African American, which limits generalizability. Despite these limitations, we attempted to control for confounding by matching. Our alcoholic subjects were heavier smokers in general, and we adjusted our analysis to control for the number of cigarettes smoked in the last month even though the unadjusted analysis does not alter the conclusion reached in this study. Second, the clinical importance of measuring intracellular zinc levels is not known and such testing is not commercially available for individuals seen by health care providers. Currently, the evaluation of pulmonary zinc status is on a research basis only and requires a relatively invasive technique (i.e. bronchoscopy) to perform. However, our experimental studies show that alveolar zinc deficiency correlates with the levels of glutathione, which can be measured non-invasively with exhaled breath condensate. It may be feasible in the future to employ such methods as surrogate markers of alveolar ‘health’.

In summary, this study furthers our understanding of the effects of alcoholism on the human lung. Specifically, we demonstrate that intracellular zinc levels are significantly
decreased in alveolar macrophages from alcoholic subjects compared to non-alcoholics even when serum zinc levels are normal. In parallel, alveolar macrophages from alcoholic subjects have impaired immune function as characterized by decreased GM-CSF receptor (β-subunit) expression and decreased phagocytic capacity. Importantly, treating these dysfunctional macrophages with zinc and GSH \textit{in vitro} restored their intracellular zinc levels and phagocytic function, providing provocative evidence that it is not the alcohol \textit{per se}, but rather the alcohol-induced zinc deficiency and oxidative stress that impairs their host immune function. While many factors contribute to the alcoholic lung phenotype, experimental and clinical evidence now implicates decreased zinc bioavailability and oxidative stress within the alveolar space as fundamental mechanisms by which alcohol impairs host immunity. Therefore, these findings should pave the way for clinical trials to evaluate the impact of dietary supplementation with zinc and glutathione precursors on lung health in individuals with chronic alcohol use disorders.
REFERENCES


42. Zarski JP, Arnaud J, Labadie H, Beaugrand M, Favier A, Rachail M. [serum and tissue concentrations of zinc after oral supplementation in chronic alcoholics with or without cirrhosis]. 


FIGURE LEGENDS

Figure 1: **Assessment of study participants.** Non-alcoholics were matched to enrolled alcoholic subjects based on age, gender, race, and smoking status.

Figure 2: **Intracellular zinc values in alveolar macrophages from alcoholic and non-alcoholic study participants.** Intracellular zinc was measured as relative fluorescence units (RFU) per cell using confocal microscopy after macrophages were incubated with 200 nM FluoZin-3AM dye. Panel A box plots illustrate the median and interquartile ranges for intracellular zinc measurements among the alcoholic and non-alcoholic subjects. The diamond shape represents the mean among alcoholics and the star shapes represent the mean and outliers among the non-alcoholics. As shown, alcoholic subjects had approximately 30% less intracellular zinc than non-alcoholic subjects (p < 0.001). Panel B shows a representative cell staining image from one alcoholic and one non-alcoholic subject.

Figure 3: **Serum zinc measurements among alcoholic and non-alcoholic subjects.** Panel A graphically represents median and interquartile ranges for serum zinc values. The diamond shape represents the mean among alcoholics and the star shape represents the mean among non-alcoholics. There was no significant difference between alcoholic and non-alcoholic subjects with regards to systemic zinc levels (p = 0.7271). Association between serum zinc value and intracellular zinc value is shown in panel B. There is no appreciable relationship between serum
zinc levels and alveolar macrophage intracellular zinc measurements among alcoholics and non-alcoholics.

**Figure 4: Evaluation of phagocytic capacity in alveolar macrophages from alcoholic and non-alcoholic study participants.** Phagocytosis was measured as relative fluorescence units (RFU) per cell using confocal microscopy after macrophages were incubated with *Staphylococcus aureus*. Panel A box plots represents median and interquartile ranges for alveolar macrophage phagocytosis among the alcoholic and non-alcoholic subjects. The diamond and star shapes represent mean values and outliers among alcoholics and non-alcoholics, respectively. As shown, alveolar macrophages from alcoholic subjects had approximately 30% less phagocytic capacity than non-alcoholic subjects (p < 0.001). Panel B shows a representative cell image from one alcoholic and one non-alcoholic subject.

**Figure 5: Assessment of GM-CSF receptor expression in alveolar macrophages from alcoholic and non-alcoholic study subjects.** GM-CSF receptor expression was measured as relative fluorescence units (RFU) per cell using confocal microscopy after alveolar macrophages were incubated with the primary antibody to the binding α-subunit and signaling β-subunit. Box plots represent the median and interquartile ranges for GM-CSF receptor subunit measurements. The diamond shapes represent the mean among alcoholics and the star shapes represent the mean and outlier among non-alcoholics. As shown in panel A, there was no significant difference between alcoholics and non-alcoholics in α-subunit expression on alveolar macrophages (p = 0.0123, p < 0.0045 considered significant after Bonferroni adjustment). Panel B illustrates about
a significant 30% decrease in expression of the signaling β-subunit in alveolar macrophages from alcoholic subjects compared to non-alcoholics (p < 0.0001).

**Figure 6: Evaluation of intracellular zinc and phagocytic capacity in alveolar macrophages from alcoholic and non-alcoholic study participants after treatment *ex vivo* with zinc acetate (10 µM), glutathione (GSH, 500 µM), or both zinc and GSH.** Intracellular zinc and phagocytosis was measured as relative fluorescence units (RFU) per cell using confocal microscopy. Panel A represents median and interquartile ranges for post-treatment intracellular zinc among alcoholic and non-alcoholic subjects, with the baseline values displayed in the top bar. Intracellular zinc increases with zinc and GSH treatment alone, and combination treatment with both zinc and GSH results in intracellular zinc levels that are modestly higher in alcoholics (p = 0.0031). Panel B represents median and interquartile ranges for post-treatment phagocytosis among alcoholic and non-alcoholic subjects. Phagocytosis increases with zinc and GSH treatment alone, and combination treatment with both zinc and GSH also results in alveolar macrophage phagocytosis that is similar between alcoholics and non-alcoholics (p = 0.1188). The diamond and star shapes represent mean values and outliers among alcoholics and non-alcoholics, respectively.
Table 1. Demographic and Health Characteristics of Study Subjects.

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\(^1\) Characteristics not related to the study design were assessed for differences between groups: BMI (p=0.7); #cigarettes in last 30 days (p=0.0003); illegal drug use (p=0.0027).

\(^2\) Missing information for 1 alcoholic.
Table 2. Alveolar macrophage measures from alcoholic and non-alcoholic subjects, with and without adjustment for the number of cigarettes smoked in the previous month.\(^1,2\)

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</tr>
<tr>
<td><strong>Phagocytosis</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline (no treatment)</td>
<td>1521 (82)</td>
<td>988 (48)</td>
</tr>
<tr>
<td>Zinc Acetate</td>
<td>1715 (35)</td>
<td>1260 (29)</td>
</tr>
<tr>
<td>GSH</td>
<td>2091 (30)</td>
<td>1775 (33)</td>
</tr>
<tr>
<td>Zinc Acetate + GSH</td>
<td>2555 (27)</td>
<td>2462 (55)</td>
</tr>
<tr>
<td><strong>Other</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GM-CSF α</td>
<td>2473 (31)</td>
<td>2630 (47)</td>
</tr>
<tr>
<td>GM-CSF β</td>
<td>2117 (38)</td>
<td>1466 (41)</td>
</tr>
<tr>
<td>Serum Zinc</td>
<td>85 (2)</td>
<td>88 (3)</td>
</tr>
</tbody>
</table>

\(^1\) General Estimating Equations were used to account for the matching of alcoholic to non-alcoholic subjects and 1-step robust regression estimator weighting to reduce the influence of outliers. Each model contains the predictor alcoholic status, and the adjusted models also contain the number of cigarettes smoked in the previous month. \(\hat{\eta}_{\text{non-alcohol}}\)=17, \(\hat{\eta}_{\text{alcohol}}\)=16 for all other models.

\(^2\) Units are µg/dL for Serum Zn and Relative Fluorescence Units (RFU)/cell for all others.

\(^3\) A family-wise Type I error rate was set at 5%; a Bonferroni adjustment for 11 tests yields a significance criteria of \(p\) values <0.0045.
Figure 1 - Recruitment

Alcoholics

175 Screened

112 Excluded
46 Declined

17 Enrolled

Non-Alcoholics

200 Screened

118 Excluded
10 Declined
55 No Match

17 Enrolled

352x352mm (300 x 300 DPI)
Figure 2 - Intracellular Zinc
197x115mm (300 x 300 DPI)
Figure 3 - Serum zinc
Figure 4 - Phagocytosis

197x115mm (300 x 300 DPI)
Figure 5 - GM-CSF
279x361mm (300 x 300 DPI)
Figure 6 - In vitro treatment studies
279x361mm (300 x 300 DPI)
SUPPLEMENTARY METHODS

Study population.

We specifically recruited test subjects whose most recent alcoholic drink was within 8 days of undergoing the bronchoscopy procedure and therefore had an active AUD and not merely a history of prior alcohol abuse. Key exclusion criteria included 1) primary substance of abuse being something other than alcohol (e.g., cocaine), 2) active medical problems, 3) HIV-positive status, and 4) an abnormal chest radiograph signifying underlying lung disease. A primary goal of the study was to recruit subjects with a current AUD but who were otherwise healthy so as to minimize the effects of confounding factors such as chronic infections or lung disease. Control subjects were recruited through advertisements placed throughout the Atlanta VAMC and were likewise healthy but did not have an AUD. Each case subject was matched to a control subject (1:1) by age (within 5 years, but still falling in the range of 18-55 years of age), gender, race, and smoking status (the majority of individuals with an AUD are cigarette smokers, and therefore we matched for smoking status to avoid possible confounding).

Subject enrollment.

A research coordinator screened subjects enrolled at the SATP clinic and discussed the study with those who met the specified inclusion criteria. Those individuals that expressed interest in participating in the study underwent more formal screening with the SMAST and the Alcohol Use Disorders Identification Test (AUDIT) as well as a review of their medical records to screen for the specified exclusion criteria. Each subject also underwent a screening chest radiograph if one had not been performed in the previous year. All control subjects were screened similarly, but in general medical clinics rather than in the SATP clinic. The majority of control
participants contacted us directly after viewing a recruitment flyer. Once test and control subjects were enrolled in the study, they had blood drawn and sent to the VA clinical laboratory for measurement of serum zinc levels. Enrolled subjects all underwent a history and physical exam prior to bronchoscopy. It was during this process that specific alcohol intake was assessed by subjective report. This subjective alcohol intake was converted to grams/day under the assumption that a standard alcoholic beverage contains 14 grams of pure alcohol. All enrolled subjects were compensated for their participation with a grocery store gift card for $150.

**Bronchoscopy Procedure.**

All subjects enrolled in the study underwent bronchoscopy using standard procedural guidelines in place at the Atlanta VA Medical Center. Subjects had topical upper airway anesthesia with nebulized 4% lidocaine and then conscious sedation with intravenous midazolam and fentanyl during which they were continuously monitored using a three-lead electrocardiogram, automatic blood pressure cuff, pulse oximetry, and capnography. All lavage samples were obtained from the right middle lobe using a total instillation of 180 ml of normal saline. The collected lavage samples were placed on ice and immediately transported to the research laboratory. Following the bronchoscopy, each subject was monitored for two hours before being discharged. There were no significant complications related to the bronchoscopic procedures in any study subjects.

**Determination of alveolar macrophage phagocytic capacity.**

The relative phagocytic capacity of alveolar macrophages at baseline and following treatment with Zn, GSH, or Zn + GSH was assessed. Cells were incubated with $1 \times 10^6$ particles of pH-sensitive pHrodo Staphylococcus aureus BioParticles conjugate (Invitrogen) for 2 hours and then
fixed with 4% paraformaldehyde. Phagocytosis of these live, biologically inactive bacteria was analyzed using an Olympus confocal microscope containing an argon/krypton laser. Cells from 10 fields per experimental condition were assessed using quantitative digital fluorescence imaging software (Olympus FluoView 300, Version 4.3). To measure S. aureus internalization, laser confocal microscopy was performed at 50% of cell depth using identical background and gain settings. Alveolar macrophages with internalized bacteria were considered positive for phagocytosis.

Statistical Analyses.

Descriptive statistics of patient characteristics (mean, standard deviation; median, first and third quartiles; n, percent) are presented for all variables by group. Distributions of serum zinc and alveolar macrophage measurements (unadjusted data) are displayed with boxplots. Statistical comparison of the alcoholic and non-alcoholic groups on illegal drug use was evaluated using a Fisher's exact test due to small expected cell counts. The relationship between serum zinc and alveolar macrophage zinc was assessed separately for alcoholic and non-alcoholic subjects using Spearman's correlation. For all other analyses, comparison of the alcoholic and non-alcoholic groups used GEE modeling methodology, as described in the main paper. While not all outcome distributions had extreme outliers, for consistency we used Huber 1-step robust regression for all models. Although subjects were matched on current smoking status (yes or no), alcoholics more commonly reported heavier smoking. As the quantity of smoking might be associated with the outcomes and could therefore potentially confound results, the number of cigarettes smoked in the previous 30 days was added as a variable in the alveolar macrophage outcome regression models to control against biased estimates. Results from unadjusted models are also reported for
comparison. A family-wise Type I error rate was set at 5%; a Bonferroni adjustment for 11 tests was applied and p values <0.0045 are reported as statistically significant.

**Missing Data.**

Values for AM phagocytosis capacity values are missing for 4 alcoholic subjects as there was insufficient reagent to perform the analyses in the research laboratory on the day of their bronchoscopy and were therefore considered missing completely at random. For one of these subjects we are also missing other lab outcomes, AUDIT score, and number of cigarettes smoked.