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Chronic Alcohol Ingestion in Rats Decreases Krüppel-like Factor 4 Expression and Intracellular Zinc in the Lung

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Abstract

Background—Chronic alcohol ingestion alters the dynamic balance between granulocyte-macrophage colony stimulating factor (GM-CSF) and transforming growth factor beta1 (TGFβ1) signaling within the alveolar space and, in parallel, impairs alveolar macrophage and epithelial cell function by inhibiting expression of the zinc importer ZIP_4 and decreasing zinc bioavailability in the alveolar compartment. Since the transcription factor Krüppel-like factor 4 (KLF_4) binds to ZIP_4, we hypothesized that alcohol exposure and consequent perturbations in GM-CSF and TGFβ1 signaling could decrease cellular KLF_4 expression and/or binding as a mechanism by which it inhibits ZIP_4 expression and decreases cellular zinc levels.

Methods and Results—Alcohol exposure *in vitro* or chronic ingestion *in vivo* decreased KLF_4 expression in alveolar macrophages and epithelial cells. Treatment with GM-CSF or TGFβ1 showed an enhancing or dampening effect on KLF_4 expression and binding, respectively. Further, treatment of a rat alveolar macrophage cell line with alcohol *in vitro* for 4 weeks decreased the expression of the zinc transporters ZIP_4 and ZNT1, and of the zinc storage protein metallothionein 1. In parallel, treating these macrophages with KLF_4 siRNA decreased ZIP_4 expression, and decreased cellular zinc and phagocytic capacity to levels equivalent to those following alcohol exposure. In epithelial monolayers, TER was significantly decreased by alcohol ingestion as compared to control diets and it was restored by *in vitro* GM-CSF treatment. In contrast, *in vitro* TGFβ1 treatment of the epithelial monolayers from control-fed rats significantly decreased TER as compared to untreated control monolayers.

Conclusions—Taken together, these results suggest that within the alveolar space, chronic alcohol exposure decreases KLF_4 and ZIP_4 expression and consequently decreases zinc transport into cells, which, in turn, impairs their function. Furthermore, the dynamic decrease in the relative influence of GM-CSF vs. TGFβ1 could mediate the zinc deficiency and consequent cellular dysfunction that characterize the ‘alcoholic lung’ phenotype.

Keywords

Krüppel-like Factor 4; lung; zinc; macrophage; type II epithelia; alcohol

INTRODUCTION

Alcohol abuse is an independent risk factor for lung infections such as *Klebsiella pneumoniae* and *Streptococcus pneumoniae* leading to lung injury (Guidot and Hart, 2005;

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Mehta et al., 2011). Using experimental models of alcohol ingestion, we have previously shown that chronic alcohol ingestion increases oxidative stress and TGFβ1 production (Bechara et al., 2004; 2005), both of which can negatively impact alveolar macrophage and epithelial function. In addition, chronic alcohol ingestion decreases expression of the zinc transporter ZIP_4 in the gut and in the lung, and decreases zinc bioavailability within the alveolar space (Joshi et al., 2009).

The essential micronutrient zinc is a co-factor for multiple enzymes and regulates many cellular processes including mitosis, apoptosis, secretion, and signal transduction (Maverakis et al., 2007; Prasad, 1983; Tudor et al., 2005; Zalewski, 2006). In fact, approximately 300 enzymes and more than 2000 transcription factors require zinc for their functional and structural integrity (Coleman, 1992; Vallee and Falchuk, 1993). As a result, many metabolic and signaling pathways are directly or indirectly dependent on zinc-requiring proteins (Beattie and Kwun, 2004). Localized intracellular oxidative stress may impede zinc binding within zinc finger proteins (Webster et al., 2001). Therefore, even relatively modest oxidative stress can affect intracellular zinc availability with deleterious effects on signaling. Within the alveolar space, zinc is transported into alveolar epithelial cells and macrophages by ZIP_4 (Dufner-Beattie et al., 2003). ZIP_4 is part of a ZIP superfamily of metal ion uptake transporters, many of which mediate zinc uptake. It is becoming increasingly recognized that zinc bioavailability is critically important to epithelial cell and immune cell function and therefore even modest zinc deficiency within the alveolar space could have a serious impact on alveolar cells (specifically, the epithelium and macrophages).

The members of the Krüppel-like family (KLF) of mammalian transcription factors have a high degree of similarity to the regulatory protein Krüppel in *Drosophila melanogaster* (Mahatan et al., 1999). All KLF family members are characterized by their Cys2-His2 zinc fingers, which bind to DNA. They are critical regulators of cellular growth and differentiation, and can be both activators and repressors of gene expression depending on the cell type and other transcription factors with which they interact (Dang et al., 2002). KLF_4, which was initially found in the gut but is also present in the lung, skin, thymus, and vascular endothelial cells (Wang et al., 2004), belongs to a 17-member zinc-finger containing family. These KLFs bind to GC or CACCC element consensus sequences, and their specificity is determined by amino acid termini and/or tissue-specific expression. KLF_4 is also expressed by monocytes/macrophages and is regulated by pro- and anti-inflammatory cytokines (Feinberg et al., 2005; 2007). In contrast, TGFβ1 decreases KLF_4 gene and protein expression in THP-1 cells (Feinberg et al., 2005). KLF_4 is essential for differentiation of inflammatory monocytes and mediates pro-inflammatory signaling in human macrophages (Alder et al., 2008; Feinberg et al., 2005). KLF_4 inhibits α-SMA gene expression by binding to the TGFβ control element (TCE) or directly to SMAD3, thereby inhibiting myofibroblast differentiation (Hu et al., 2007). TGFβ1, which is increased in the lung by chronic alcohol ingestion, is both an inhibitor and a target of KLF_4 (Feinberg et al., 2005). Interestingly, KLF_4 binds to the ZIP_4 promoter and regulates its expression (Liuzzi et al., 2009).

Granulocyte-macrophage colony stimulating factor (GM-CSF) is an important growth factor in the lung. It is produced by the alveolar epithelium and binds to specific GM-CSF receptors on the membranes of both alveolar macrophages and alveolar epithelial cells (Trapnell and Whitsett, 2002). This binding activates an intracellular signaling pathway leading to expression and nuclear binding of the master transcription factor PU.1 (Shibata et al., 2001). We have previously shown that alcohol ingestion decreases alveolar macrophage and epithelial function by decreasing GM-CSF receptor expression and downstream PU.1 nuclear binding in both the alveolar macrophage and in the alveolar epithelium (Joshi et al.,

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2005; 2006). Importantly, recombinant GM-CSF treatment in the airway *in vivo* restores alveolar macrophage function in alcohol-fed rats (Joshi et al., 2005). Interestingly, KLF_4 is a PU.1 target gene. Specifically, $PU.1^{-/-}$ cells do not express KLF_4, whereas over-expression of PU.1 restores KLF_4 expression in HL-60 cells (Deaton et al., 2009). In addition, KLF_4 has a PU.1 binding site and PU.1 induces the KLF4 promoter 7 (Deaton et al., 2009; Feinberg et al., 2007).

With this background, we hypothesized that alcohol decreases alveolar KLF_4 expression and/or activity, which in turn decreases alveolar ZIP_4 expression and thereby decreases zinc bioavailability to the alveolar epithelium and macrophages. To test this hypothesis, we extended our previously published studies and examined the effects of chronic alcohol exposure, as well as the direct effects of GM-CSF or TGFβ1 independently of alcohol, on KLF_4 expression in alveolar epithelial cells and macrophages. In parallel, we determined if RNA silencing of KLF_4 reproduced the alcohol-induced inhibition of ZIP_4 and the consequent decrease in intracellular zinc that mediates cellular dysfunction in the alcoholic lung.

MATERIALS AND METHODS

Animals and alcohol feeding

Adult Male Sprague-Dawley rats (initial weights 150–200 g; Charles River Laboratory, Wilmington, MA) were fed the Lieber-DeCarli liquid diet (Research Diets, New Brunswick, NJ) containing either alcohol (36% of total calories) or an isocaloric substitution with maltose-dextrin *ad lib* for 6 weeks as previously published (Guidot and Brown, 2000; Guidot et al., 1999). All work was performed with the approval of the Institutional Use and Care of Animals Committee (IACUC) at Emory University.

Brochoalveolar Lavage and Isolation of Alveolar Macrophages

Rats were anesthetized with 0.8ml Euthasol containing pentobarbital sodium and phenytoin sodium (Vibac AH Inc, Fort Worth, TX), a tracheotomy tube was placed and lungs were lavaged four times with 10 ml of sterile PBS (pH 7.4). The recovered lavage solution was centrifuged (405 g, 7 min) and the cell pellet re-suspended in sterile F12-K medium for functional studies. This procedure routinely yields cells that are >95% viable by Trypan blue exclusion test (Joshi et al., 2005).

Isolation of primary alveolar type II epithelial cells

Alveolar epithelial cells from control- and alcohol-fed rats were isolated using our established protocol (Fan et al., 2011; Fernandez et al., 2007; Guidot et al., 2000). Briefly, lungs and trachea were removed as one unit and flushed with 40 ml solution containing 16 mg of elastase to dissociate cells from lungs. Lung lobes were cut and finely minced in a solution containing DNase I and newborn calf serum. The lung tissue suspension was shaken at 37°C for 10 minutes and filtered through 100μm and 20μm nylon mesh. The filtered lung suspension was then centrifuged and re-suspended in 30ml of DMEM/F12 containing antibiotics and plated on IgG coated dishes. Cells were then incubated at 37°C 5% CO2 for 1 hour, and non-adherent type II epithelial cells were gently removed and resuspended in DMEM/F12 containing 10% fetal bovine serum and antibiotics. Cell viability was determined by Trypan blue exclusion test and was always >96%.

Measurement of transepithelial electrical resistance (TER)

Epithelial cells were plated on permeable membranes (0.4 μM pore; Corning Corp, Lowell, MA) and cultured for 5–7 days in DMEM/F12 complete medium. Monolayers were treated with or without GM-CSF or TGFβ1, and the TER of these monolayers was measured using

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an EVOM volt/ohm meter with electrodes (World Precision Instruments, Sarasota, FL (Fan et al., 2011).

Cell lines

In some experiments, the rat alveolar macrophage cell line NR8383 (ATCC CRL-2192) was used. These cells were maintained in F-12K complete medium with 10% fetal bovine serum and antibiotics. In some experiments, these cells were treated with or without 0.2% alcohol for 4 weeks.

RNA isolation and Real-time PCR

RNA was extracted from cells using Qiagen RNeasy Mini Kit (Valencia, CA). Reverse transcription was performed using 1 μg RNA using iScript cDNA synthesis kit from Bio Rad (Hercules, CA), and real-time PCR was performed using primers for the target gene and 18S as housekeeping gene (Otis et al., 2007). Quantum RNA class II 18S primers were purchased from Ambion (Austin, TX). All the primers for the target genes (Table 1) were designed in our laboratory and were obtained from Invitrogen (Carlsbad, CA). All samples were run in triplicate. Messenger RNA expression for each gene of interest was normalized to 18S and then expressed as the change relative to the control group.

Western blots

Nuclear protein lysates from freshly isolated cells were prepared using NucBuster protein extraction kit (Novagen, Madison, WI). Thirty micrograms of protein was loaded onto a 10% polyacrylamide gel and electrophoresed at 150 V for 60 min. The separated proteins were transferred to a nitrocellulose membrane at 15 V for 45 min. Membranes were blocked at RT for 1 h in Tris-buffered saline with 0.2% Tween 20 containing 5% nonfat dry milk and incubated with anti-KLF_4 (Santa Cruz Biotechnology) at 1:100 at 4°C overnight followed by incubation at room temperature with horseradish peroxidase-labeled secondary antibody at 1:1000 for 2h. β-actin antibody (Santa Cruz) at 1:2000 was used to normalize KLF_4 levels.

RNA silencing

Silencer select siRNA for KLF_4 was purchased from Applied Biosystems. We first tested different concentrations of two different siRNA oligonucleotides designed for rat KLF_4. We chose the concentration of a specific siRNA that consistently decreased KLF_4 gene expression. Sequence for this siRNA is as follows: sense AGGCACACCUGCGAACUCAtt, antisense UGAGUUCGGUGUGCCUtg. We used Qiagen HiPerFect transfection protocol (Qiagen, Valencia, CA) to transfect specific siRNA or Stealth RNAi duplex negative control from Invitrogen. Briefly, 0.2×10^6 NR8383 cells were plated in 24 wells in the presence of either 5nM of KLF_4 siRNA, negative control, or HiPerFect transfection reagent (mock control) for 72 hr. Block it GFP fluorescent oligo (Invitrogen) was used to ensure the efficiency of transfection within this period. Seventy two hours post transfection, fluorescence signal for transfection efficiency was assessed before cells were used for mRNA analysis.

Flow cytometric detection of intracellular zinc

NR8383 cells were treated with or without KLF_4 siRNA or alcohol and then stained with a membrane-permeable form of a flurochrome, FluoZin-3 AM (Invitrogen), for one hour. FluoZin-3 AM has a high affinity for zinc and gives a green fluorescence after binding to intracellular zinc. The labeled cells were analyzed by FACScan flow cytometer (Becton Dickinson, San Jose, CA) and data are expressed as percentage of positive cells after each treatment.

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Phagocytosis

After siRNA or alcohol treatment, NR8383 cells were incubated for 1 hr with FITC-labeled *Staphylococcus aureus* (Wood strain without protein A; Molecular Probes, Eugene, OR). Cells were vigorously washed with PBS containing Trypan blue to quench extracellular fluorescence and FITC-labeled bacteria containing cells were measured by flow cytometry. The percentage of positive cells was multiplied by the mean channel fluorescence and divided by 100 to calculate the phagocytic index.

NoShift transcription factor assay

The binding of KLF_4 to the specific DNA sequences was identified by the NoShift transcription factor assay, which is an ELISA-based colorimetric assay. Briefly, the nuclear extracts were prepared from alveolar macrophages and epithelial cells using the NucBuster protein extraction kit (Novagen). Nuclear proteins were bound to the double-stranded biotinylated KLF_4 consensus oligonucleotides, and these protein-DNA complexes were captured on a streptavidin-coated plate (Novagen). Rabbit anti-KLF_4 antibody (Santa Cruz Biotechnology) followed by HRP-conjugated donkey anti-rabbit antibody were added to the plate. Color was developed with ABTS substrate (Vector Labs) and absorbance at 405 nm was measured.

Statistics

Data are presented as mean ± SEM. Data analysis was done by Student's unpaired t-test or ANOVA with Student-Newman-Keuls test for group comparisons, and differences between and/or among experimental groups were considered statistically significant at a P value of <0.05.

RESULTS

Chronic alcohol ingestion *in vivo* or alcohol exposure *in vitro* decreased KLF_4 gene and protein expression in alveolar macrophages and epithelial cells

We first examined KLF_4 gene expression in alveolar macrophages and epithelial cells isolated from control- and alcohol-fed rats. As shown in Figure 1, panel A, chronic alcohol ingestion decreased ($P<0.05$) KLF_4 gene expression in alveolar macrophages and epithelial cells when compared to cells from control-fed rats. We next determined the direct effects of long-term alcohol exposure on alveolar macrophages *in vitro*. NR8383 cells, which are a rat alveolar macrophage cell line, were maintained in culture for 4 weeks ± alcohol (0.2%), at which time both KLF_4 gene and protein expression were measured. As shown in Figure 1, chronic alcohol exposure significantly ($P<0.05$) decreased KLF_4 gene expression (panel B) and KLF_4 protein expression (panel C).

Treatment of primary cells or alveolar cell lines *in vitro* with TGFβ1 decreased KLF_4 expression to levels comparable to those caused by chronic alcohol ingestion, and KLF_4 expression in alveolar macrophages and epithelial cells isolated from alcohol-fed rats was restored by GM-CSF treatment *in vitro*

Through direct exposure of both cell line and primary cells we sought to link TGFβ1, the expression of which increases in the lung during alcohol consumption, as a mechanism for decreased alveolar macrophages and epithelial cell KLF_4. First we compared KLF_4 gene expression in untreated NR8383 cells with NR8383 cells treated with alcohol (0.2%), TGFβ1 (10 ng/ml), or the combination of alcohol (0.2%) and GM-CSF (10 ng/ml). As shown in Figure 2, panel A, there was a decrease ($P<0.05$) in KLF_4 expression when treated with alcohol or TGFβ1. Specifically, treatment with TGFβ1 or alcohol decreased KLF_4 gene expression in NR8383 cells (Figure 2, panel A). In contrast, cells treated with alcohol plus

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GM-CSF had the same (P>0.05) KLF_4 expression as untreated cells (Figure 2, panel A). Cells treated with GM-CSF alone showed no significant difference (p<0.05) as compared to untreated cells (data not shown in the figure). We also treated primary alveolar epithelial cells and macrophages from control- and alcohol-fed rats with either TGFβ1 (10 ng/ml) or GM-CSF (10 ng/ml) *in vitro* for 48 hours and then assessed KLF_4 protein expression by western blot analyses. As shown in Figure 2, panel B, treating macrophages from control-fed rats with TGFβ1 decreased (P<0.05) KLF_4 protein expression and this was comparable to macrophages from alcohol-fed rats in which KLF_4 expression was decreased (P<0.05) compared to cells from control-fed rats. In contrast, macrophages isolated from alcohol-fed rats and then treated with GM-CSF had the same (P>0.05) level of KLF_4 expression as macrophages from control-fed rats (Figure 2, panel C). As shown in Figure 2, panel C, similar effects were observed in alveolar epithelial cells. Specifically, treating alveolar epithelial cells isolated from control-fed rats with TGFβ1 showed a trend toward decreased KLF_4 protein expression, although this was not statistically significant (P>0.05) in these experiments. However, alveolar epithelial cells from alcohol-fed rats had decreased KLF_4 protein expression (P<0.05) by compared to cells from control-fed rats. Finally, and consistent with the findings in alveolar macrophages, GM-CSF treatment increased (P<0.05) KLF_4 protein expression in alveolar epithelial cells isolated from alcohol-fed rats; in fact, KLF_4 protein expression in these cells was even higher than in cells from control-fed rats (Figure 2, panel C).

Chronic alcohol exposure decreased KLF_4 DNA binding in alveolar macrophages and epithelial cells, and these effects could be reproduced by treating cells with TGFβ1 and abrogated by GM-CSF treatment

To further examine the role of TGFβ1 in modulating KLF_4 function during chronic alcohol exposure, we analyzed DNA binding of KLF_4 in NR8383 treated with and without alcohol for four weeks. Alcohol exposure decreased (P<0.05) KLF_4 DNA binding compared to cells from control-fed rats (Figure 3, panel A). We then assessed KLF_4 DNA binding in alveolar macrophages and epithelial cells isolated from control-and alcohol-fed rats; in addition, as the adverse effects alcohol appear to be mediated by TGFβ1 and restored by GM-CSF, we treated some of these cells with either GM-CSF or TGFβ1 for 48 hours. As shown in Figure 3, panels B&C, TGFβ1 treatment decreased (P<0.05) KLF_4 DNA binding in both alveolar macrophages and epithelial cells isolated from control-fed rats, and these effects were comparable to those assessed in alveolar macrophages and epithelial cells isolated from alcohol-fed rats. In contrast, and consistent with its effects on KLF_4 gene and protein expression, GM-CSF treatment increased (P<0.05) KLF_4 DNA binding in alveolar macrophages and epithelial cells isolated from alcohol-fed rats Figure 3, panels B&C), and in fact back to the same levels as those observed in untreated cells from control-fed rats.

Chronic alcohol exposure decreased expression of the zinc transporters ZIP_4 and ZNT1, and the zinc storage protein metallothionein 1, in the NR8383 macrophage cell line

We have previously shown that chronic alcohol ingestion in rats alters gene expression of the zinc transporters ZIP_4 and ZNT1, and the zinc storage protein, metallothionein 1 (MT1) in the alveolar macrophages and epithelial cells (Joshi et al., 2009). Since alcohol decreased KLF_4 expression after four weeks of treatment, and KLF_4 is known to bind to the ZIP_4 promoter, we explored the effects of alcohol on the gene expression of these zinc homeostasis-related genes. ZIP_4 is one of the transporters that mediates zinc uptake by cells and as shown in Figure 4, panel A, chronic alcohol exposure decreased (P<0.05) ZIP_4 expression. By comparison, ZNT1 is one of the transporters that mediates zinc export by cells and as shown in Figure 4, panel B, chronic alcohol exposure also decreased (P<0.05) ZNT1 gene expression. In parallel, MT1 is the principal intracellular zinc storage protein, and as shown in Figure 4, panel C, its expression was also decreased (P<0.05) by chronic

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alcohol exposure. Taken together, the results in Figure 4 show that prolonged alcohol exposure decreases the expression of zinc transporters and MT1 in alveolar macrophages.

Treatment with KLF_4 siRNA decreased ZIP_4 gene expression, intracellular zinc, and phagocytic function in the NR8383 alveolar macrophage cell line

To test if alcohol-induced alveolar macrophage dysfunction is mediated via defects in zinc transport that reflect, at least in part, decreased KLF_4 expression and/or DNA binding, we next examined the effects of targeted inhibition of KLF_4 expression (independent of alcohol exposure) on zinc homeostasis and bacterial phagocytic function. NR8383 cells were treated with either 5nM KLF_4 siRNA, a Stealth duplex negative control, or a mock transfection reagent for 72 hours, at which time KLF_4 and ZIP_4 gene expression were determined. As shown in Figure 5, panel A, KLF_4 gene expression was effectively decreased ($P<0.05$) by the siRNA construct, whereas the other treatments had no effect ($P>0.05$) as expected. Consistent with the previously described effects of chronic alcohol exposure, this targeted decrease in KLF_4 expression produced a significant ($P<0.05$) decrease in ZIP_4 gene expression as shown in Figure 5, panel B suggesting a direct relationship between KLF_4 and ZIP_4 gene expression. Next, we used flow cytometry to quantify intracellular zinc levels using the zinc-specific indicator, FluoZin-3, in untreated and KLF_4 siRNA-treated cells. As a comparison in these experiments, we treated some cells with alcohol (60 mM). As shown in a representative Figure 5, panel C, alcohol exposure decreased the percentage of cells that were positive for FluoZin-3 from 74% to 40%, with a clear shift of the peak to the left indicating decreased intensity of FluoZin-3 (and therefore decreased intracellular zinc levels). As shown in a representative Figure 5, panel D, KLF_4 siRNA treatment produced comparable effects in that it decreased the percentage of cells that were positive for FluoZin-3 from 74% to 52% and caused a shift of the peak to the left. The actual percentage of positive cells and mean channel fluorescence (brightness of cells) for FluoZin-3 AM stained cells were as follows: control or untreated :72±3.6 (42.3±1.9), siRNA treated: 55±1.8(15±1.2), and alcohol treated: 43±4.6 (9.1±2.0), $p<0.05$ for both siRNA and alcohol groups as compared to control/untreated group (data is shown here as mean±SEM for the percentage of positive cells (mean channel fluorescence) for three samples. As alcohol-mediated zinc deficiency decreases innate immune function of the alveolar macrophage, we next examined bacterial phagocytic capacity in KLF_4 siRNA-treated macrophages. As shown in Figure 6, KLF_4 siRNA treatment or alcohol exposure each significantly ($P<0.05$) and comparably decreased macrophage bacterial phagocytic capacity. Taken together with the results shown in the previous figures, the data shown in Figure 5&6 are consistent with the interpretation that chronic alcohol exposure decreases KLF_4 expression and DNA binding, which in turn disrupts zinc homeostasis and impairs bacterial phagocytic capacity in the alveolar macrophage.

Decreased KLF_4 expression in epithelial cells correlated with alcohol-induced alveolar epithelial barrier function and was restored by GM-CSF treatment *in vitro*

Chronic alcohol ingestion causes alveolar epithelial barrier disruption, as reflected by increased paracellular permeability and decrease in transepithelial resistance. As shown in Figure 1, panel A and previously discussed, freshly isolated type II epithelial cells showed a significant decrease in KLF_4 gene expression. Further and as previously presented, alveolar epithelial cells from alcohol-fed rats had decreased KLF_4 protein expression ($P<0.05$) compared to cells from control-fed rats (Figure 2, panel C), and GM-CSF treatment significantly increased KLF_4 protein expression in alveolar epithelial cells isolated from alcohol-fed rats (Figure 2, panel C) suggesting that it is possible to restore KLF_4 expression in epithelial cells from alcohol-fed rats. To examine whether or not GM-CSF treatment would also repair the epithelial barrier dysfunction, we measured transepithelial resistance of the type II cell monolayers (from alcohol-fed rats) that were treated *in vitro* with or

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without 10 ng GM-CSF. As shown in Figure 7, type II monolayers from alcohol-fed rats showed a significant decrease ($p<0.05$) in TER when compared to control monolayers, and GM-CSF treatment reversed this defect in epithelial barrier function. In contrast, treating monolayers established from control-fed rats with TGFβ1 significantly decreased ($p<0.05$) their TER.

DISCUSSION

In this study, we determined that chronic alcohol ingestion decreased the expression and nuclear binding of KLF_4, thereby revealing a novel mechanism by which chronic alcohol abuse causes zinc deficiency and susceptibility to opportunistic infections. Specifically, we determined that chronic alcohol ingestion in an experimental rat model decreased the expression of the transcription factor KLF_4 in the alveolar epithelial cells and macrophages. In parallel, treatment of NR8383 cells (a rat alveolar macrophage cell line) with 0.2% alcohol *in vitro* for four weeks also decreased KLF_4 gene and protein expression. Further, treatment with TGFβ1, which is increased in the alcoholic lung, showed a direct effect on cells by decreasing KLF_4 expression and DNA binding. Interestingly, the treatment of alcohol-exposed alveolar macrophages and epithelial cells with GM-CSF, a key growth factor in the airways whose signaling is dampened by alcohol, restored KLF_4 expression and DNA binding. To explore the functional consequences of these effects of alcohol on KLF_4 expression and DNA binding, we focused on the alveolar macrophage. Consistent with our previous studies in a rat model of chronic alcohol ingestion, alveolar macrophages treated with alcohol *in vitro* also had decreased expression of the zinc transporters ZIP_4 and ZNT1 and of the zinc storage protein MT1. In addition, treating alveolar macrophages with KLF_4 siRNA decreased ZIP_4 gene expression and, intracellular zinc levels, and impaired their bacterial phagocytic capacity. Since alcohol-mediated zinc deficiency decreases phagocytic function of the alveolar macrophage, these results reveal for the first time that chronic alcohol exposure decreases KLF_4 expression in the lung and, as at least one consequence, decreases phagocytic function in the alveolar macrophage. Although we did not silence KLF_4 in epithelial cells in this study, the decrease in its expression and DNA binding in primary alveolar epithelial cells from rats fed alcohol suggests that KLF_4 may have a role in alcohol's effects on epithelial cell barrier function as well. Interestingly, GM-CSF treatment increased both the expression and the DNA binding of KLF_4, whereas TGFβ1 decreased it. Further, GM-CSF and TGFβ1 treatment *in vitro* had antagonistic effects on the permeability of monolayers derived from primary alveolar type II epithelial cells, suggesting a role for this transcription factor in the integrity of the alveolar epithelial barrier.

These findings are important, as individuals with alcohol use disorders are prone to respiratory infections and experimental animal models have demonstrated that chronic alcohol ingestion adversely affects the functional integrity of both the alveolar epithelium and the alveolar macrophage (Brown et al., 2001; Guidot et al., 2000; Joshi et al., 2005), and increases the risk for acute lung injury (Holguin et al., 1998; Velasquez et al., 2002). Various studies have reported specific mechanisms by which alcohol ingestion down-regulates innate immune defenses in the airways. These include depletion of the antioxidant glutathione (Holguin et al., 1998), an increase in reactive oxygen species formation (Guidot and Hart, 2005; Polikandriotis et al., 2006), and increased expression and activation of TGFβ1(Bechara et al., 2004). Our laboratory has previously reported that six weeks of alcohol ingestion in a rat model lowers the expression of zinc transporters in the small intestine and the alveolar epithelium and macrophages (Joshi et al., 2009). This results in a decrease in zinc bioavailability within the alveolar space and makes rats susceptible to respiratory infections (Mehta et al., 2011). In the present study, we focused on an upstream mediator, specifically KLF_4, which may be responsible for alcohol's effects on the expression of zinc transporters in alveolar macrophages and epithelial cells.

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There are 17 mammalian KLFs and they all bind to consensus GC-rich or CACCC sequences in the gene promoters through their zinc fingers and act as either transcriptional repressors and/or activators (Bieker, 1996; Feinberg et al., 2004). Post-translational modifications such as acetylation and phosphorylation also regulate the function of KLFs (Hu and Wan, 2011). KLF_4 was originally identified in the epithelium of the gut and the skin (Katz et al., 2002; Yang et al., 2005). Like other KLFs, it has diverse regulatory functions in many biological processes including cell growth, proliferation, differentiation, apoptosis, embryogenesis, and reprogramming of the stem cells (Katz et al., 2002). Previous research by Liuzzi et al. has shown that KLF_4 regulates ZIP_4 in mouse small intestine (Liuzzi et al., 2009). These investigators used a mouse model with zinc adequate or zinc deficient diet and showed a direct link between KLF_4 and ZIP_4 expression using polymerase chain reaction, western blot analysis, immunochemistry, and siRNA technique (Liuzzi et al., 2009). However, no correlation was found between KLF_4 and ZIP_4 in the lung of these animals. We have previously shown that chronic alcohol ingestion has a unique down-regulating effect on the expression of ZIP_4. In the present study, we have shown that treatment with alcohol *in vivo* down regulates KLF_4 expression in the alveolar macrophages and epithelial cells. This alcohol-induced decrease in KLF_4 expression is interesting as KLF_4 plays an important role in cell proliferation and apoptosis, and we had previously determined that chronic alcohol ingestion renders the alveolar epithelium susceptible to apoptosis (Brown et al., 2001; Holguin et al., 1998) Therefore, one consequence of alcohol-mediated inhibition of KLF_4 expression could be the loss of alveolar epithelial cell and macrophage viability during inflammatory stresses. In addition, treating macrophages with KLF_4 siRNA directly affects ZIP_4 expression suggesting a link between these two in the alcoholic lung.

Expression of KLF_4 is regulated by inflammatory cytokines and oxidative stress. For example, KLF_4 expression was up-regulated in the lungs of mice after endotoxin challenge (Liu et al., 2008), and KLF_4 in macrophages was induced by LPS, IFN-gamma, and TNFα (Feinberg et al., 2005). In contrast, TGFβ1 inhibited KLF_4 expression in macrophages (Feinberg et al., 2005), and recently Hu and Wan reported (Hu and Wan, 2011) that KLF_4 is profoundly degraded in response to TGFβ signaling. Thus, KLF_4 responds to cytokines and growth factors present in the microenvironment and regulate cell signaling pathways through distinct mechanisms. We have previously published that chronic alcohol ingestion decreases GM-CSF receptors, and transcription factor PU.1 in the alveolar macrophages and epithelial cells (Joshi et al., 2005; 2006), and impairs phagocytic function of macrophages and disrupts epithelial barrier function. Recombinant GM-CSF treatment restores the signaling and normalizes alveolar macrophage phagocytosis (Joshi et al., 2005) and epithelial barrier function (Pelaez et al., 2004). These beneficial effects of GM-CSF in the lower airways can be compromised by the presence of immunosuppressive TGFβ1. As previously shown, the alcoholic lung has increased levels of TGFβ1 (Bechara et al., 2004), and TGFβ1 is deleterious to the epithelial barrier (Bechara et al., 2004). In addition, TGFβ1 has direct effect on the GM-CSF receptor expression on the alveolar macrophage (Joshi, 2009). Together these studies show that in the lower airways, GM-CSF and TGFβ1 have antagonistic effects on the function of alveolar macrophages and epithelial cells. In the present study, we have shown that GM-CSF and TGFβ1 have opposite effects on the expression of KLF_4 and other downstream effectors such as ZIP_4. Further, the transepithelial resistance of the epithelial cells was significantly affected by GM-CSF and TGFβ1in an opposite manner (Figure 7). Together, through these antagonistic effects, TGFβ1 and GM-CSF can regulate the function of alveolar macrophages and epithelial cells in KLF_4-dependent manner. Interestingly, KLF_4 is a downstream target of the GM-CSF and master transcription factor PU.1 (Alder et al., 2008). As per our knowledge, this is the first report of KLF_4 expression in the rat alveolar macrophages and epithelial cells and its modulation by alcohol, GM-CSF, and TGFβ1.

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In summary, we report that chronic alcohol ingestion, most likely through increased TGFβ1 expression, affects the expression and DNA binding of the transcription factor KLF_4 in alveolar macrophages and epithelial cells (Figure 8). The findings in this study provide evidence for a novel mechanism by which alcohol adversely impacts zinc homeostasis in the lung. Further, it appears that the growth factors GM-CSF and TGFβ1, whose balance within the alveolar space is disrupted by chronic alcohol ingestion, regulate KLF_4 expression in an antagonistic manner in both alveolar macrophages and epithelial cells. Taken together, these new findings extend our previous observations about alcohol's effects on the pulmonary host defenses and provide new insights into the potential mechanisms by which chronic alcohol abuse renders patients susceptible to opportunistic respiratory infections.

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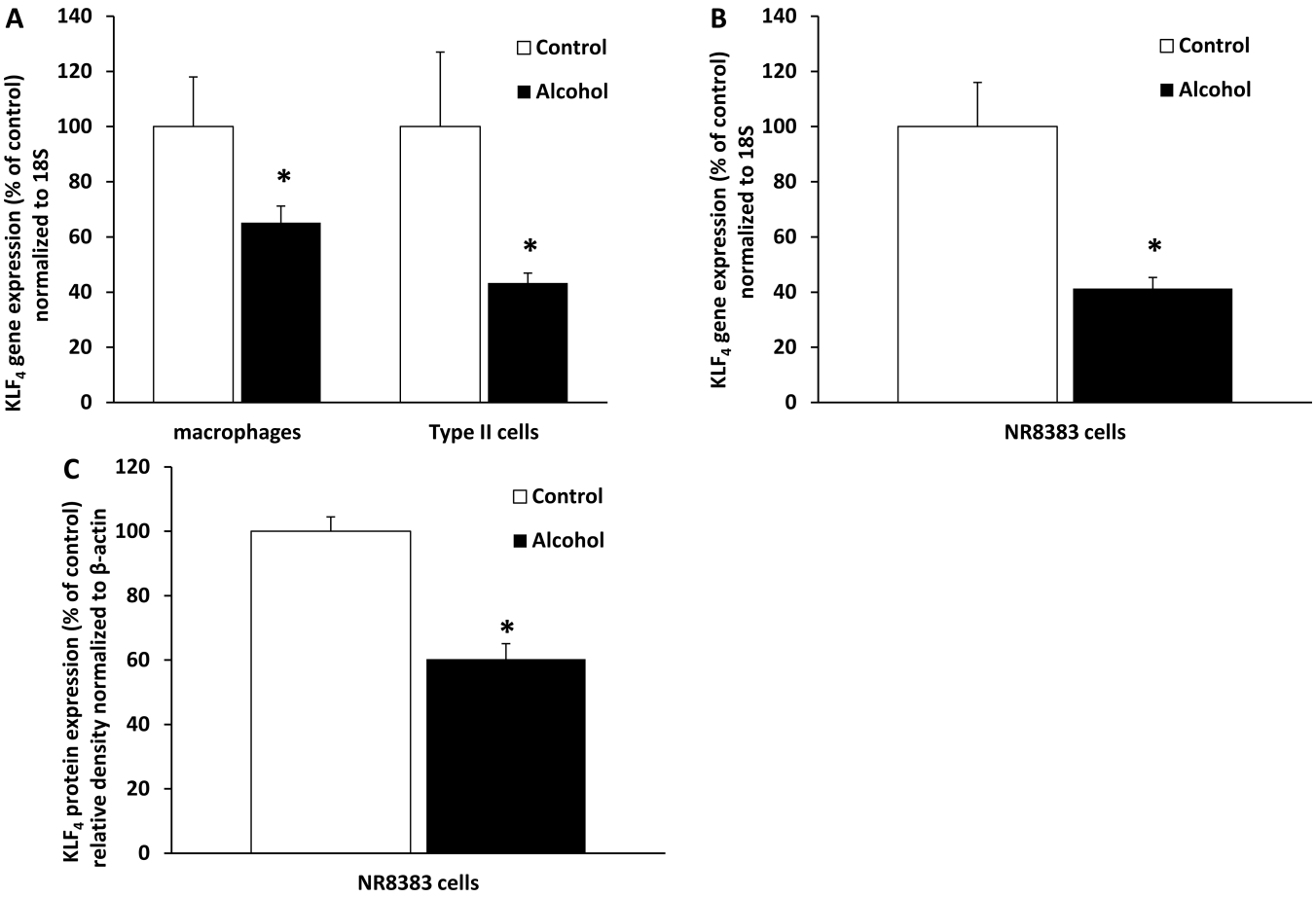

Figure 1. *In vivo* and *in vitro* effects of alcohol on KLF_4 expression in the alveolar macrophages and epithelial cells

(A) Gene expression of KLF_4 in rat alveolar macrophages and type II epithelial cells from Sprague-Dawley rats fed the Lieber-DeCarli liquid diet containing either alcohol or an isocaloric substitution with maltin-dextrin (control diet) for 6 weeks was determined by real time PCR. N=3; * $p<0.05$ compared with control. Data are presented as gene expression relative to control and normalized to 18S. (B&C) NR8383 cells were treated with or without 0.2% alcohol for 4 weeks and used for gene and protein analysis. Gene expression of KLF_4 was normalized to18S and presented as % of control. N=6; * $p<0.05$ compared with control. The relative protein expression as determined by Western blot analysis of KLF_4 in NR8383 cells treated with and without 0.2% alcohol for 4 weeks. The relative amount of protein was quantified by determining the densitometry of the bands on the blots, and then normalized to the densitometry of the β-actin band from the same sample. N=3; * $p<0.05$ compared with control (no alcohol) group. Each value (A–C) represents the mean ± SEM.

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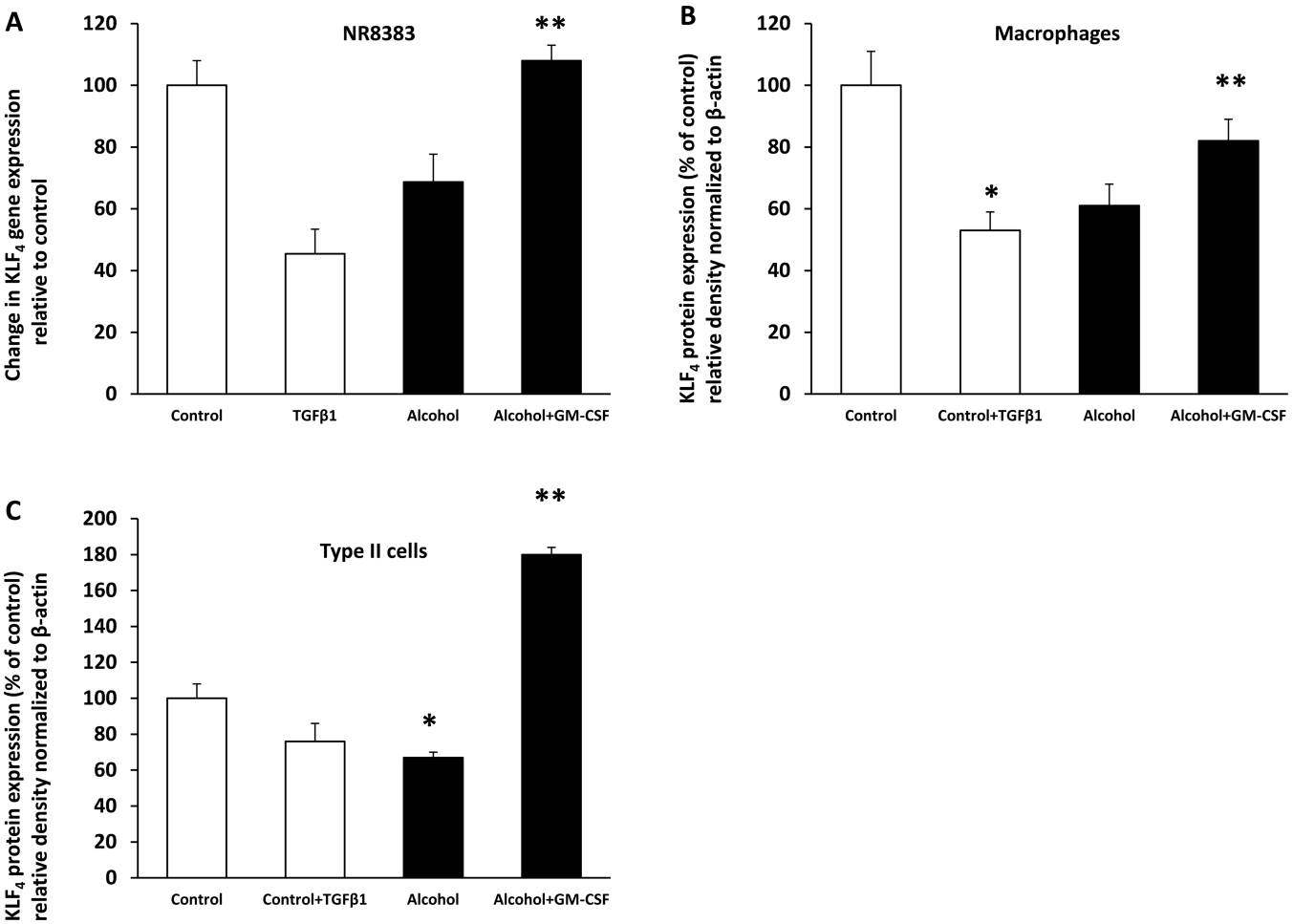

Figure 2. Effects of TGFβ1 and GM-CSF on KLF_4 expression in the alveolar macrophages and epithelial cells

(A) Gene expression of NR8383 without treatment (control) or treatment with 10ng of TGFβ1, 0.2% alcohol, or 0.2% alcohol + 10ng of GM-CSF for 72 hours. N=6 for control and alcohol; N=12 for TGFβ1 and alcohol + GM-CSF. * $p<0.05$ compared with control group. (B&C) The relative amount of nuclear protein expression of KLF_4 as determined by Western blot analysis in alveolar macrophage (B) or Type II epithelial cells (C) from rats fed alcohol or control diet (see methods for details). Cells were treated *in vitro* with either 10ng of TGFβ1 (control group) or GM-CSF (alcohol group) for 48 hours. The relative amount of protein was quantified by determining the densitometry of the bands on the blots, and then normalized to the densitometry of the β-actin band from the same sample. N=3–6, * $p<0.05$ compared with control group, ** $p<0.05$ compared with alcohol group Each value (A–C) represents the mean ± SEM.

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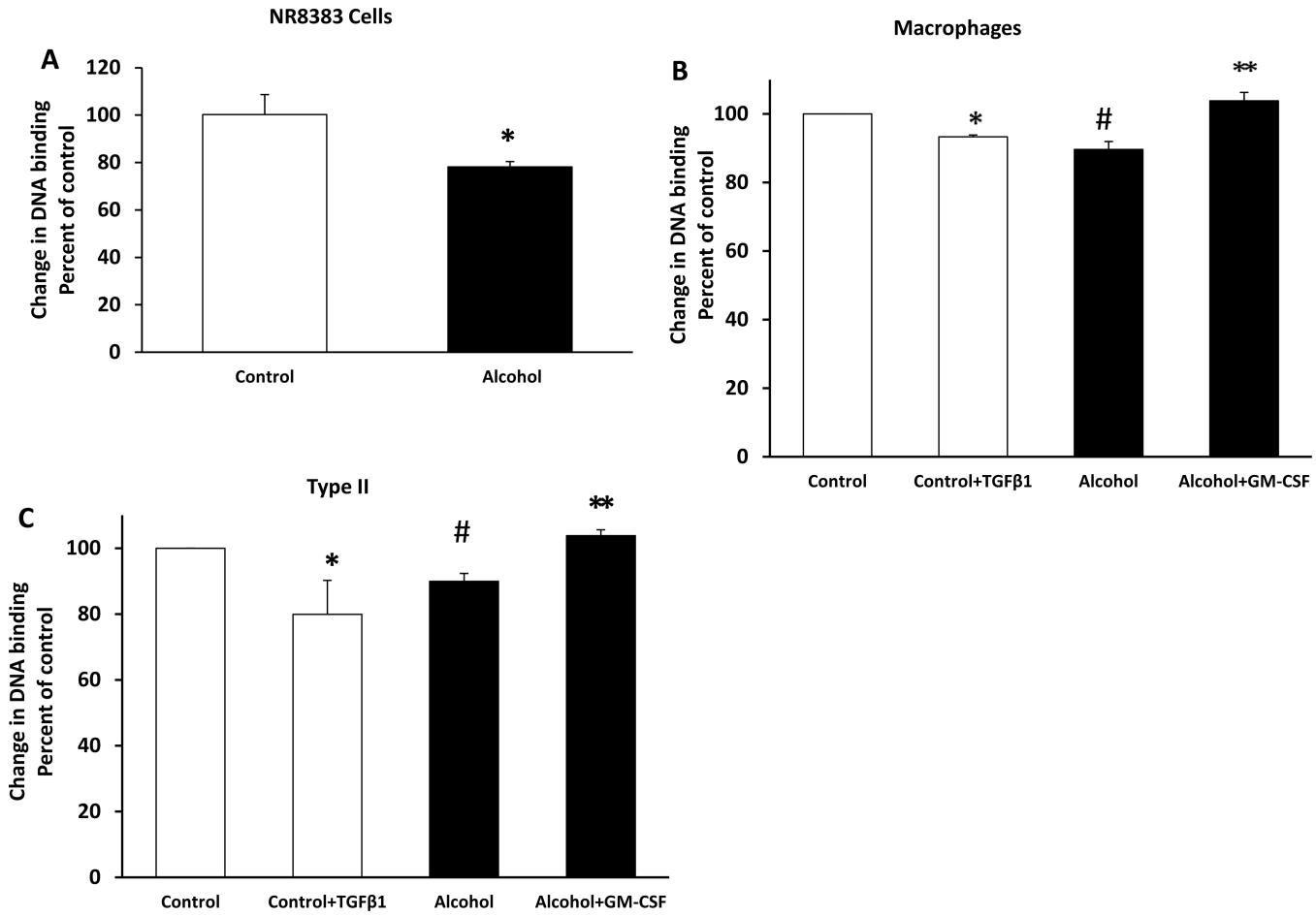

Figure 3. Effect of alcohol, GM-CSF, and TGFβ1on KLF_4 DNA binding

NR8383 cells treated with or without 0.2 % alcohol for 4 weeks (A). Primary alveolar macrophages and epithelial cells were isolated from rats fed diet with or without alcohol for 6 weeks and then treated with either 10ng of TGFβ1 (control group) or GM-CSF (alcohol group) for 48 hours (B&C). The nuclear extracts were prepared from alveolar macrophages and type II epithelial cells using NucBuster protein extraction kit (Novagen). The binding of KLF_4 to the specific DNA sequences was identified by an ELISA-based colorimetric assay as described in details in the Methods. N=3 for NR8383 cells (A), * $p<0.05$ compared with control group. N=3–5 for alveolar macrophages (B) and N=6–9 for alveolar type II cells (C); * $p<0.05$ compared with control group, $^{\#}$ $p<0.05$ compared with control group, ** $p<0.05$ compared with alcohol group. Each value (A–C) represents the mean ± SEM.

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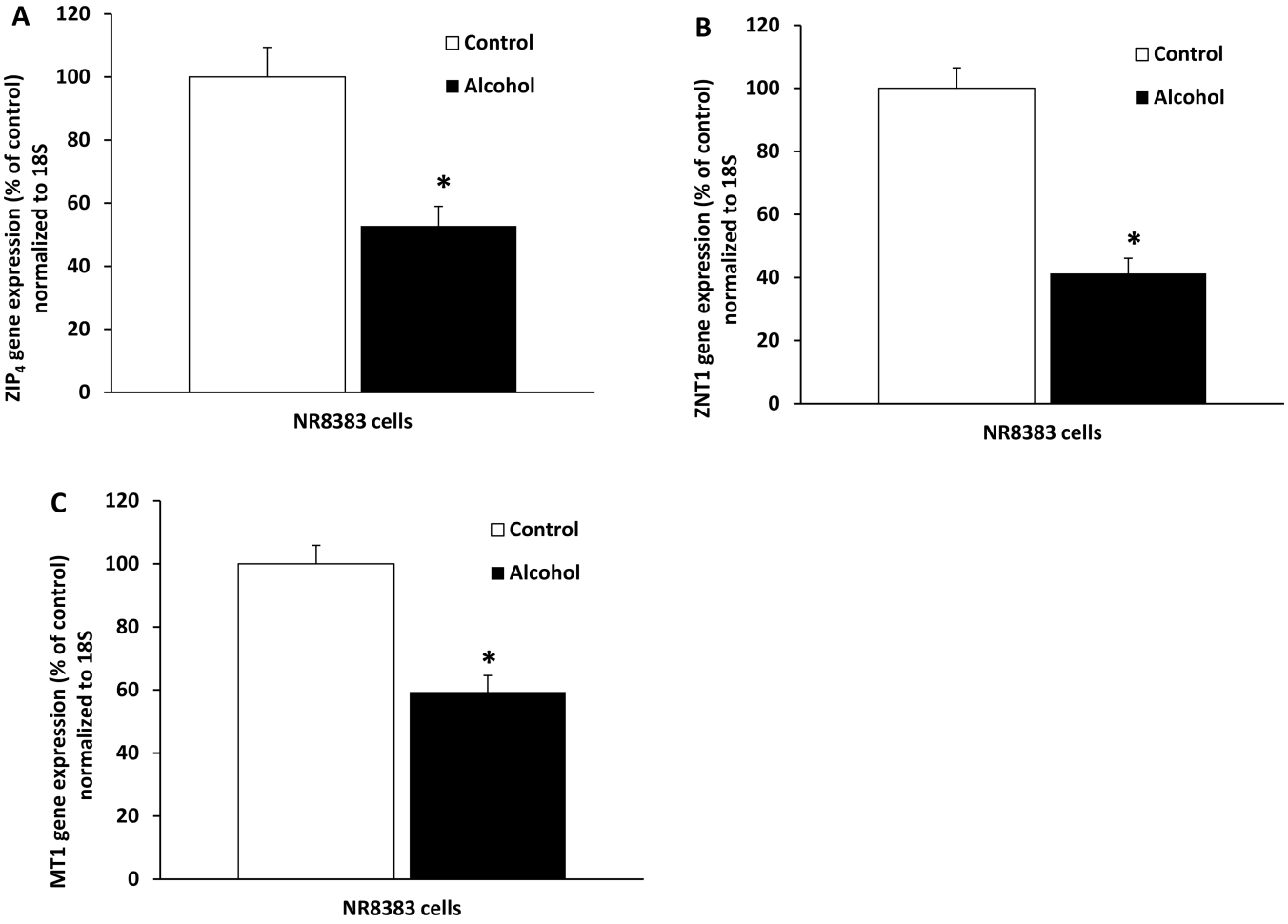

Figure 4. Changes in gene expression of ZIP_4, ZNT1, and MT1 in NR8383 cells in response to alcohol treatment

NR8383 cells were treated with or without 0.2% alcohol for 4 weeks. Gene expression of (A) ZIP_4, (B) ZNT1, and (C) MT1 was determined by real time PCR. Data are presented as gene expression relative to control and normalized to 18S. N=6, * $p<0.05$. Each value (A–C) represents the mean ± SEM.

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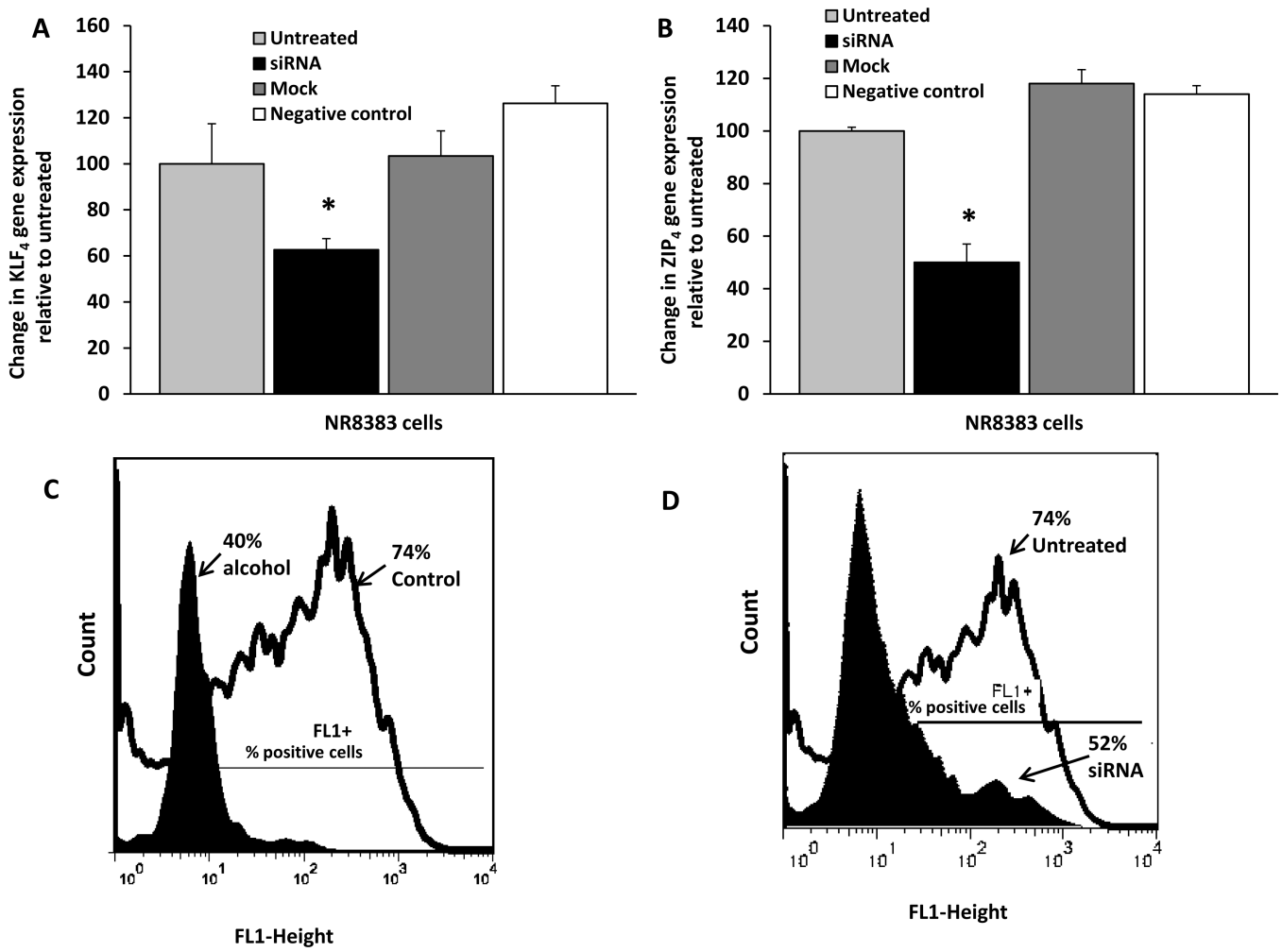

Figure 5. The effect of silencing KLF_4 on alveolar macrophage gene expression and intracellular zinc

(A) Gene expression of KLF_4 in cells untreated or treated with either siRNA for KLF_4, transfection reagent only (mock), or a negative control. Data are presented as gene expression relative to control and normalized to 18S. N=9,* $p<.05$. (B) Gene expression of ZIP_4 in the same samples. N=9,* $p<.05$. (C&D) Flow cytometric analysis of FluoZin-3 AM (zinc specific dye) stained NR8383 cells. Briefly, cells were either treated with or without 60 mM alcohol (C) or 5nM siRNA for KLF_4 (D) for 72h and then stained with FluoZin-3 AM. Representative histograms for percentage of positive cells for FluoZin-3 AM are shown: (C) not filled: control or without alcohol, filled: 60mM alcohol treatment and (D) not filled: untreated or without siRNA for KLF_4, filled: KLF_4 siRNA treatment. In panels C and D, a leftward shift in the peak means a decrease in the percentage of cells that stain positively for intracellular zinc.

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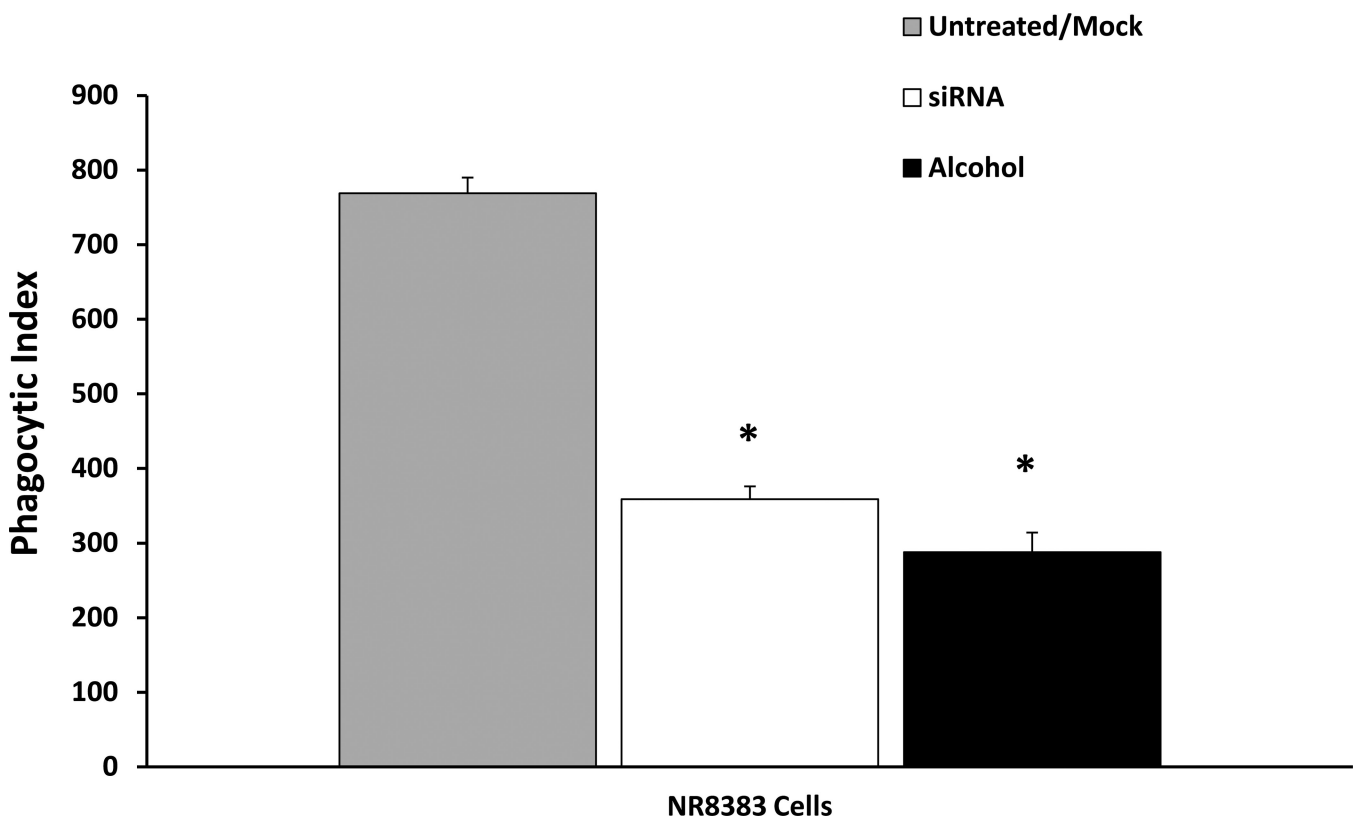

Figure 6. The effect of silencing KLF_4 on alveolar macrophage phagocytosis

Phagocytic index of NR8383 cells before and after the treatment with siRNA for KLF_4 or alcohol *in vitro*. Macrophages were incubated with FITC-labeled inactivated *S. aureus* and the percentage of macrophages that ingested fluorescent bacteria was measured by flow cytometry. N=3; * $p<0.05$ compared with untreated group. Each value represents the mean ± SEM.

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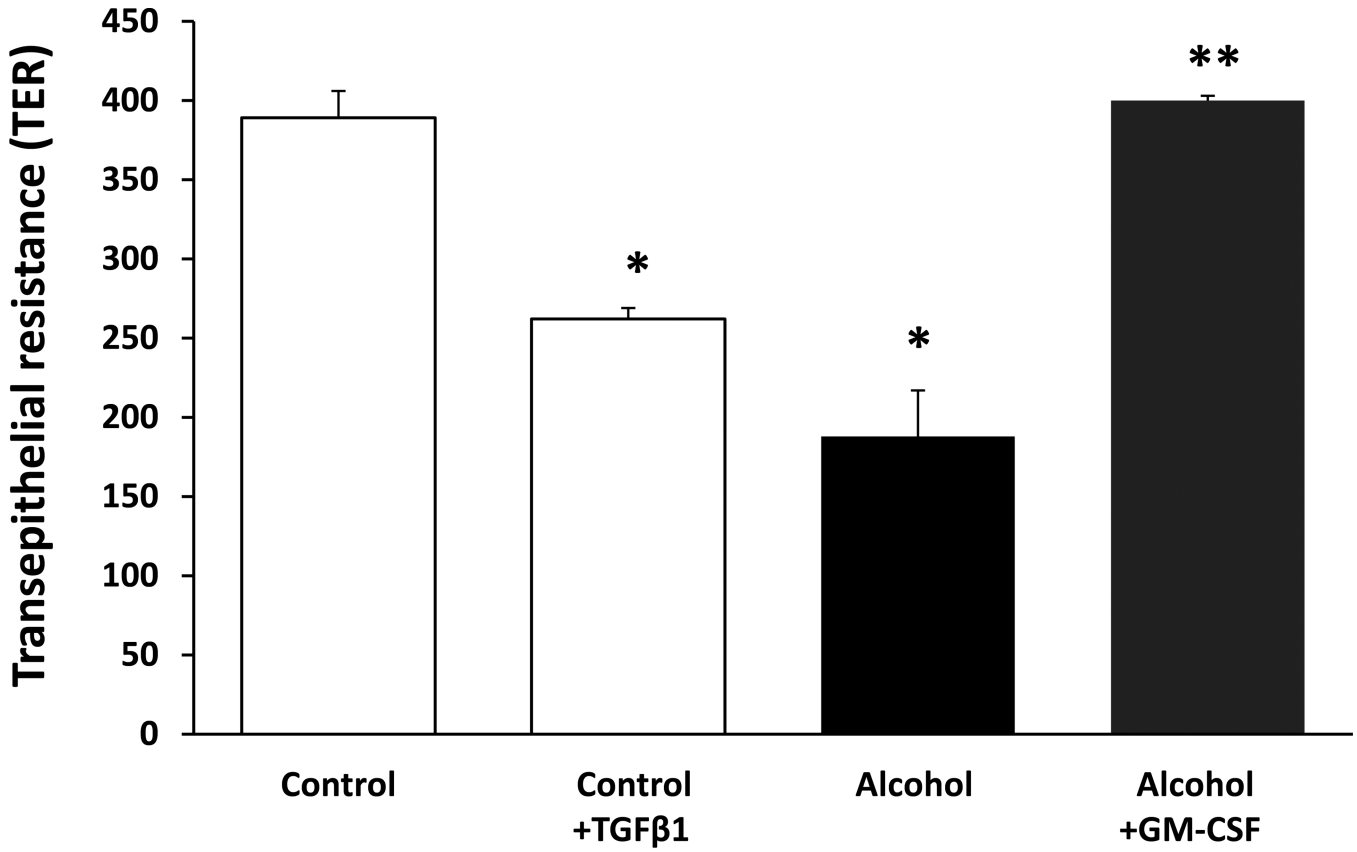

Figure 7. Effect of GM-CSF and TGFβ1on transepithelial resistance of type II epithelial monolayers from control- and alcohol-fed rats

Type II alveolar epithelial cells were isolated from control- and alcohol-fed rats and cultured in transwell plates to establish monolayers. Monolayers established from control- and alcohol-fed rats were treated with TGFβ1 or GM-CSF, respectively. The transepithelial electrical resistance was measured as described in Methods. Each value represents the mean ± SEM of 4 determinations. *p<0.05 compared to control, **p<0.05 compared to alcohol.

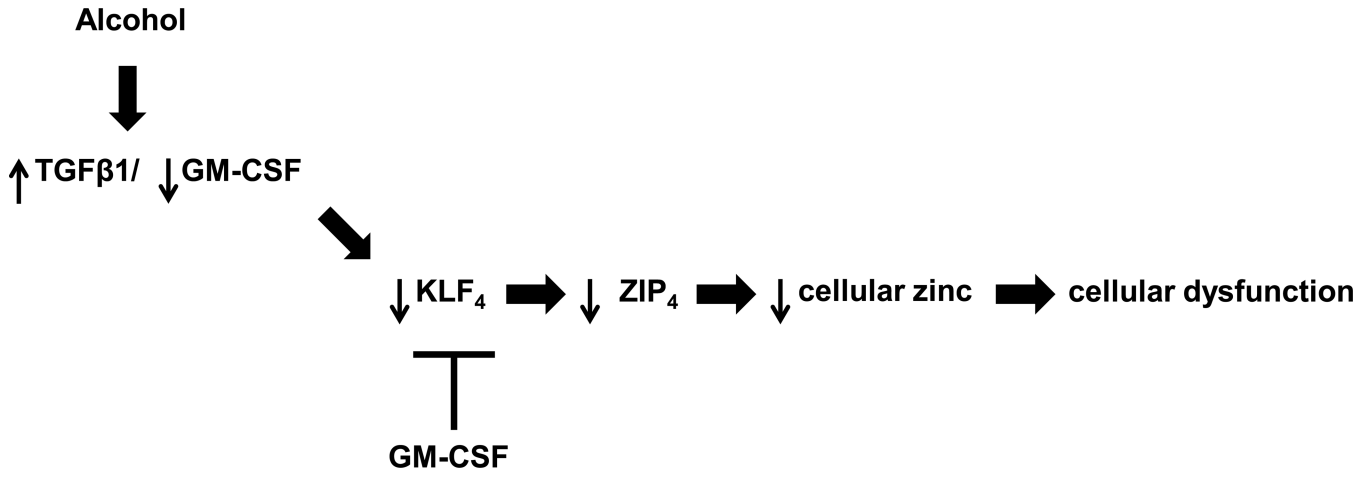

Figure 8. Schematic representation of proposed hypothesis

Alcohol ingestion increases TGFβ1 (as previously established in this animal model) and causes a decrease in KLF_4. This leads to a decrease in zinc importer ZIP_4. The resultant decrease in cellular zinc causes dysfunction of the alveolar macrophage and epithelial cell function. The effects of alcohol can be abrogated by GM-CSF, which returns KLF_4 expression and DNA binding to the control levels in macrophages and epithelial cells.

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Table 1

PCR primer sequences for KLF_4, ZIP_4, ZNT1, and MT1used for gene detection in these studies.

Primer	Forward	Reverse
$KLF_{4\ rat}$	5’- CTGAACAGCGGGACTGTCA	5’- GTGTGGGTGGCTGTTCTTTT
$ZIP_{4\ mouse}$	5’-CTTGGCTCTAGGCAAACCTG	5’-AGTGTGGCCAGGTAATCGTC
$ZNT1_{\ mouse}$	5’-GCTCTCGAGTTGGTCCTGTC	5’-GCCTCATGGTGAGGTAGGAA
$MT1_{\ rat}$	5’-GAACTGCAAATGCACCTCCT	5’- ACTTGTCCGAGGCACCTTT