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TITLE: Cadmium-mediated toxicity of lung epithelia is enhanced through NF-κB-mediated transcriptional activation of the human zinc transporter ZIP8

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Abstract

Cadmium (Cd), a toxic heavy metal and carcinogen that is abundantly present in cigarette smoke, is a cause of smoking-induced lung disease. SLC39A8 (a.k.a. ZIP8), a zinc transporter, is a major portal for Cd uptake into cells. We have recently identified that ZIP8 expression is under the transcriptional control of the NF-κB pathway. Based on this, we hypothesized that cigarette-smoke induced inflammation would increase ZIP8 expression in lung epithelia thereby enhancing Cd uptake and cell toxicity. Herein we report that ZIP8 is a central mediator of Cd-mediated toxicity. TNFα treatment of primary human lung epithelia and A549 cells induced ZIP8 expression resulting in significantly higher cell death due to both apoptosis and necrosis following Cd exposure. Inhibition of the NF-κB pathway and ZIP8 expression significantly reduced cell toxicity. Zinc (Zn), a known cytoprotectant, prevented Cd-mediated cell toxicity via ZIP8 uptake. Consistent with cell culture findings, a significant increase in ZIP8 mRNA and protein expression was observed in the lung of chronic smokers compared to non-smokers. From these studies, we conclude that ZIP8 expression is induced in lung epithelia in an NF-κB-dependent manner thereby resulting in increased cell death in the presence of Cd. From this we contend that ZIP8 plays a critical role at the interface between micronutrient (Zn) metabolism and toxic metal exposure (Cd) in the lung microenvironment following cigarette smoke exposure. Further, dietary Zn intake, or a lack thereof, may be a contributing factor in smoking-induced lung disease.

Keywords: cigarette smoke, COPD, zinc transporters, inflammation

Introduction

Chronic obstructive pulmonary disease (COPD) is a major cause of morbidity and mortality in the U.S. and worldwide, affecting 4 to 6 percent of all people over the age of 45 years. The prevalence of COPD is expected to rise and the World Health Organization (WHO) predicts that it will be the third leading cause of death by 2020[35,39]. COPD is characterized by progressive airflow obstruction, which is not fully reversible, resulting from an abnormal inflammatory response in the lungs to noxious gases and particles[11]. Cigarette smoking is the single most important risk factor for developing COPD in men and women in the United States[23]. Individuals that develop COPD also have a much higher likelihood of developing lung cancer[36,53]. Cadmium (Cd), a major component of cigarette smoke, contributes to both disorders. Each cigarette contains approximately two micrograms of Cd, of which, ten percent is directly transferred to lung tissue following inhalation of primary cigarette smoke[37]. Of the Cd present in primary inhaled cigarette smoke, nearly fifty percent is absorbed from the lungs into the systemic circulation during active smoking[24,47]. Cd, relative to other components in cigarette smoke, is unique with a biologic half-life in humans of 15 to 20 years[25,52]. Therefore, it is not surprising that smokers typically carry Cd blood and whole body burdens more than double those of nonsmokers. Importantly, it remains unclear how Cd enters the lung thereby causing pathological manifestations that lead to COPD and lung cancer.

Analysis of over 6,700 subjects derived from the third National Health and Nutrition Examination Survey (NHANES III) revealed that adult smokers with low zinc (Zn) intakes were significantly more likely to have a higher Cd burden and increased risk of

developing COPD[31]. Based on these findings, the investigators postulated that a dynamic interplay exists between Zn and Cd; however, the mechanism(s) by which this occurs “warrant further study”. Importantly, malnutrition is common in COPD patients[45] and small case controlled studies have shown an association between Zn deficiency and COPD[22,27] although this has never been thoroughly investigated. Relative to Zn metabolism and COPD pathogenesis, we propose a novel role for the mammalian Zn transporter, ZIP8, as a key mediator of Cd-mediated toxicity in lung epithelia, target cells in COPD pathogenesis. Recently ZIP8 was identified as a primary transporter of Cd, in addition to Zn[12,20,32]. Our group reported that ZIP8 is unique, relative to all other ZIP family members, in that ZIP8 expression in lung epithelia is induced by pro-inflammatory cyokines[6] via transcriptional up-regulation by NF-κB, a signal transduction pathway that is persistently activated in the lung of chronic smokers[49]. Taken together, we contend that chronic cigarette exposure, which creates an inflammatory microenvironment in the lung[35,39,45], up-regulates ZIP8 expression in lung epithelia thereby increasing the extent of Cd uptake, tissue burden, and lung-related pathology. Based upon the work of Lin[31], we predict that Zn, a known cytoprotectant[2], will also influence ZIP8-mediated Cd uptake such that nutritional Zn deficiency will augment lung injury whereas Zn sufficiency will minimize injury by preventing Cd uptake into lung tissue through ZIP8.

In the present study, using primary human lung epithelia and a related cell line, we determined to what extent Zn metabolism impacts Cd-induced toxicity. Accordingly, we sought to determine whether TNFα, a cytokine present in the lung of chronic smokers, induced changes in ZIP8 expression in lung epithelia and whether changes in

transporter expression was necessary to mediate Cd-induced toxicity. Knowing that Zn and Cd compete for cellular uptake through ZIP8, we further modified Zn concentrations to simulate both Zn deficient and sufficient states and observed that the relative abundance of this key micronutrient is vital in maintaining cell viability. Cell culture findings were validated following inspection of lung specimens obtained from chronic smokers and nonsmokers. Consistent with emerging clinical evidence obtained from the NHANES III Trial, our findings indicate that Zn and a factor central to its metabolism impact the extent by which Cd enters the lung subsequently causing cell death. We believe that these basic observations begin to fill a critical gap in our understanding of how Cd contributes to COPD pathogenesis.

Materials and Methods

Cell Culture

The human lung epithelial A549 cell line (catalog no. CCL-185, American Type Culture Collection) was maintained under standard culture conditions in DMEM supplemented with 10% FBS, 0.1 mg/ml streptomycin, 100 IU/ml penicillin and 1% non-essential amino acids at 37°C in a 5% CO_2-humidified incubator. All cells were used between passages 6 and 20. Cells were maintained in serum-free conditions 24 hours before and throughout the duration of experiments to minimize absorptive cadmium loss due to serum protein binding. Primary, differentiated, polarized human upper airway epithelial cells (HUAECs) were isolated and cultured as previously reported[2,28]. Results in this investigation are derived from three different donors. HUAECs were maintained in 1:1 DMEM and Ham's F-12 media (DMEM/F-12) supplemented with 2% Ultroser G (BioSepra, Villeneuve, France) and antibiotics unless otherwise stated. Human lungs were collected with approval from The Ohio State University Institutional Review Board.

Cadmium Exposure and Analysis of Cytotoxicity

A549 cells were seeded and then serum starved for 24 hours followed by overnight treatment with 100 ng/ml TNFα or vehicle control. Cultures were then exposed to increasing concentrations of $CdCl_2$ for 24 hours. All experiments, unless otherwise stated, were performed in triplicate. Culture supernatants were then collected and lactate dehydrogenase (LDH) activity was measured using the Cytotoxicity Detection Kit (Roche Diagnostics, Mannheim, Germany). Samples were compared to a positive control treatment group, generated for each experiment by treatment with 2% Triton-X (Sigma Chemical Company, St. Louis, MO) in DMEM for 10 minutes to yield 100% cell

death. The same protocol was conducted in primary cultures however, TNFα was administered to the basolateral surface of polarized cultures whereas cadmium was administered either basolaterally or apically. In addition to LDH release, the integrity of primary cultures was monitored by measuring transepithelial electrical resistance (TEER) using a portable ohmmeter (Millicell-ERS, Millipore). For TEER measurements, 200 µl of media was placed on the apical surface and then immediately removed following recording. Baseline TEER measurements were determined at the beginning of each experiment and then following cadmium exposure. In our model, fully differentiated cultures that maintain an air-to-liquid interface typically exhibit TEER measurements > 400 Ω.

Inhibition of NF-κB and ZIP8 Expression

A549 cells were initially seeded and serum starved for 24 hours followed by treatment with 20 µM Bay 11-7082, a pharmacologic inhibitor of the NF-κB pathway that irreversibly binds to the phosphorylation site of IκB-α, or DMSO as a vehicle control. Following a one hour exposure, fresh medium was then replaced containing Bay 11-7082 or DMSO and TNFα and then cells were incubated for an additional 24 hours. Cultures were then exposed to increasing concentrations of $CdCl_2$ (0 to 25 µM) for an additional 24 hours after which cytotoxicity was determined by measuring LDH release.

ZIP8 expression was inhibited using a 21-mer short interfering (si)RNA target sequence (QIAGEN, Valencia, California) following transfection into A549 cells using HiPerFect transfection reagent (QIAGEN). ZIP8 expression was typically decreased by > 70% following TNFα stimulation. ZIP8 siRNA treated cultures were compared to a

nonsilencing control siRNA that did not affect ZIP8 expression. Following siRNA treatment, cultures were exposed to TNFα and then increasing concentrations of $CdCl_2$ (0 to 25 μM) for an additional 24 hours after which cytotoxicity was determined by measuring LDH release.

Zinc co-culture study

Cells were seeded and serum starved for 24 hours, then stimulated with TNFα or vehicle control for 24 hours. Cultures were then exposed to a fixed concentration of $CdCl_2$ (10 μM) with or without increasing concentrations of $ZnCl_2$ (0, 10, 20, and 40 μM) and then incubated for an additional 24 hours. Cytotoxicity was again determined by measuring LDH release.

Western Analysis

Membrane protein fractions were generated by suspending cells in a lysis buffer composed of Buffer A (20 mM Tris/HCl pH 7.5, 5 mM $MgCl_2$, 1 mM ethylene glycol tetraacetic acid, 20 mM β-glycerophosphate, 1 mM phenylmethanesulfonylfluoride, 2 μg/ml aprotinin, 2 μg/ml leupeptin, 1 mM sodium vanadate) followed by sonication for 5 seconds four times while on ice. The lysate was then centrifuged at 2900 rpm for 5 minutes. The supernatant was then centrifuged at 55,000 rpm for 30 minutes with a TLA 120.2 rotor using the Optima TLX-120 Ultracentrifuge (Beckman Coulter, Brea, California). The pellet was resuspended in Buffer A and centrifuged for 5 minutes at 2900 rpm. The remaining pellet was resuspended in Buffer A containing 1% NP-40 and agitated for 1 hour at 4°C followed by centrifugation at 55,000 rpm for 30 minutes giving

yield to the membrane extract in the resulting supernatant. The whole cell and membrane lysates were quantified using a protein assay (Bio-Rad, Hercules, CA) and then mixed in Laemmli buffer (Bio-Rad) containing 5% (vol/vol) 2-mercaptoethanol, boiled for 5 minutes, separated on 10% SDS-PAGE gel (Bio-Rad), and then transferred to a nitrocellulose membrane (Amersham Biosciences, Little Chalfont, UK). Membranes were blocked with 5% milk (w/v) in PBS 0.1% Tween 20 (PBS-T) for 1 hour at room temperature and then incubated with primary antibody overnight at 4°C. After washing, the membranes were incubated with secondary antibody for 1 hour at room temperature. The signal was detected with an ECL Kit (Amersham Biosciences) and a Fluor-S Multi-Imager Max/Bio-quantity one (Bio-Rad). The following antibodies were used in our experiments: rabbit anti-ZIP8 (1:2000, Covance, Princeton, NJ), mouse anti-β-actin (1:2000, MP Biomedicals, Aurora, OH), goat anti-rabbit IgG-HRP (1:3000, Cell Signaling), and horse anti-mouse IgG-HRP (1:3000, Cell Signaling).

Intracellular cadmium measurements

A549 cells were subjected to a 24 hour TNFα stimulation with or without ZIP8 siRNA, and then treated with increasing concentrations of Cd as previously described. Cell supernatants were then collected and centrifuged to further collect detached cells. The Measure-iT™ Lead and Cadmium Assay Kit (Invitrogen, Carlsbad, CA) was used to measure intracellular cadmium concentration. DMSO was added directly to each well and then cells were scraped and combined with pelleted cells in the supernatant, followed by vortexing to lyse the cells. Then 10 µl of sample was added to a 96 well plate followed by 200 µl of the Measure-iT™ kit reagent. The fluorescence intensity

was recorded for each well at 520 nm (λ_{ex}: 490). Samples were analyzed in triplicate and three readings were performed for each sample. A cadmium calibration curve with a range between 5 to 200 nM $CdCl_2$ was used per manufacturer guidelines to determine intracellular Cd concentration within each sample. Cd content of samples was standardized to protein content as measured in the lysates using the Pierce BCA Protein Assay Kit (Thermo Scientific, Rockford, Illinois).

Analysis of Apoptosis and Necrosis

A549 cells were detached with trypsin and pooled with cells already suspended during culture, and then fixed using 100% methanol and stained with an M30 CytoDEATH, Fluorescein conjugated antibody (Boehringer Mannheim, Indianapolis, IN), a monoclonal antibody that specifically detects caspase-cleaved human cytokeratin-18 (CK-18). Concomitant nuclear staining was also conducted using 0.5 mg/ml 4'6-diamidino-2-phenylindole dihydrochloride (DAPI; Roche Molecular Biochemicals, Indianapolis, IN). Upon staining, cells were collected and cytospun onto slides and analyzed by fluorescent microscopy. Apoptotic cells (M30-positive cells with fragmented nuclei) were enumerated by a blinded observer who randomly selected 14 fields of view per treatment condition. Data are presented as the average percentage of apoptotic cells divided by the total number of cells per viewing area. Lung epithelial cultures were also evaluated by flow cytometric analysis to evaluate cell death. Briefly, A549 cells were detached with trypsin and combined with cells already suspended during culture and then pelleted. The pellet was resuspended and washed with PBS and again pelleted. Pellets were then resuspended in Annexin V binding buffer with an

Annexin V antibody and then incubated in the dark for 15 minutes. An additional volume of Annexin V binding buffer was added and then samples were filtered through 0.2 μm filters into tubes suited for flow cytometry. Propidium iodide was added immediately before flow analysis.

Immunohistochemical Analysis of ZIP8 expression

The apical surface of HUAEC cultures grown on 24-well transwells were first washed with PBS to remove debris and then fixed with 4% formaldehyde. Monolayers were then blocked with 10% goat serum for two hours at room temperature in permeabilizing buffer and then incubated with primary rabbit anti-ZIP8 antibody overnight at 4°C. Following washing, membranes were incubated with secondary antibody (Alexa Fluor 488 goat anti-rabbit antibody, Invitrogen) for one hour. Nuclear DNA was detected with DAPI. Slides were mounted with Citifluor antifadent mounting medium (AF1, Electron Microscopy Science) and then examined using a disk scanning confocal microscope at 600x (Olympus BX61). The magnification of all images was performed using X10 (WHN10X) and X60 (Olympus 60X/1.42 Oil PlaneApon or 60X/0.90N LUMPLANF1) objectives. The *z*-section images were obtained and analyzed using Slidebook (Intelligent Imaging Innovations Inc., Denver, CO) software.

mRNA Analysis of Human Lung Tissue

Total RNA was isolated using Trizol reagent from human lung tissue samples that were received from the Lung Tissue Research Consortium of the National Heart Lung and Blood Institute that included 7 chronic smokers (COPD Stage 0) and 5 control, life-time

nonsmokers. The ThermoScript RT-PCR kit (Invitrogen) was used to generate cDNA. Primer pairs were designed for ZIP8 and GAPDH using Primer Express software version 2.0 (Applied Biosystems) as previously reported[8]. The 7900HT Fast Real-Time PCR system (Applied Biosystems) using SYBR Green Master Mix (2x, Applied Biosystems, Foster City, CA) was used to perform Real-Time quantitative PCR. All samples were standardized to average cycle threshold number of the GAPDH gene. Messenger expression was reported as the average relative copy number (RCN) as follows: $2^{-\Delta C}{}_t \times 100$, where ΔC_t is the C_t value standardized to GAPDH[18].

Immunodetection of ZIP8 in Human Lung Tissue

A portion of the same human specimens described above were also fixed and mounted on slides. After deparaffinization, endogenous peroxidases were blocked for 15 minutes with hydrogen peroxide, then blocked with 10% milk in TBS for 10 minutes, and then with 10% normal goat serum in TBS for 2 hours. Primary antibody (anti-hZIP8) was then applied in 5% BSA in TBS and incubated overnight at 4°C. Following washing three times with PBS-T, slides were incubated with goat anti-rabbit biotin conjugated antibody for 45 minutes at room temperature. Slides were washed with PBS-T four times and then incubated in avidin-biotin-horseradish peroxidase in PBS-T for 45 minutes. After being washed three times in PBS-T, slides were developed using diaminobencidine (DAB) diluted in 0.05M Tris-HCl and then rinsed in distilled water and counterstained with hematoxylin. After dehydration with alcohols and xyline, slides were mounted on a permanent coverslip and evaluated by light microscopy.

Statistical analysis

All data are expressed as mean ± standard deviation (SD). For comparisons involving multiple variables and observations, a two- and three-way ANOVA (GraphPad, La Jolla, California) were used. Having passed statistical significance by ANOVA, individual comparisons were made with the Bonferroni multiple-comparison test. Statistical significance was defined as a p value < 0.05.

Results

TNFα enhances Cd toxicity in lung epithelia

ZIP8 expression is typically low in lung epithelia but highly induced by pro-inflammatory mediators [3,6]. Based on this and knowing that ZIP8 is a transporter of Cd, we predicted that induction of ZIP8 expression by TNFα, a relevant pro-inflammatory factor present in the lung of smokers[11], would increase Cd toxicity in lung epithelia. To investigate the transporter's contribution to Cd-induced toxicity, A549 cells were first stimulated with TNFα for a time sufficient to increase ZIP8 expression and then exposed to increasing concentrations of Cd for 24 hours. A549 cells stimulated with TNFα prior to Cd challenge had a significant increase in cell death, as determined by LDH release, in comparison to cultures that were exposed only to Cd (Figure 1a). Western analysis confirmed a 6.5-fold induction of membrane-bound ZIP8 following TNFα stimulation (Figure 1b). These results indicate that lung epithelia become more vulnerable to Cd-mediated toxicity following activation by TNFα. There was no toxicity associated with just TNFα exposure in agreement with our previously reported findings[2].

NF-κB and ZIP8 inhibition decrease Cd-induced cell toxicity

We next determined whether Cd-induced toxicity in lung epithelia is dependent on the induction of ZIP8 expression. Our group recently reported that ZIP8 expression is transcriptionally activated by NF-κB (RelA/p65) (manuscript submitted, Liu MJ). Knowing this, we stimulated lung epithelia cultures with TNFα but in the presence of Bay 11-7082, a compound that prevents IκB-α phosphorylation thereby inactivating the canonical NF-κB pathway. Inhibition of the NF-κB pathway resulted in a significant

decrease in cell toxicity in cultures exposed to a combination of TNFα and Cd when compared to the DMSO vehicle control treatment group (Figure 2a). Knowing that Cd-mediated toxicity is NF-κB-dependent, we then pursued a similar study in conjunction with siRNA designed to suppress the induction of ZIP8 expression. A549 cells were first treated with ZIP8-specific siRNA or a corresponding scrambled siRNA control, then stimulated with TNFα and then once again subject to increasing concentrations of Cd. Cultures in which ZIP8 expression was inhibited exhibited a significant decrease in cell toxicity relative to cultures treated with the siRNA control (Figure 2b). Western blotting confirmed a ~70 % knockdown of ZIP8 in cultures treated with either Bay 11-7082 or siRNA (Figure 2c). Collectively, these results support our hypothesis that the induction of ZIP8 expression by a pro-inflammatory factor associated with chronic cigarette smoke exposure significantly increases lung epithelial vulnerability to Cd.

Zn decreases Cd-induced cell toxicity

ZIP8 was first identified as a Zn importer and then subsequently discovered to also be an avid transporter of Cd[5,12]. Knowing that Zn acts as a cytoprotectant in lung epithelia[6], we wanted to determine whether physiologically relevant concentrations of extracellular Zn can prevent Cd-mediated toxicity. A549 cells were again stimulated with TNFα and then exposed to a constant concentration of Cd but in the presence of increasing concentrations of Zn. Strikingly, lung epithelial cell toxicity induced by Cd was decreased in the presence of increasing concentrations of Zn which was most notable when the molar ratio between Cd and Zn was in favor of Zn (Figure 3c). Cell toxicity was completely inhibited with a 4-fold molar excess of zinc relative to Cd. Consistent

with these findings, we observed an increase in intracellular Cd concentrations in TNFα treated cells, and a decrease in intracellular concentrations when treated in conjunction with a ZIP8 siRNA (Figure 3a, 3b). Based on the minimum detection threshold of intracellular Cd measurements (5 nM) we were not able to evaluate the impact of Cd doses below 10 μM. Taken together these findings further demonstrate that ZIP8 is an important regulator of Cd uptake and that the vital micronutrient Zn plays an important role in preventing Cd-mediated lung epithelia toxicity, which we believe to be relevant when considering that a substantial number of COPD subjects are also malnourished.

Cd induces apoptosis and necrosis

Having established that Cd induces toxicity in lung epithelia in a ZIP8-dependent manner we also wanted to determine whether cell death was a consequence of necrosis, apoptosis, or both under these conditions. We first evaluated cells for the presence of caspase-cleaved cytokeratin-18 to identify apoptotic cells following combined TNFα and Cd exposure using the M30 apoptotic marker and DAPI. Cells were considered apoptotic only in the presence of diffuse M30 staining throughout the cytosol and in the presence of a condensed nucleus (Figure 4a). Combined TNFα and Cd exposure increased the frequency of apoptotic cells, achieving statistical significance at the highest Cd exposure (Figure 4b). Using flow cytometry in conjunction with annexin V (AV) and propidium iodide (PI) staining, we next determined the extent of both necrotic and apoptotic cell populations. Briefly, cells that did not stain positive for either dye were considered viable. AV-positive/ PI-negative cells were considered early apoptotic, AV-positive/PI-positive were identified as mixed late apoptotic/necrotic

and PI-positive/ AV-negative cells were considered to be necrotic. Consistent with previous findings, the combination of TNFα and Cd exposure resulted in an increase in both the necrotic and apoptotic populations (Figure 4c). Combined TNFα and Cd exposure increased the frequency of PI and AV positive cells, achieving statistical significance at both Cd concentrations (Figure 4d).

ZIP8 is preferentially expressed at the apical surface and mediates Cd-induced toxicity in primary human lung epithelia

Initial studies were conducted in fully differentiated and polarized human upper airway epithelial cells (HUAECs) monolayers to determine whether ZIP8 preferentially translocates to the apical or basolateral membranes following transcriptional activation by TNFα. Confocal analysis of TNFα stimulated HUAEC cultures established that ZIP8 protein preferentially but not completely localized to the apical membrane upon cell activation (Figure 5a) whereas, little if any ZIP8 was observed in nonstimulated cultures. Next, we determined whether polarized cultures were more sensitive to Cd following apical or basolateral exposure. Treatment conditions were similar to past studies; however, TNFα was administered basolaterally, as previously reported by our group[7], followed by Cd exposure at either the apical or basolateral surface. Consistent with past observations, basolateral and apical Cd exposure resulted in increased cell toxicity and more so in TNFα stimulated cells as measured by LDH release. Strikingly, Cd exposure at the apical surface resulted in significantly more cell damage when compared to basolateral exposure (Figure 5b). A similar significant reduction in transepithelial electrical resistance (TEER; a.k.a R_t) was also observed demonstrating that apical Cd

exposure in TNFα activated cultures resulted in the largest reduction in epithelial barrier function (Figure 5c). Taken together, our findings demonstrate that ZIP8 protein expression is rapidly induced in primary lung epithelia and preferentially localizes to the apical membrane, an anatomical location ideally suited for Cd uptake following cigarette exposure.

ZIP8 is increased in the lungs of chronic smokers

Based upon our findings obtained from human lung epithelial cell models, we next sought to evaluate the expression of ZIP8 in human lung tissue. Lung tissue samples were obtained from lifetime non-smokers (n = 5) and chronic smokers (n = 7) through the National Institute's of Health sponsored Lung Tissue Research Consortium. Quantitative analysis of ZIP8 mRNA levels revealed a consistent and significant increase in ZIP8 mRNA transcripts in the lungs of smokers when compared to non-smokers (Figure 6a). Consistent with RNA findings, immunohistochemical analysis of the same biopsy specimens revealed an increase in ZIP8 protein throughout parenchymal tissue that appeared to be most prominent in upper airway and alveolar epithelia (Figure 6b). The increase of both ZIP8 protein and mRNA transcripts in the lungs of smokers strongly supports our cell culture findings indicating that chronic cigarette smoke exposure increases ZIP8 expression thereby enhancing the capacity of lung tissue to obtain cadmium.

Discussion

Smoking is responsible for 90% of all COPD cases in the United States. Cigarette smoke itself contains over 2000 xenobiotic compounds that can damage lung tissue resulting in chronic bronchitis and emphysema. Specifically, chronic smoke inhalation creates an inflammatory environment within the lung through the elaboration of cytokines and chemokines by parenchymal cells and alveolar macrophages. Activation of resident and recruited cell populations result in a significant increase in TNFα production, as well as other inflammatory mediators, both within the lung and throughout the body[4,9,10,11,17]. Consistent with these observations, the lungs of smokers and COPD patients exhibit a persistent increase in NF-κB activity, a transcriptional pathway that is central to the innate immune system and activation of the inflammatory response[49,55]. Whether persistent inflammation in the lung directly impacts the uptake of xenobiotics present in tobacco smoke, particularly Cd, remains unknown. Importantly, recent analysis of the NHANES III study that involved 6,726 patients revealed that higher urine Cd and therefore body levels positively correlate with smoking-related lung dysfunction and even more so in subjects with insufficient Zn intakes[31]. Taken together, these findings suggest that a dynamic interplay between Cd and Zn may exist in the context of cigarette smoke-induced lung disease.

Zn is an essential micronutrient and it's metabolism in humans is primarily controlled by Zn transporters, a composite of 24 proteins that are further divided into two families based on their ability to transport zinc into or out of the cytosol. There are 14 SLC39A importers (a.k.a ZIPs) and 10 SLC30A exporters (a.k.a. ZnTs) of which all 24 are highly conserved between humans and mice[13,33]. Together, Zn transporters

438 function coordinately throughout the body to direct Zn biodistribution in response to
439 nutritional intake and body demand. ZIP8 is unique, relative to most other transporters,
440 in that its expression is under the transcriptional control of inflammatory mediators[6].
441 Specifically, our group was the first to discover that the ZIP8 promoter is activated by
442 NF-κB (p65) (manuscript submitted, Liu MJ), a transcription factor that, as already
443 mentioned, plays a central role in coordination of innate immune function and host
444 defense. With this in mind, it is plausible to consider that ZIP8 expression is induced in
445 the lung as a consequence of chronic cigarette smoke exposure. Consistent with this
446 postulate, we observed an increase in ZIP8 mRNA and protein expression in the lungs
447 of smokers when compared to non-smokers (Figure 6). Further, ZIP8 was most
448 prominent throughout epithelia lining both the airway and alveolar regions. This
449 observation is remarkable when considering that ZIP8 was first identified as the Cd
450 toxicity gene[12]. Indeed, in addition to ZIP8's unique ability to become transcriptionally
451 activated by inflammatory stimuli, it is a transporter of Cd (K_m = 0.48 μM), exhibiting a
452 K_m comparable to its endogenous ligand Zn (K_m=0.26 μM)[20,32]. Further, consistent with
453 our own observations (Figure 5a), ZIP8 is preferentially expressed on the apical surface
454 of polarized epithelia as a plasma membrane protein making it ideally situated to
455 transport recognizable trace metals into lung epithelia[12,20,21,32]. Taken together, this
456 would suggest that the induction of ZIP8 expression in lung epithelia of a smoker
457 creates a potential disadvantage by enhancing Cd uptake into tissues following its
458 repeated inhalation subsequent to cigarette smoke exposure.
459 Zn deficiency as a consequence of insufficient nutritional intake affects nearly 2
460 billion people worldwide primarily within developing nations[43]. Adolescence, aging, and

lower socioeconomic status are significant risk factors for poor nutrition and Zn deficiency[1,42,43]. Perhaps not coincidentally, COPD is also associated with lower socioeconomic status and age[8,23], and often occurs with comorbidities, of which nutritional deficiency is a common manifestation[22,27,45]. In particular, muscle wasting, cachexia and appetite suppression are common symptoms associated with COPD linked to nutritional deficiencies. Although the incidence of Zn deficiency has never been formally studied within the COPD population, we predict that its prevalence may be relatively high considering the incidence of malnutrition. Clearly, epidemiologic evidence exists within the U.S. to indicate that lower Zn intakes are commonly encountered within smokers and that it increases the risk of developing COPD following prolonged cigarette smoke[31]. It then becomes plausible to consider that Zn supplementation in smokers has the potential to prevent or delay the progression of Cd-dependent pathogenesis within the lung. Our findings (Figure 3c) and others[32] demonstrate that Cd and Zn are both substrates for uptake via ZIP8 into cells and that differences in the uptake of either trace metal has profound influence on cell viability. In particular, when Zn concentrations are in relative excess, Zn uptake relative to Cd is most likely favored thereby enhancing cell survival. Joshi et al. reported a reduction of Zn within the bronchoalveolar lavage (BAL) fluid of alcohol-fed rats that was restored upon dietary Zn supplementation[26]. This suggests that there exists an available pool of Zn within the airway that is dependent on dietary intake. However, little is known regarding homeostatic regulation of Zn within the airway in humans and requires further study. Although not yet investigated in our model, we believe that the preferential uptake of Zn results in beneficial intracellular affects by influencing signaling events and

redox balance that maintain a more favorable environment, unlike Cd. Indeed, ZIP8-mediated Zn uptake acts as a cytoprotective in lung epithelia in the setting of inflammation whereas Cd has been shown to alter formation of ROS and derivatives leading to cell toxicity in the setting of inflammation[29,41,56].

Zn plays a critical role in immune function, redox signaling and regulation of inflammation[38,44,48]. Consistent with this, our group revealed that Zn deficient mice exhibit greater NF-κB activation and subsequent inflammation in response to polymicrobial sepsis[3]. Further, intracellular zinc content has been shown to function as a cytoprotectant and maintain cellular homeostasis in response to inflammation[6,51]. Based on these findings, we postulate that intracellular Zn status plays an important role relative to the cellular response following Cd uptake. Importantly, Cd has been shown to induce TNFα production in monocytes, macrophages, renal and liver tissue[15,16,19,30,54]. Taken together, this would suggest that ZIP8-mediated uptake of Zn relative to Cd is critical in determining the overall cellular response, in this case, to the inflammatory environment within a smoker's lung. In our model, we provide evidence that ZIP8 expression is a critical event in determining cell survival and that the amount of Zn present in the extracellular environment relative to Cd is critical in determining cell fate. Indeed, if Cd accumulation was favored over Zn then it is plausible to consider that intracellular Cd would alter cellular signaling events, NF-κB-mediated transcription, and more ZIP8 production thereby creating a vicious cycle of further Cd accumulation and cellular dysfunction. In our model, we did not observe substantial cell toxicity with just Cd exposure alone as shown (Figure 1a and Figure 3a). A major difference between past studies and findings presented herein is that Cd-mediated changes were studied in

monocytes and macrophages, both principal producers of TNFα. Epithelial cells are not considered to be significant producers of TNFα. Further, our group and others have shown that ZIP8 expression in monocytes and macrophages, similar to lung epithelia, is highly inducible in response to proinflammatory stimuli such as TNFα[5,7,46]. This raises important questions regarding the impact of Cd within alveolar macrophages in the setting of Zn deficiency and chronic cigarette smoke exposure and how cellular crosstalk may contribute to epithelial cell toxicity. Future investigations within our laboratory will focus on ZIP8-mediated effects of both epithelial and mononuclear cellular function in the setting of COPD pathogenesis.

This investigation provides novel insight into a mechanism whereby Zn metabolism influences Cd-mediated toxicity in the lung; however, findings from our model are limited in that COPD develops over decades. Disruption of the balance of cell turnover results in alveolar septal loss but the rate and extent of cell turnover is very low. Clearly, the balance between micronutrient and trace metal uptake is more complicated in humans. In consideration of our observations we postulate that ZIP8 may contribute to COPD pathogenesis through additional mechanisms. In particular, Cd accumulation increases epithelial injury and turnover, either via apoptosis or necrosis, wherein the latter can result in the release of damage-associated molecular patterns (DAMPs). In this scenario, ZIP8-mediated cadmium uptake would further promote inflammation[14,34,40]. In addition, Cd entry into lung cells has been shown to disturb multiple signaling pathways thereby altering normal physiological functions[50]. Given the long half-life of Cd in relation to the life expectancy of lung epithelia, it is

plausible that increased uptake into lung epithelia would have a sustained impact on cell function thereby causing permanent impairment.

In conclusion, we have identified that ZIP8 is a major regulator of Cd-mediated lung toxicity in the setting of inflammation. The expression of ZIP8 is upregulated by the NF-κB pathway that in turn enhances Cd uptake into lung epithelia causing apoptosis and necrosis. We believe that these findings fill a current gap in our understanding of Cd-mediated toxicity in the lung through interaction with a unique Zn transporter thereby potentially influencing pathogenesis associated with COPD and possibly cancer.

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Disclosures

No conflicts of interest, financial or otherwise, are declared by the authors.

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Figure Legends

Figure 1. A549 cell toxicity in response to Cd and TNFα treatment. A. A549 cells were first exposed to TNFα (100 ng/ml) for 24 hours and then exposed to increasing concentrations of Cd for an additional 24 hours. A significant increase in cell toxicity was observed in TNFα stimulated cultures exposed to Cd. Toxicity was standardized relative to detergent-lysed control cells (100% cell death). Data is expressed in triplicate and representative of at least 3 experiments (***$p < 0.001$, Two-way ANOVA). **B.** A549 cells were exposed to TNFα for 24 hours and cell membrane fractions were analyzed by Western blotting for human ZIP8. Autoradiographs were quantified by standard densitometry and standardized to β-Actin for each sample in order to measure fold induction of ZIP8 expression compared to untreated control samples.

Figure 2. ZIP8 inhibition reduces Cd-mediated toxicity. A. A549 cultures were first treated with the NF-κB inhibitor Bay 11-7082 (20 μM) or DMSO as vehicle control for 60 minutes and then exposed to TNFα for 24 hours followed by exposure to increasing concentrations of Cd for an additional 24 hours. NF-κB inhibition resulted in a significant decrease in cell toxicity when compared to the DMSO (vehicle) control in TNFα + Cd treated cultures. Data is expressed in triplicate and representative of 3 separate experiments (***$p < 0.001$; Two-way ANOVA). **B.** Similarly inhibition of ZIP8 expression with a ZIP8-specific siRNA resulted in a significant decrease in cell toxicity when compared to the siControl treatment group. Data expressed in triplicate and representative of 3 experiments (***$p < 0.001$, Two-way ANOVA). **C**. Membrane fractions were also obtained from samples in A. and B. and analyzed by Western

blotting with a primary antibody against ZIP8. Densitometry was standardized to actin and used to determine the percent knockdown of ZIP8.

Figure 3. ZIP8 increases Cd and Zn uptake in lung epithelia. A. A549 cells were stimulated with TNFα for 24 hours and then exposed to increasing amounts of Cd (0 - 25 μM) for 24 hours. Intracellular Cd was significantly increased in TNFα treated cells. Data expressed in triplicate and representative of 3 experiments (***$p < 0.001$, *$p < 0.05$, Two-way ANOVA). **B**. A549 cells were transfected with scrambled or ZIP8 siRNA and then stimulated with TNFα for 24 hours. Cells were then exposed to Cd (0-25 μM) for 12 hours. A decrease in intracellular Cd was observed in cells treated with the ZIP8 siRNA, though not statistically significant. Data expressed in triplicate and representative of 3 experiments. **C.** A549 cells were first exposed to TNFα and then increasing concentrations of Zn (10 to 40 μM) in combination with a fixed amount of Cd (10 μM). A significant decrease in cell toxicity was observed as Zn concentration increased, relative to Cd, in TNFα stimulated cultures. Data expressed in triplicate and representative of 4 experiments (***$p < 0.001$, **$p < 0.01$, Two-way ANOVA).

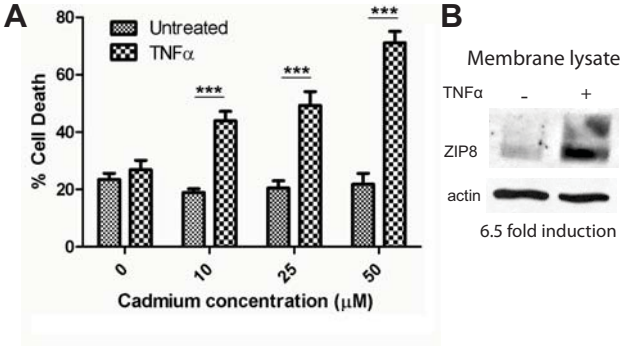
Figure 4. ZIP8-mediated Cd uptake induces apoptosis and necrosis. A. Cells were treated with TNFα and increasing concentrations of Cd (0 to 25 μM) for 24 hours and subsequently stained with an M30 antibody specific for caspase-cleaved cytokeratin 18. Representative photomicrographs of M30 positive A549 cells exposed to TNFα and Cd are shown. Cells were designated as apoptotic if they stained green with condensed nuclei (M30-positive green, DAPI blue). **B**. An increase in apoptosis was observed in cells exposed to TNFα and Cd. Percent apoptosis was calculated by dividing the number of apoptotic cells by the total number of cells in the field of view. Data is

representative of 4 experiments. A statistically significant increase in apoptotic cells was observed in cells exposed to higher Cd concentrations in combination with TNFα (***p < 0.001, Two-way ANOVA). **C**. A549 cells treated under the same conditions with TNFα and Cd were also stained with Annexin V (AV) and Propidium Iodide (PI) and enumerated by flow cytometry. The incidence of both necrosis (N) (PI+/AV+, top right quadrant) and apoptosis (A) (PI-/AV+, bottom right quadrant) was highest in cells exposed to both TNFα and Cd. Data in each figure is representative of 5 experiments. **D.** An increase in cell death was observed in cells exposed to TNFα and Cd. There was a statistically significant increase in PI+/AV+ cells following exposure to Cd in combination with TNFα (***p < 0.001, Two-way ANOVA). PI+/AV+ cells represent a mixed population of late apoptotic and necrotic cells.

Figure 5. Polarized ZIP8 expression in primary lung epithelia increases cell toxicity in response to TNFα and Cd treatment. A. Immunofluorescent staining was conducted on confluent polarized primary HUAEC cultures using an hZIP8 antibody and then visualized by *z*-stack using a disc-scanning confocal microscope. TNFα treated cells exhibited an increase in ZIP8 expression that was preferentially localized to the apical membrane whereas nonstimulated cultures showed minimal evidence of ZIP8 expression. ZIP8 staining is indicated by red fluorescence and DAPI nuclear staining is indicated by blue fluorescence. **B**. Fully differentiated, polarized, primary human upper airway epithelial cells were treated with TNFα for 24 hours, followed by Cd exposure for 48 hours. LDH release was measured and percent cell death was determined using a detergent positive control (100 % cell death). Toxicity was significantly increased in cells treated with TNFα and then apically exposed to cadmium. Data expressed in

triplicate and representative of four experiments (*p < 0.05, Two-way ANOVA). **C.** In a similar experiment, cultures were treated as previously described and trans-epithelial membrane resistance (TEER; R_t) was measured (Ω). The decrease in R_t, indicative of compromised membrane integrity, was more significant following apical Cd exposure when compared to basolateral Cd exposure. Data expressed in triplicate and representative of three experiments (*p < 0.05, **p < 0.01, Two-way ANOVAs).

Figure 6. ZIP8 mRNA and protein expression is elevated in smokers. A. Total RNA was extracted from human lung tissue samples that were obtained from chronic smokers (stage 0 COPD) and lifetime non-smokers and ZIP8 mRNA expression levels were measured. Quantitative RT-PCR analysis consistently revealed higher ZIP8 mRNA levels in the lung tissue of smokers (n=7) when compared to tissues obtained from non-smokers with the exception of one outlier in the control group (n=5) (Student's t-test; **p value < 0.01). **B**. Immunohistochemical staining for ZIP protein was then conducted on tissue sections obtained from two subjects within the chronic smoker group. Consistent with mRNA expression, both specimens exhibited an increase in ZIP8 throughout the lung epithelia (staining indicated by brown areas at 200x and 400x magnification of donor 224671). ZIP8 immunostaining of two lifetime non-smokers did not reveal evidence of increased ZIP8 expression (donor 013011 shown at 200x and 400x magnification). As a negative control, the same specimens were stained with the secondary rabbit IgG control antibody (donors 224671; smoker, and 013011; non-smoker), at 200x.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+
ZIP8
actin
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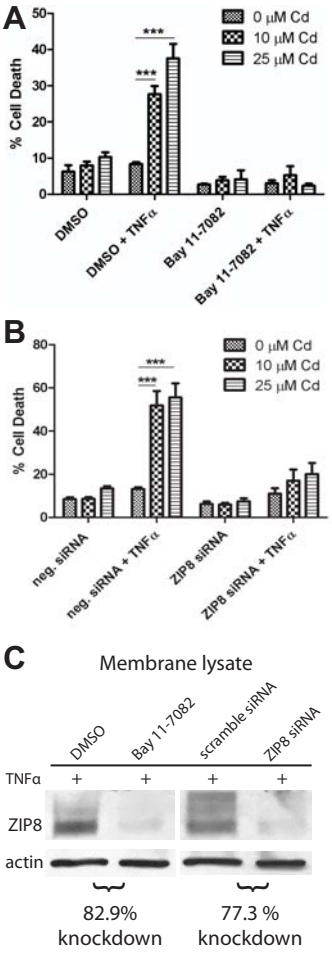
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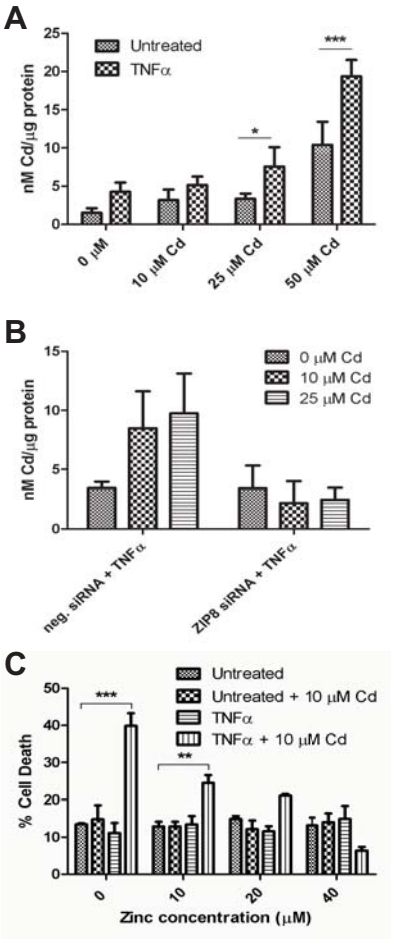
knockdown
77.3 %
knockdown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neg. siRNA + TNFα
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% Cell Death
Untreated
Untreated + 10 μM Cd
TNFα
TNFα + 10 μM Cd
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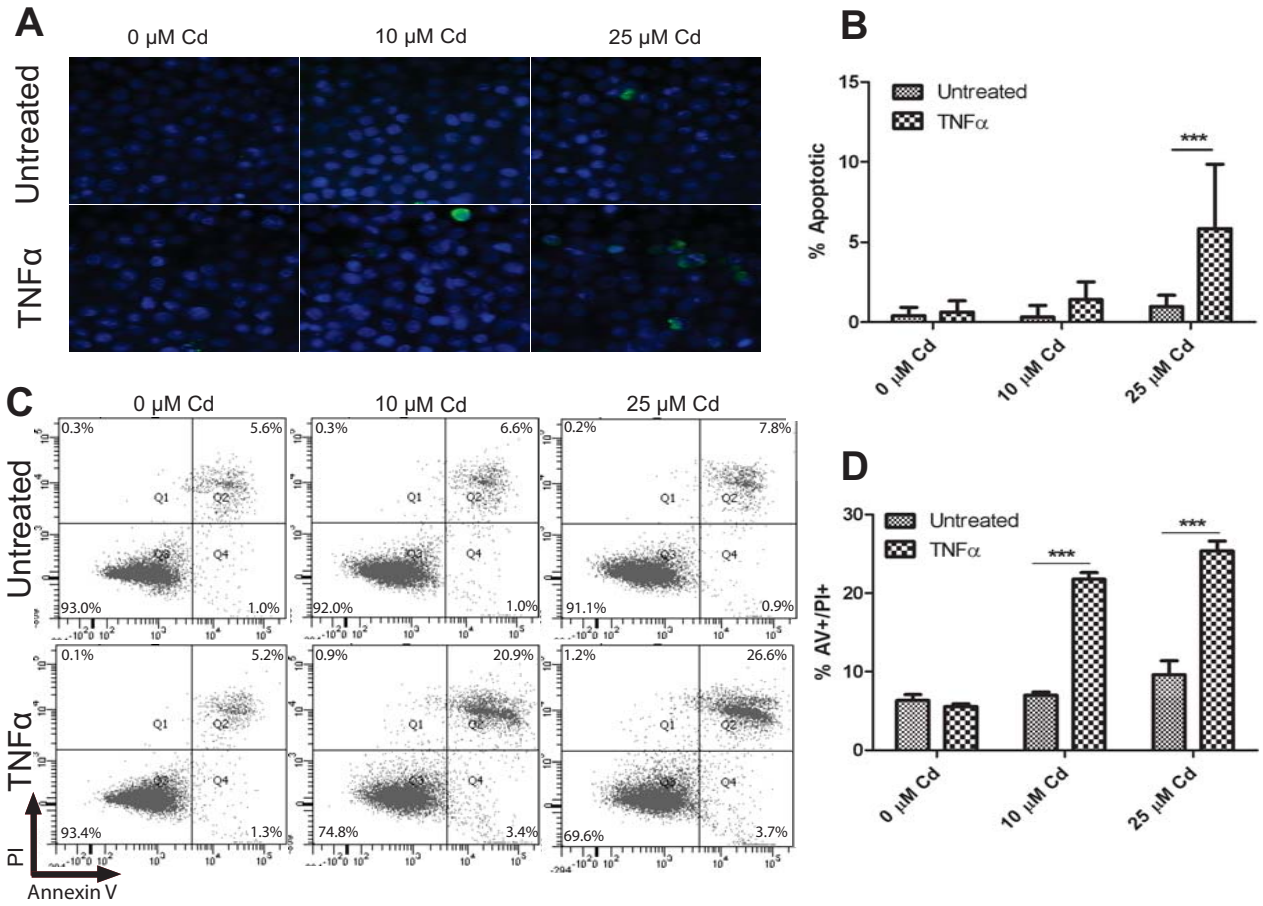
Zinc concentration (μM)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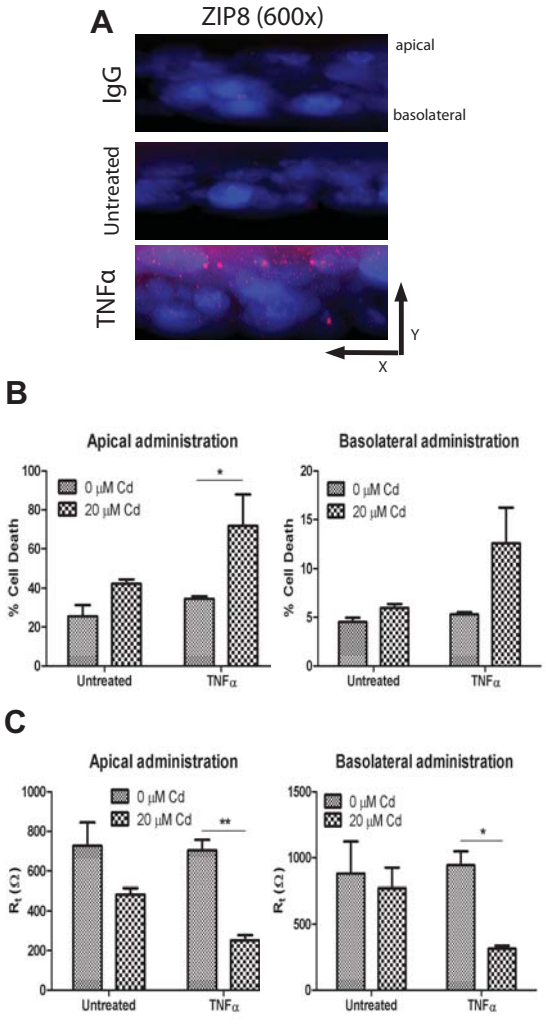
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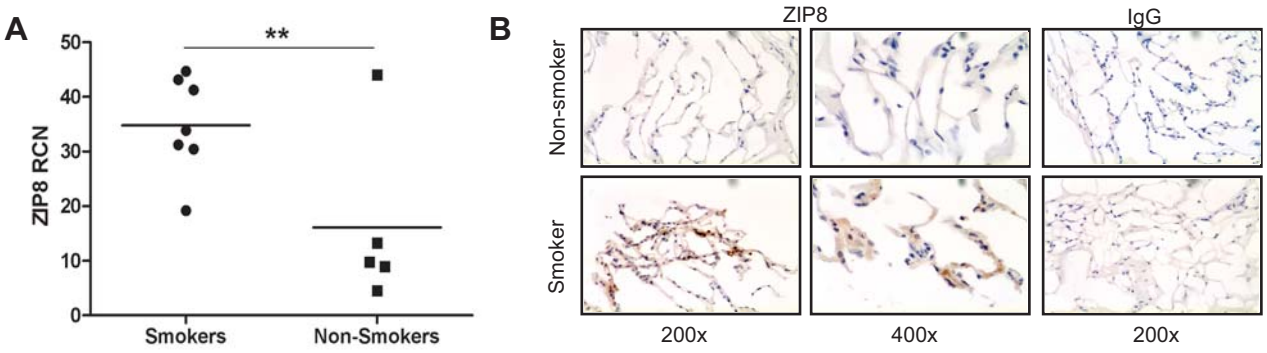
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