Cadmium attenuates the macrophage response to LPS through inhibition of the NF-κB pathway

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Cox JN, Rahman MA, Bao S, Liu M, Wheeler SE, Knoell DL. Cadmium attenuates the macrophage response to LPS through inhibition of the NF-κB pathway. Am J Physiol Lung Cell Mol Physiol 311: L754–L765, 2016. First published August 5, 2016; doi:10.1152/ajplung.00022.2016.—Chronic obstructive pulmonary disease (COPD) in the U.S. is primarily caused by cigarette smoking. COPD patients are highly susceptible to respiratory infections in part due to alveolar macrophage dysfunction despite a substantial increase in macrophages in the lung. Cadmium (Cd) is a toxic metal that is concentrated within tobacco and accumulates in the lung of smokers. We hypothesized that Cd uptake into macrophages alters immune function thereby impairing the macrophage response to invading pathogens. Our hypothesis was tested by comparing primary human monocytes and macrophages, primary mouse bronchoalveolar lavage myeloid cells, and related cell lines. Strikingly, Cd exposure followed by LPS stimulation resulted in a dose-dependent, significant decrease in nuclear p65 activity in macrophages that was not observed in monocytes. This corresponded with Cd-mediated inhibition of IKKβ and an impaired ability to transcribe and release cytokines in response to LPS challenge in vivo. These findings provide novel evidence that Cd has the capacity to disrupt macrophage immune function compared with monocytes. Importantly, Cd results in immune dysfunction in macrophages through inhibition of the NF-κB signaling pathway. Based on these findings, we provide new evidence that Cd contributes to immune dysfunction in the lung of COPD subjects and may increase susceptibility to infection.

In particular, alveolar macrophages isolated from the lungs of smokers and COPD patients subjected to ex vivo endotoxin challenge have impaired capacity to produce cytokines (21, 43). In contrast, circulating monocytes from COPD patients exhibit an increase in cytokine release (1). The induction of cytokines, a key component of the innate immune response, is in part dependent on the NF-κB signaling pathway (7). The IKK complex (composed of IKKβ, IKKα, and IKKγ) is a central component of the canonical NF-κB signaling pathway and when activated induces the translocation of the transcription factor p65 into the nucleus, which then induces the transcription of specific cytokine genes. Taking these findings together, we reasoned that cadmium (Cd), a toxic metal abundant in cigarette smoke, may have the capacity to alter this signaling pathway, and, if so, impair the ability of alveolar macrophages to mount an effective innate immune response upon pathogen exposure.

Cd ranks as one of the top 20 most lethal components of cigarette smoke as determined by the FDA. It is also recognized as a contributor to respiratory disease (26, 27, 49a). Cd is an inorganic metal abundant within the earth’s crust and absorbed into tobacco plants (53). Within each cigarette there exists ~0.5 μg of Cd (10), of which ~10% is absorbed through the lungs into the circulation (32). It is well established that Cd accumulates over time with repeated oral ingestion of contaminated dietary sources; however, the extent to which Cd is retained in the lung of smokers remains less clear. Smokers have an average twofold increase in blood and urinary Cd concentrations, reflective of its unusually long biological half-life estimated between 10 and 30 years (11, 12, 40).

Cd has multiple mechanisms that cause toxicity in humans, many of which have been observed in the lung of COPD patients (24, 50). Cd has a high affinity to bind thiol groups, leading to irreversible inactivation of proteins (19). Relative to this investigation, Cd can provoke or suppress the proinflammatory response depending upon the dose, stimulus, and model studied (45, 52). Our group previously reported that zinc inhibits the NF-κB pathway by binding directly to IKKβ (30). Given this and the high level of chemical similarity between zinc and Cd, we hypothesized that Cd contributes to macrophage immune dysfunction in a similar fashion. The purpose of this study was to determine whether Cd inhibits NF-κB signaling in macrophages compared with monocytes, as exhibited by differences in the response to endotoxin.

Knowing that chronic cigarette exposure alters alveolar macrophage phenotype in a manner that inhibits their capacity to clear respiratory pathogens (4, 48), we also wanted to examine the functional consequences of Cd-mediated impact on cell signaling events. Recent studies have revealed that the...
alveolar macrophage phenotype is shifted away from the classically activated M1 phenotype toward the “alternatively activated” M2 phenotype (21, 43). This is important because polarization toward M2 alters the capacity of macrophages to respond to danger signals as well as assist with repair of damaged tissue (16, 17, 33). Therefore, we further reasoned that macrophage reprogramming may occur following Cd exposure.

Herein we reveal that Cd has a dose-dependent suppressive effect on macrophage immune function but enhances monocyte immune activation. Cd-mediated immune suppression was caused by inhibition of NF-κB signaling. This corresponded with an inability of macrophages to induce NF-κB genes in response to LPS. In addition, Cd inhibited macrophage M1-type behavior with less effect on M2 phenotype. Taking these findings together, we have identified an important mechanism by which Cd may contribute to macrophage dysfunction and COPD progression.

MATERIALS AND METHODS

Cell culture and maintenance. The human acute monocytic leukemic THP-1 cell line (catalog no. TIP-202, American Type Culture Collection, Manassas, VA) was maintained under standard culture conditions in RPMI supplemented with 10% FBS at 37°C in a 5% CO2-humidified incubator. The rat alveolar macrophage cell line (NR8383) was obtained from the American Type Culture Collection (ATCC). The cells were maintained in F12K medium supplemented with 15% fetal bovine serum (FBS) in 1% penicillin and streptomycin. The cells were grown in a humidified incubator at 37°C and 5% CO2 atmosphere. All cells were used between passages 2 and 20.

THP-1-derived monotypic and macrophage models. THP-1 monocytes were maintained in 2% FBS and RPMI ± 10 μM CdCl2 overnight (~16 h). Serum concentration was reduced to 2% to minimize absorptive loss of Cd and at the same time maintain cell viability during Cd exposure. Cells were then stimulated with 100 ng/ml LPS for periods ranging from 15 min up to 12 h. To establish a cell line model of THP-1-derived macrophages (TDMs), THP-1 monocytes were resuspended in 10% FBS and RPMI with 50 ng/ml of phorbol myristate acetate (PMA) (39). After 24 h, cells were evaluated for adherence to the plate, and medium was replaced with 10% FBS and RPMI to facilitate differentiation over a period of 5 days. On the fifth day, medium was replaced with 2% FBS and RPMI ± 10 μM CdCl2 overnight. Similar to the monocyte model, the following day cells were stimulated with 100 ng/ml LPS for up to 12 h.

Primary human monocyte and macrophage models. Blood was drawn from healthy, nonsmoking, consenting human donors with approval from the Ohio State University Institutional Review Board. Briefly, blood was collected into heparin-coated tubes and then diluted with PBS. This mixture was then layered onto Histopaque-1077 (Sigma-Aldrich, St. Louis, MO) and centrifuged at room temperature for 20 min at 800 g. The mononuclear fraction was collected and washed with RPMI. Cells were incubated with CD14+ magnetized beads and run through a magnetic column (Miltenyi Biotec, San Diego, CA). After washing, the column was flushed to collect CD14+ cells. Following collection, CD14+ monocytes were separated into two groups. Half of the cells were resuspended and maintained in 2% FBS in RPMI ± CdCl2. Freshly isolated monocytes were immediately used in experiments. The other aliquot was resuspended in 10% FBS in RPMI with 20 ng/ml macrophage colony stimulating factor (M-CSF) (Peprotech, Rocky Hill, NJ) to initiate differentiation into macrophages. Two days after isolation, cell supernatant was collected and replaced with fresh medium containing a second dose of M-CSF. On day 6 following isolation, cells were adherent and fully differentiated macrophages. At this time, identical to treatment of monocytes, medium was replaced with 2% FBS and RPMI ± 2 μM CdCl2 and stimulated with 100 ng/ml LPS for up to 4 h the following day. Utilizing this approach we were able to routinely evaluate intra- and interindividual comparisons between monocyte and macrophage behavior under similar experimental conditions.

Cd quantification. Cells were thoroughly washed with PBS and a fraction was collected for protein quantification (Bio-Rad) and cell counting. The remaining cell fraction was digested in 1% nitric acid and Cd was quantified by using atomic absorption (Varian AA575, Palo Alto, CA) compared with known amounts of Cd used to generate a standard curve.

Cytotoxicity. Supernatant was collected and centrifuged, and lactate dehydrogenase activity was measured using the Cytotoxicity Detection Kit (Roche Diagnostics, Mannheim, Germany). Samples were compared with a positive control treatment group generated for each experiment by treatment with 2% Triton X-100 for 10 min to yield 100% death. Cell death was measured as percent of positive control. ELISA. Cells were stimulated with LPS for 4 h or 12 h following overnight Cd incubation. Supernatant was collected and centrifuged to remove debris. ELISA kits were used according to manufacturer’s instructions (BioLegend, San Diego, CA) for TNFα, IL-6, and IL-8. All samples were quantified following comparison to a standard curve with known amounts of recombinant protein.

mRNA analysis. Total RNA was isolated from samples by using TRIzol reagent. The ThermoScript RT-PCR kit (Invitrogen, Carlsbad, CA) was used to generate cDNA. Primer pairs were designed for TNFα, IL-6, IL-8, ZIP8, ZIP14, Nmmp2, megalin, cubulin, and GAPDH using Primer Express software version 2.0 (Applied Biosystems, Foster City, CA) as previously reported (6). Primer sequences for M1 and M2 phenotype-related genes included CXCL9, CXCL10, CXCL11, CCL5 and MMP2, MMP7, ADORA3, respectively, were obtained from the literature (22, 28, 29, 37, 44). The 7900HT Fast Real-Time PCR system (Applied Biosystems) using SYBR Green Master Mix (2X, Applied Biosystems) 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) described elsewhere (14). Briefly, RCN = 2^[-ΔΔCt] * 100, where ΔCt is the Ct value standardized to GAPDH.

Western blotting. Nuclear and cytosolic membranes were harvested by using the NE-PER Nuclear Protein Extraction Kit (Pierce, Rockford, IL) according to manufacturer’s instructions. Briefly, cells were washed and harvested and then subjected to cytoplasmic and/or nuclear buffers in concert with sequential centrifugation steps. Whole cell, nuclear, and cytosolic proteins were quantified by a protein assay (Bio-Rad, Hercules, CA) and then mixed in Laemmli buffer (Bio-Rad). The following antibodies were used in our experiments: rabbit anti-β-actin (1:3,000, Cell Signaling), rabbit anti-β2-microglobulin, mouse anti-p-IκBα, rabbit anti-p-IκBβ, mouse anti-p-p65, rabbit anti-p-p65 (all 1:1,000, Cell Signaling), mouse anti-β-actin (1:2,000, AMP Biomedicals, Aurora, OH), goat anti-anti-rabbit IgG-HRP (1:3,000, Cell Signaling), and horse anti-mouse IgG-HRP (1:3,000, Cell Signaling).

NF-κB activity assay. Nuclear p65 activity was determined by using the TransAM Transcription Factor ELISA Kit (Active Motif, Carlsbad, CA). Nuclear protein was isolated from samples and analyzed for activity according to manufacturer’s instructions. Briefly, nuclear lysate was incubated on plates coated with a consensus binding sequence specifically recognized by p65, Phast
ELISA was then used to measure differences in activity between different treatment groups by spectrophotometric methods. Activity was measured as fold change over baseline (unstimulated samples).

**IKKβ activity assay.** IKKβ kinase activity was measured with the HTScan kinase assay kit (Cell Signaling). Briefly, recombinant human IKKβ was incubated with increasing concentrations of Cd and then incubated with ATP and a biotinylated IkBα peptide. Kinase activity was measured using an antibody specific to phosphorylated IkBα at serine residue 32, followed by a secondary Europium-labeled secondary antibody. Dissociation-enhanced lanthanide fluorescent immunoassay (DELFIA) measures were used to calculate kinase activity. The IC₅₀ value was calculated by nonlinear regression curve fitting.

**Analysis of endogenous IKKβ activity.** THP-1 monocytes and TDMs treated with or without 10 μM CdCl₂ were lysed with NP-40 lysis buffer containing protease inhibitors. Cell lysates were incubated with anti-IKKβ antibody (Cell Signaling) overnight. Protein A-agarose beads were added to pull down the IKK complex, which was then analyzed by Western blotting and a IKKβ activity assay, as described above. Kinase activity was measured by standard ELISA methods.

**Cd exposure animal model.** Adult female C57BL/6 mice, 10 to 12 wk old, were subject to nebulization using an Aeroneb Lab Nebulizer System with either a saline solution (NaCl 0.9%) as control or a 0.1% CdCl₂ saline solution. Aerosol was dispersed at a flow rate of 0.5 l/min. Three mice per group were nebulized for 2 h per day under identical exposure conditions for 7 days. After 7 days of Cd exposure, mice were exposed for 20 min to an aerosol of LPS (500 μg/ml). At 4 h after LPS exposure mice were anesthetized for collection of total bronchoalveolar lavage (BAL) (9) cells. All animal studies were IACUC approved.

**BAL preparation and determination of inflammatory response.** Mice were euthanized and the thoracic cavity trachea was exposed. Then lungs were lavaged using a 18G Exel Safelet catheter (Exelint International) that was inserted into the trachea. Lungs were washed with 1 ml of cold, sterile 1× PBS. The recovery of lavage fluid was reproducibly greater than 80% of total volume input. BAL fluid was pooled and centrifuged at 400 g for 5 min at 4°C and then cells were resuspended in 200 μl 1× PBS. Tritol was used to harvest mRNA from total BAL cells; this mRNA was then made into cDNA for RT-PCR and gene expression profiles were then obtained for IL-6, CXCL-2, and TNFα.

**Statistical analyses.** All data are expressed as means ± SD. For comparisons involving multiple variables and observations, an ANOVA (GraphPad, La Jolla, CA) was used. Having passed statistical significance by ANOVA, individual comparisons were made with the Tukey’s post hoc or Bonferroni multiple-comparison test. Statistical significance was defined as a P value < 0.05.

**RESULTS**

**Cd inhibits NF-κB-dependent cytokine transcription in macrophages.** THP-1 monocytes, TDMs, or rat alveolar macrophages were initially exposed to increasing concentrations of Cd (0.1, 1.0, 2.5, 5.0, 10, 20, 40, or 100 μM) overnight followed by 6 h LPS (100 ng/ml) treatment. Strikingly, Cd significantly inhibited cytokine gene expression in a dose-dependent manner in macrophages (Fig. 1, B and C) but not in monocytes (Fig. 1A). Based on the dose-response curves obtained from THP-1 monocytes and TDMs, we chose to use a Cd concentration of 10 μM for subsequent studies to further examine differences between monocytes and macrophages relative to Cd-mediated inhibition of NF-κB-dependent cytokine gene expression.

TNFα gene expression was increased in monocytes by LPS regardless of Cd exposure at all time points analyzed (Fig. 2A). In contrast, expression was significantly decreased in Cd-exposed macrophages within 4 h of LPS exposure (Fig. 2B). Similarly, IL-6 transcription was significantly increased in Cd-treated monocytes at 4 h and 12 h but was significantly decreased in Cd-exposed macrophages at 12 h post-LPS stimulation. Macrophages also exhibited a peak in transcription at 4 h which was more prominent in non-Cd-treated cultures. Most important, the differences between monocyte and macrophage mRNA expression profiles revealed that Cd has an immunosuppressive impact of NF-κB-responsive cytokines on macrophages but not monocytes.

**Cd inhibits macrophage cytokine protein production.** Based on mRNA findings we next determined to what extent THP-1 and TDMs respond to endotoxin challenge following Cd exposure at the protein level. THP-1 cells were collected and designated to either remain as suspended monocytes or differentiated into adhered macrophage cultures. Experiments were conducted in parallel from the same passage or same donor. Following overnight Cd exposure, cells were stimulated with 100 ng/ml LPS for either 4 or 12 h, and cytokine production was determined. We observed marked differences in cytokine profiles in response to Cd exposure between THP-1 monocytes (Fig. 3A) and THP-1-derived macrophages (Fig. 3B). Monocytes released increased amounts of TNFα at 4 h and at 12 h post-LPS stimulation. Macrophages also exhibited an increase in TNFα in response to LPS in Cd-unexposed cultures; however, Cd treatment resulted in near complete ablation of TNFα production. Similar to TNFα, IL-6 release by monocytes peaked at 12 h post-LPS exposure and was significantly increased in the presence of Cd. As before, macrophages exhibited decreased production in the presence of Cd. Identical trends between monocytes and macrophages were also observed with respect to IL-8 and IL-10 production.

**Cd inhibits NF-κB signaling in macrophages.** Knowing that the gene expression and production of NF-κB driven cytokines is significantly decreased by Cd in macrophages, we examined whether deficits, if any, occurred in signal transmission within this pathway. We first determined to what extent differences exist in p65 phosphorylation and nuclear translocation. Nuclear protein was isolated from cells within 1 h following LPS exposure. Cd-treated monocytes exhibited nuclear translocation of phosphorylated p65 that increased over time following LPS exposure. Cd-treated macrophages exhibited nuclear translocation of phosphorylated p65 that increased over time following LPS exposure which was comparable to non-Cd-treated, LPS-exposed cultures (Fig. 4). In contrast, Cd-treated macrophages exhibited a decrease in the amount of phosphorylated p65 present in the nucleus compared with Cd-untreated, LPS-exposed controls. Western blotting for IkBα, a protein whose phosphorylation and subsequent degradation is required for p65 nuclear mobilization, revealed a substantial decrease in IkBα phosphorylation in Cd-treated macrophages (Fig. 4A). Consistent with previous findings, Cd-treated monocytes exhibited phosphorylation comparable to non-Cd-exposed cultures. The observed IkBα phosphorylation correlated with the extent of degradation of total IkBα protein that occurs upon NF-κB pathway activation. Western blotting of IKKβ, which is an essential component of the IKK complex and functions directly upstream of IkBα, also demonstrated phosphorylation patterns consistent with prior findings whereby Cd-treated macrophages exhibited less IKKβ phosphorylation following LPS activation (Fig. 4A). Taken together, these observations provide strong supportive evidence that Cd uptake by macro-
phages but not monocytes causes significant inhibition of the NF-κB pathway resulting in immune dysfunction.

Based on this, we next determined whether these changes resulted in defects in p65 protein function in THP-1 and TDM cultures following Cd exposure through use of an activity assay. There was no observable difference in p65 activity between Cd-treated and Cd-untreated monocytes following LPS exposure (Fig. 4B). Consistent with previous findings, we observed a highly significant decrease in p65 activity within Cd-treated macrophages as measured by fold change over untreated controls (Fig. 4C). We then compared p65 activity between primary human monocytes and monocyte-derived macrophages from the same donor. Most important, and consistent with THP-1 monocyte and TDM cultures, similar changes in p65 activity were observed between primary monocytes whereas p65 activity was significantly decreased following Cd treatment in primary monocyte-derived macrophages (Fig. 4C), an effect that was highly reproducible using different human donors. Taken together, we conclude that NF-κB activity is inhibited by Cd in LPS-stimulated macrophages but not LPS-stimulated monocytes.

Cd inhibits IKKβ activity. Zn has been shown to modulate NF-κB activity by directly binding to and inhibiting IKKβ (30). Given the chemical similarity between Zn and Cd and our observations that indicate a strong inhibitory effect of Cd upon this pathway, we hypothesized that Cd may directly inhibit IKKβ. As predicted, recombinant IKKβ kinase activity was inhibited by Cd in a dose-responsive manner with an IC50 in the nanomolar range (≈130 nM) characteristic of a high-affinity interaction (Fig. 5A). We then sought to determine whether endogenous IKKβ was also a molecular target for Cd by examining IKKβ kinase activity in Cd-treated, LPS-stimulated THP-1 monocytes and TDMs. As shown, 10 μM Cd blocked LPS-induced endogenous IKKβ kinase activity in macrophages but not in monocytes, as determined following measurement of kinase activity in immunoprecipitated IKKβ complex extracts (Fig. 5B). Taken together, these results indicate that Cd blocks IkB phosphorylation in macrophages but not in monocytes through inhibition of IKKβ kinase activity.

Comparison of Cd content, cytotoxicity, and MT1 expression. Based on the finding that monocytes and macrophages exhibit a markedly different response to Cd relative to cytokine production, we next determined whether differences exist in intracellular Cd content in monocytes and macrophages before and following 4-h LPS exposure. LPS stimulation did increase intracellular Cd content but, surprisingly, there was no

Fig. 1. Impact of different doses of Cd on inflammatory cytokines genes expression from THP-1 monocytes (A), PMA-derived THP-1 macrophages (TDMs) (B), and rat alveolar (rat AM) macrophages (C). Cells were treated with CdCl2 (0.0, 0.1, 1.0, 2.5, 5.0, 10, 20, 40, and 100 μM) overnight followed by 6-h LPS (100 ng/ml) treatment. TRIzol was used to harvest mRNA, which was then made into cDNA for RT-PCR analysis of IL-6, IL-8, or CXCL2 and TNFα genes. Messenger expression was reported as the average relative copy number (RCN). The bars shown are expressed as means of 3 independent experiments for THP-1 and TDMs, and a representative experiment for rat AMs from 2 independent experiments.
Increased at 12 h in monocytes (**). IL-6 release was significantly increased in Cd-treated monocytes (**/H9251P/IL-8, and TNFα genes. In general, gene transcription was increased in non-Cd-treated, LPS-stimulated monocytes and macrophages at 4 and 12 h. Specifically, IL-6 transcription was significantly increased in Cd-treated monocytes (*P < 0.05) but decreased in macrophages at 4 h and more significantly so at 12 h (**/P < 0.01). IL-8 transcription was significantly decreased in Cd-treated monocytes at 12 h (*P < 0.05). Cd-exposed macrophage IL-8 transcription was decreased significantly at both 4 (**/P < 0.01) and 12 h (***/P < 0.001). Cd did not impact TNFα expression in monocytes but was significantly decreased in Cd-treated macrophages (**/P < 0.01). Figures are representative of 3 independent experiments, all conducted in triplicate. Significance was determined by 2-way ANOVA and Bonferroni posttests.

A difference in intracellular Cd accumulation between monocytes and macrophages (Fig. 6A). We also determined whether differences in Cd-mediated cytotoxicity exist between monocytes and macrophages by measuring LDH release. Both monocytes and macrophages exhibited a modest increase in LDH release following overnight Cd exposure, indicating that Cd has a similar albeit mild toxic impact on both monocytes and macrophages under the conditions studied (Fig. 6B).

Metallothionein (MT) is an intracellular protein that has the capacity to bind Cd and protect cells from cytotoxicity (25).

Fig. 2. Cd inhibits transcription of NF-κB-dependent cytokines in macrophages. THP-1 monocytes (A) and PMA-derived THP-1 macrophages (TDMs) (B) were treated overnight with 10 μM CdCl₂. The following morning cultures were stimulated for 4 or 12 h with 100 ng/ml LPS. TRIzol was used to harvest mRNA, which was then made into cDNA for RT-PCR analysis of IL-6, IL-8, and TNFα genes. In general, gene transcription was increased in non-Cd-treated, LPS-stimulated monocytes and macrophages at 4 and 12 h. Specifically, IL-6 transcription was significantly increased in Cd-treated monocytes (*P < 0.05) but decreased in macrophages at 4 h and more significantly so at 12 h (**P < 0.01). IL-8 transcription was significantly decreased in Cd-treated monocytes at 12 h (*P < 0.05). Cd-exposed macrophage IL-8 transcription was decreased significantly at both 4 (**P < 0.01) and 12 h (***P < 0.001). Cd did not impact TNFα expression in monocytes but was significantly decreased in Cd-treated macrophages (**P < 0.01). Figures are representative of 3 independent experiments, all conducted in triplicate. Significance was determined by 2-way ANOVA and Bonferroni posttests.

Fig. 3. Cd inhibits cytokine production in macrophages. THP-1 monocytes (A) and PMA-derived macrophages (B) were treated overnight with 10 μM CdCl₂. The following morning cultures were stimulated for 4 or 12 h with 100 ng/ml LPS. Supernatants were collected and analyzed by ELISA for the release of IL-6, IL-8, and TNFα. IL-6 release was significantly increased in Cd-treated monocytes (**P < 0.01) at 12 h but significantly decreased in macrophages (***P < 0.001). IL-8 release was increased in Cd-treated monocytes (***P < 0.01) but significantly decreased at both 4 (**P < 0.001) and 12 h (***P < 0.01) in macrophages. Based on mRNA and protein findings a distinct pattern emerged in which Cd enhances cytokine production and release in monocytes but almost completely attenuates cytokine production and release in macrophages. Figures are representative of 3 independent experiments, all conducted in triplicate. Significance was determined by 2-way ANOVA and Bonferroni posttests.
Based on our previous findings, we determined whether variations in MT expression between monocytes and macrophages in response to Cd could account for altered immune function. We first observed a pattern of MT mRNA expression as previously reported (47) showing that MT-1 is significantly suppressed during THP-1 cell differentiation by PMA (data not shown). However, even higher mRNA levels of MT-1 were detected in Cd-induced macrophages compared with monocytes.

Fig. 4. Cd inhibits the NF-κB pathway in TDMs and MDMs. A: nuclear and cytosolic protein fractions of Cd-treated THP-1 monocytes and PMA-derived macrophages (TDMs) were harvested up to 60 min following LPS exposure, and then subjected to Western blotting. Nuclear phospho-p65 (p-p65) detection was unaltered by Cd following LPS stimulation in monocytes but was decreased in Cd-treated LPS-exposed TDMs. Detection of phosphorylated IkBα was also unaltered by Cd in LPS-exposed monocytes; however, IkBα phosphorylation was decreased in Cd-treated macrophages following LPS exposure, which was complemented by an increase in total IkBα that is typically degraded upon phosphorylation. Detection of phosphorylated IKKβ, a component of the IKK complex that is directly upstream of IkBα, revealed no difference in Cd-treated monocytes whereas IKKβ phosphorylation was significantly and sustainably inhibited in TDMs. Analysis of phosphorylated and total protein levels were conducted from the same blots and are representative of 3 independent experiments.

B: THP-1 monocytes and TDMs were treated overnight with or without 10 μM CdCl₂ followed by 1 to 4 h of 10 ng/ml LPS. Nuclear lysate was prepared and subject to an ELISA-based p65 activity assay. LPS induced p65 activity in THP-1 monocytes but without changes in p65 activity attributable to Cd exposure at any time point. In contrast, Cd-treated TDMs exhibited a significant decrease in p65 activity at each time point (*** P < 0.001). Data are representative of 3 independent experiments, all conducted in duplicate. The same experiment was conducted in freshly isolated human monocytes and monocyte-derived macrophages. Similar to THP-1 monocytes, there were no differences in primary monocytes in p65 activation as a result of Cd exposure. As previously observed in TDMs, there was a significant decrease in p65 activity at 2 (** P < 0.01) and 4 h (*** P < 0.001) in Cd-treated monocyte-derived macrophages. Data presented are from 1 donor, representative of 3 separate donors. Statistical analysis was conducted using a 2-way ANOVA and Bonferroni posttests. NS, nonstimulated; 15, 30, and 60 min.

C: THP-1 monocytes and TDMs were treated overnight with or without 10 μM CdCl₂ followed by 1 to 4 h of 10 ng/ml LPS. Nuclear lysate was prepared and subject to an ELISA-based p65 activity assay. LPS induced p65 activity in THP-1 monocytes but without changes in p65 activity attributable to Cd exposure at any time point. In contrast, Cd-treated TDMs exhibited a significant decrease in p65 activity at each time point (*** P < 0.001). Data are representative of 3 independent experiments, all conducted in duplicate. The same experiment was conducted in freshly isolated human monocytes and monocyte-derived macrophages. Similar to THP-1 monocytes, there were no differences in primary monocytes in p65 activation as a result of Cd exposure. As previously observed in TDMs, there was a significant decrease in p65 activity at 2 (** P < 0.01) and 4 h (*** P < 0.001) in Cd-treated monocyte-derived macrophages. Data presented are from 1 donor, representative of 3 separate donors. Statistical analysis was conducted using a 2-way ANOVA and Bonferroni posttests. NS, nonstimulated; 15, 30, and 60 min.
overnight (O/N) with 10 and THP-1-derived macrophage cells was then analyzed. Cells were treated/H9252-specific antibody. The activity of immunoprecipitated IKK/LPS stimulation. Extracts of treated cells were then immunoprecipitated with/Cd content in macrophages compared with monocytes.

decreased MT-1 expression accounts for elevated intracellular/Cd inhalation suppresses BAL cells inflammatory response to LPS. We next wanted to determine whether Cd has the capacity to recapitulate macrophage paralysis in the lung in vivo. To do so, we exposed adult mice to Cd inhalation over a

7-day period. Cd exposure resulted in a substantial increase in alveolar macrophages and neutrophils (Fig. 7A). Alveolar macrophages comprised ~50% of the total cell population extracted by BAL, which was almost exclusively myeloid cells (Fig. 7B). In mice that were exposed to a one-time aerosol dose of LPS, following 7 days of Cd exposure, we observed significant impairment in the expression of inflammatory cytokines mRNA levels in BAL cells compared with the saline-exposed, LPS-treated group (Fig. 7C), consistent with in vitro observations. Further analysis of lung tissues revealed that Cd accumulation in the lung occurred only in Cd-exposed mice with Cd content being similar between LPS-exposed and nonexposed cohorts (Fig. 7D).

Cd deactivates M1 polarization in macrophages. Knowing that Cd is a potent inhibitor of NF-κB signaling in macrophages, we next determined whether Cd could alter the capacity to exhibit an M1 or M2 phenotype following endotoxin challenge. This is important because chronic cigarette smoke exposure has been shown to deactivate the capacity of alveolar macrophages to exhibit an M1 phenotype in the lung of COPD patients (43). Accordingly, we examined the transcription profile of both M1- and M2-related genes (43) by qPCR to determine to what extent, if any, Cd could recapitulate the findings previously obtained in humans. M1-related genes included the type I chemokines CXCL9, CXCL10, CXCL11, and CCL5. The M2-related genes examined were matrix metalloproteinases MMP2 and MMP7 and the adenosine A3 receptor (ADORA3). Cd exposure resulted in a substantial reduction in all M1-related genes examined in response to LPS stimulation (Fig. 8A). In contrast, LPS treatment alone exhibited a differential capacity to either inhibit or induce M2-related genes (Fig. 8B); however, Cd treatment in addition to LPS stimulation did not result in any significant increase or decrease in gene expression beyond that of LPS alone.

DISCUSSION

Cd exposure prior to endotoxin challenge impaired the capacity of macrophages to elicit a proper immune response. Our novel findings herein demonstrate that Cd impairs macrophage immune function through inhibition of the NF-κB pathway, specifically through inhibition of IKKβ, and that it alters macrophage programming in response to LPS relative to M1 or M2 status. In direct comparison, monocytes exhibited an increase in NF-κB pathway activation following combined Cd and LPS exposure. These findings were consistently observed in relevant cell lines, primary monocytes, monocyte-derived macrophages, and myeloid cells including alveolar macrophages obtained from the lungs of Cd-exposed mice. Alteration of immune function between monocytes and macrophages were not attributed to differences in intracellular Cd accumulation or cell toxicity. These findings are consistent with past studies that have examined monocyte and macrophage function in chronic smokers (49). Collectively, we postulate that these findings are significant when considered in the context of chronic cigarette smoke exposure, a rich source of Cd, and the known difficulty that COPD patients have in clearing respiratory pathogens.

COPD patients are at an increased risk for contracting both viral and bacterial respiratory infections that perpetuate inflammation in the lung, thereby further increasing morbidity and

Fig. 5. Cd inhibits IKKβ kinase activity in macrophages. A: first, using a cell-free system, we determined whether Cd inhibits IKKβ kinase activity. Recombinant human IKKβ was incubated with increasing concentrations of Cd and a substrate peptide. The reaction mixture was then placed into streptavidin-coated 96-well plates and incubated with a phospho-IκBα antibody, followed by a Europium-labeled secondary antibody and then read with a fluorescent plate reader. The IC50 was ~130 nM indicative of a strong inhibitory effect of Cd on IKKβ. Data were obtained from 1 experiment using replicate samples. B: endogenous IKKβ activity in THP-1 monocytes and THP-1-derived macrophage cells was then analyzed. Cells were treated overnight (O/N) with 10 μM CdCl2 followed by 0, 15, or 30 min of 100 ng/ml LPS stimulation. Extracts of treated cells were then immunoprecipitated with an IKKβ-specific antibody. The activity of immunoprecipitated IKKβ was measured utilizing a standard kinase assay with IκBα peptide as substrate (see MATERIALS AND METHODS). The relative expression of IKKβ activity was then normalized. Consistent with previous findings, endogenous IKKβ kinase activity was inhibited by Cd in LPS-stimulated TDMs but not THP-1 monocytes. Experiments were repeated 3 times with similar results. *P < 0.05 by 1-way ANOVA with Tukey’s post hoc comparison.

Fig. 6. Cd impairs NF-κB expression and protein release in macrophages. Cd inhibits MT-1 expression accounts for elevated intracellular Cd content in macrophages compared with monocytes.

cytes (Fig. 6C), which does not support the hypothesis that decreased MT-1 expression accounts for elevated intracellular Cd content in macrophages compared with monocytes.
mortality. COPD patients have a sixfold higher incidence of pneumonia compared with healthy peers (39). Approximately 30% of COPD patients with stable disease are colonized with Haemophilus influenzae, Streptococcus pneumoniae, and Moraxella catarrhalis (34, 42). Rhinovirus is the most commonly detected virus in COPD patients, followed by influenza and respiratory syncytial virus (38). Acute exacerbations of the disease, characterized by sudden worsening of dyspnea and respiration, typically correlate with contraction of viral or bacterial infections (51). In the setting of moderate to severe disease, it is common for patients to suffer from more than three exacerbations a year (46). While the pathogenesis of COPD has long been considered to be driven by a hyperinflammatory state brought upon by chronic cigarette smoking, a growing body of evidence has emerged that demonstrates the immunosuppressive effects of tobacco smoke, which has been postulated to increase susceptibility to respiratory infections.

Alveolar macrophages are the principal phagocytic cell in the lung responsible for the removal of pathogens and cellular debris. Macrophages also play a vital role in coordinating host defense between other cell types in part through the release of cytokines. Alveolar macrophages accumulate in the lungs of COPD patients, and more so in subjects with severe disease (9). Despite the increased presence of alveolar macrophages, smokers are more susceptible to infection. Interestingly, we observed a similar increase in alveolar macrophages following 7 days of aerosol exposure of Cd. Whether the immune paralysis observed in the myeloid cell population obtained by BAL following LPS exposure generated a deficit in bacterial clearance remains to be studied.

In comparison to macrophages, we observed that Cd-treated monocytes exhibit an exaggerated proinflammatory phenotype when exposed to LPS. This finding is consistent with previous studies that have documented systemic inflammation in COPD patients (1). These investigations proposed that exaggerated inflammation may perpetuate monocyte influx within the lung space leading to production of dysfunctional macrophages that are incapable of mounting a proper immune response. Based on this, we postulate that respiratory infections superimposed upon COPD subjects in the Cd-laden lung microenvironment may exacerbate macrophage dysfunction, thereby creating a dangerous cycle that enhances COPD pathogenesis through immune dysfunction, altered macrophage phenotype, and limited capacity to clear pathogens and facilitate effective tissue repair.
Cd can disrupt oxidative balance within cells through generation of reactive oxygen species (ROS) (23). It is well established that acute ROS production can stimulate the NF-κB pathway. In contrast, Cd has also been shown to inhibit the NF-κB pathway (45, 52). Based on findings from our own studies, we observed that Cd-treated macrophages exhibit decreased nuclear localization of phosphorylated p65, resulting in a substantial reduction in transcriptional activity (Fig. 3). Additionally, we observed that this effect is at least in part accounted for through defects in protein phosphorylation of intermediate proteins through inhibition of IKKβ. The discrepancy that exists between our study and others may be a consequence of study design and model. In particular, evidence that Cd is an activator of NF-κB was derived from acute exposure models that utilized higher Cd concentrations (13). Our model deliberately utilized physiologically relevant concentrations of Cd and allowed sufficient time for uptake before LPS challenge. To our knowledge, our study is the first to evaluate the effects of prolonged Cd exposure in primary monocytes compared with macrophages. By doing so, we were able to reveal substantial differences between monocytes and macrophages.

No measurable differences in the extent of intracellular Cd uptake between monocytes and macrophages were observed despite profound differences in NF-κB signaling. Furthermore, MT-1 mRNA levels were higher in Cd exposed, LPS-treated macrophages, which does not support the concept that available pools of Cd are higher in macrophages. Previous work has shown that, once Zn and Cd permeate the cell, that Cd is trapped due to insufficient export pathways, unlike Zn (20). This scenario agrees well with a much narrower distribution and longer retention of Cd2+ in mammalian tissues, compared with the rapid turnover of dietary Zn. With regard to uptake, three transport proteins have emerged as leading Cd transporters. The divalent metal transporter 1 (a.k.a. DMT1 or Nramp2) is abundant within the brain and gut and is responsible for the import of multiple divalent cations (2). More recently, the zinc transporters ZIP8 and ZIP14, which function primarily to
import zinc, have been recognized as Cd transporters (8, 15, 18). Zinc and Cd have similar biochemical properties, which in part explain how Cd can gain cellular entry through exploitation of zinc transporters. Relative to this, ZIP8 expression is elevated in the lung of cigarette smokers (35) and also been shown to be induced by danger signals in both monocytes and macrophages (5, 31). In preliminary studies, knockdown of ZIP8 and ZIP14 expression in TDM cultures did not alter Cd accumulation (data not shown). Based on this evidence, we predict that Cd translocation with regard to access of the ZIP14 and ZIP8 zinc transporters. Relative to this, ZIP8 expression is shown to be induced by danger signals in both monocytes and macrophages (5, 31). In preliminary studies, knockdown of ZIP8 and ZIP14 expression in TDM cultures did not alter Cd accumulation (data not shown). Based on this evidence, we predict that Cd translocation with regard to access of the ZIP14 and ZIP8 zinc transporters.

We believe that Cd-mediated changes on cell signaling may further impact the lung microenvironment by further influencing macrophage cell behavior in response to LPS toward an M2 phenotype. This is important because chronic cigarette exposure in humans has been shown to potentiate development of an M2 state and prohibit the clearance of respiratory pathogens, thereby increasing susceptibility to repeated upper respiratory tract infections. Furthermore, once Cd obtains entry inside the macrophage, it has the potential to dynamically alter cells in favor of the alternative M2 phenotype.

In conclusion, we have identified major differences in the response to Cd exposure between monocytes and macrophages. Cd is able to prevent macrophages from mounting a proper immune response to a classic TLR4 agonist (endotoxin) by directly inhibiting the NF-κB pathway. We believe these findings may provide insight into mechanisms that underlie the inability of COPD patients to effectively clear pathogens, thereby increasing susceptibility to repeated upper respiratory tract infections. Furthermore, once Cd obtains entry inside the macrophage, it has the potential to dynamically alter cells in favor of the alternative M2 phenotype.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Fig. 8. Cd downregulates M1-related genes in TDMs. M1 (A)- and M2 (B)-associated gene expression profiles. PMA-derived macrophages were treated overnight (O/N) with 10 μM CdCl₂. The following morning cultures were stimulated for 4 or 12 h with 100 ng/ml LPS. TRizol was used to harvest mRNA, which was made into cDNA for quantitative RT-PCR analysis for M1 (CXCL9, CXCL10, CXCL11, and CCL5) and M2 (MMP2, MMP7, and ADORA3) related genes. Transcription of all M1-related genes was inhibited by Cd in TDMs at 12 h post-LPS exposure. At both 4 and 12 h, the transcription of M2-related genes was not altered by Cd in TDMs. We also observed that LPS exposure alone decreased the expression of MMP2 and ADORA3. Figures are from 3 independent experiments. *P < 0.05 by 1-way ANOVA with Tukey’s post hoc comparison.
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