Vitamin D Treatment Modulates Organic Dust–Induced Cellular and Airway Inflammatory Consequences

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ABSTRACT: Exposure to organic dusts elicits airway inflammatory diseases. Vitamin D recently has been associated with various airway inflammatory diseases, but its role in agricultural organic dust exposures is unknown. This study investigated whether vitamin D reduces organic dust–induced inflammatory outcomes in cell culture and animal models. Organic dust extracts obtained from swine confinement facilities induced neutrophil chemokine production (human IL-8, murine CXCL1/CXCL2). Neutrophil chemokine induction was reduced in human blood monocytes, human bronchial epithelial cells, and murine lung slices pretreated with 1,25-$(OH)_2D_3$. Intranasal inhalation of organic dust extract induced neutrophil influx, and CXCL1/CXCL2 release was also decreased in mice fed a relatively high vitamin D diet as compared to mice fed a low vitamin D diet. These findings were associated with reduced tracheal epithelial cell PKCα and PKCε activity and whole lung TLR2 and TLR4 gene expression. Collectively, vitamin D plays a role in modulating organic dust–induced airway inflammatory outcomes.
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INTRODUCTION

Pulmonary disease is an established occupational hazard of agricultural work [1]. In the United States and worldwide, agriculture systems have increased in size to maximize production, which has led, in part, to the creation of largely indoor swine animal feeding confinement facilities [2]. Agriculture workers in these facilities are at high risk to develop respiratory diseases including chronic bronchitis, obstructive lung disease, and asthma symptoms as a result of their exposure to the organic dust environments [3,4]. However, there is variability in disease occurrence and severity among workers, which has not been entirely explained by levels of dust and/or endotoxin concentrations within the operations [3], suggesting that other host and/or environmental factors may be important. Vitamin D is a potential immunomodulator that may play a role in agricultural-induced lung disease as recent studies have found that low serum vitamin D levels are associated with increased risk of asthma severity and chronic bronchitis [5,6]. Moreover, vitamin D may have a relevant role in indoor farming work because others have found that indoor work correlates with diminished sunlight exposure and lower serum 25-hydroxy (25OHD) vitamin D levels [7–9]. However, the role of vitamin D in agricultural organic dust exposure-induced respiratory disease has not been described.

Organic dust from swine confinement environments represents a complex mixture of particulate matter and a wide diversity of microbial components from Gram-positive and Gram-negative bacteria, archeabacteria, and fungi [3]. Organic dust exposures can elicit airway inflammatory responses marked by neutrophil influx and release of proinflammatory mediators in humans and animals including neutrophil

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chemoattractants, human interleukin (IL)-8, and murine homologs, CXCL1 and CXCL2 [10, 11]. Several important mechanisms to explain organic dust–induced airway inflammatory response have been demonstrated. Activation of protein kinase C (PKC) isoforms is important in mediating dust-induced epithelial cell responses and activation of PKCα and PKCε parallels dust-induced airway inflammatory consequences [11–13]. Innate immune pattern recognition receptors, specifically Toll-like receptor 2 (TLR2) and TLR4, have also been implicated in mediating large animal (swine) confinement organic dust–induced airway disease [10,14,15]. Namely, mice deficient in TLR2 and TLR4 demonstrate reduced airway inflammatory responses following swine confinement organic dust exposures [10, 15]. However, practical strategies other than physical strategies to prevent and/or alleviate airway disease from exposure to complex organic dusts are lacking.

There is mounting evidence to support a potential role for vitamin D to reduce inflammatory respiratory diseases. Mice deficient in the vitamin D receptor show increased NF-κB activity [16], and, moreover, this increased NF-κB activity leads to an increase in lung inflammation, cytokine production, and increased fibroblast cells [17]. In addition, exposure to 1,25-$(OH)_2D_3$ (active vitamin D) potentiates the beneficial effects of allergen immunotherapy in the mouse model of allergic asthma [18]. Importantly, treatment with 1,25-$(OH)_2D_3$ has been shown to reduce peripheral blood mononuclear cell TLR2 and TLR4 expression [19] and decrease lipopolysaccharide (LPS)-induced inflammatory cytokine production in bone marrow-derived monocytes [20]. Finally, in severe asthmatics, lower serum concentrations of 25OHD have been associated with impaired lung function, increased airway hyperresponsiveness, and reduced response to glucocorticoids [5].

On the basis of these observations, we hypothesized that vitamin D would reduce organic dust–induced inflammatory consequences in vitro and in vivo and that vitamin D would modulate organic dust–induced PKC isoform activation and TLR2 and TLR4 expression.

METHODS

Swine Facility Organic Dust Extract

Organic dust was collected from settled dust (~3 feet above floor level) at local swine confinement feeding operations housing more than 500–700 hogs as previously described [11]. Briefly, aqueous dust extracts were prepared by incubating 1 g of the dust in 10 mL of Hank's balanced salt solution (Life Technologies, Grand Island, NY) at room temperature for 1 h. The mixture was centrifuged twice at $500\times g$, and final supernatant was filter (0.22 μm) sterilized, which is a process that also removes coarse particles. Batches of stock (100%) aqueous extracts (organic dust extract **(**ODE)) were frozen at –80°C, and ODE was diluted (vol/vol) in growth media or sterile phosphate-buffered saline (PBS) for in vitro and in vivo experimental studies, respectively. The diluted 1%–5% ODE has previously been determined to elicit optimal proinflammatory chemokine release from cultured cells [13, 21], and the diluted 12.5% ODE has been shown to elicit maximal airway inflammation in mice and is well tolerated [10]. Complete analysis of the dust extract has been previously published [14]. Briefly, stock (100%) ODE contains 23.3–31.1 mg/mL of total protein as measured by nanodrop spectrophotometry (NanoDrop Technologies, Wilmington, DE). Endotoxin levels in stock ODE range from 184 to 760 EU/mL as assayed using the limulus amebocyte lysate assay according to manufacturer's instruction (Sigma, St. Louis, MO).

Cell Culture

BEAS-2B cells (American Tissue Culture Collection, Manassas, VA), an SV40-transformed human bronchial epithelia cell line, were plated on type I collagen-coated (Celtrix Laboratories, Palo Alto, CA) 100 mm × 155 mm dishes and maintained in serum-free LHC-9-RPMI (Sigma-Aldrich, St. Louis, MO) supplemented with Fungizone and penicillin/streptomycin (Invitrogen, Grand Island, NY) at 37°C in 5% CO_2. Confluent monolayers (~80% confluency) were pretreated with or without 1,25-$(OH)_2D_3$ (100 nM) (Sigma-Aldrich) for 18 h. Next, cells were washed and fresh control or vitamin D-supplemented media was reapplied and epithelial cells were simulated with 5% ODE or saline for 24 h. Cell-free supernatants were collected and frozen at –80°C for later chemokine analysis. Cells were harvested to determine protein concentrations by a nanodrop spectrophotometer. Viability of cell cultures was assured by lactate dehydrogenase assay (Sigma-Aldrich).

Human peripheral blood monocytes were collected from volunteer donors at the institution's Elutriation Core Facility as previously described [21]. Briefly, monocytes were isolated by countercurrent centrifugal elutriation of mononuclear leukocyte-rich fractions and purity of monocyte populations confirmed by determination of CD14 cell surface expression by flow cytometry (>99%). Peripheral blood was taken with written informed consent, and studies were approved by the University of Nebraska Medical Center

Institutional Review Board. Cells (1×10^6/mL) were cultured in complete RPMI supplemented with Fungizone (Invitrogen) and penicillin/streptomycin (Invitrogen). Consistent with epithelial cell experimental studies for chemokine production assessment, monocytes were pretreated with or without 100 nM of 1,25-$(OH)_2D_3$ for 18 h, whereupon cells were washed, and stimulated with 1% ODE in the presence or absence of freshly supplemented vitamin D media for 24 h. Cell-free supernatants were collected and frozen at –80°C until later chemokine analysis. Cell viability was assured by trypan blue exclusion method.

Preparation of Murine Lung Slices

Precision-cut murine lung slices from control C57BL/6 mice were prepared in cultures as previously described [22]. Lung slices were pretreated with 1,25-$(OH)_2D_3$ (100 nM) for 18 h, and subsequently restimulated with or without 5% ODE for 24 h. Cell-free culture supernatants were collected and stored at –80°C for later analysis.

Animal Model and Exposure

Male C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME) at 3–4 weeks of age. Upon arrival, mice were randomly assigned to a specially ordered rodent chow (Land O' Lakes-Purina; Minneapolis, MN) diet of standard soy-based (soy naturally contains small amounts of vitamin D) rodent chow supplemented with 0 IU/g or 5 IU/g of 1,25 hydroxyvitamin D. Manufacturer-provided samples of the diet were independently tested by N-P Analytical Laboratories (St. Louis, MO), which reported concentrations of 2.3 IU/g and 5.55 IU/g of 1,25-$(OH)_2D_3$ in the low and high concentration diets, respectively. Mice were weighed twice weekly to assure proper and equal growth between groups. At age 8 weeks (chosen because the half-life of the active form 1,25-$(OH)_2D_3$ is approximately 7 days) [23], mice were anesthetized by isoflurane and treated with 50 μL of ODE (12.5%) or sterile saline via an intranasal route as previously described [11]. All experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Nebraska Medical Center.

Whole Lung Lavage

Five hours postexposure, animals were euthanized by intraperitoneal injection of 50 mg/kg of sodium pentobarbital (Nembutal; Abbott Labs, Chicago, IL). The trachea was exposed, and a cannula was intraluminally placed just below the larynx. Bronchoalveolar lavage fluid (BALF) was collected by whole lung lavage with 3×1 mL of sterile PBS as previously described [11]. Cell-free BALF supernatant from first lavage was frozen at –80°C for later chemokine analysis, and cells from all three lavages were pooled. Total cells were enumerated and spun onto slides with Cytopro cytocentrifuge (Wescor, Logan, UT) and stained with DiffQuick (Dade Behring, Newar, DE). Counts of the cells determined the differential ratio of cell types in 200 cells per slide per mouse.

Chemokine Assays

Murine neutrophil chemoattractants, KC/CXCL1 (cytokine-induced neutrophil-attracting chemokine), and MIP-2/CXCL2, (macrophage inflammatory protein 2-alpha), were quantitated in cell-free BALF supernatants according to the manufacturer's instructions using commercially available ELISA kits (R&D Systems, Minneapolis, MN) with sensitivities of 15.6 and 7.8 pg/mL, respectively. The human neutrophil chemoattractant, interleukin-8 (IL-8)/CXCL8, was quantitated from cell culture supernatant by sandwich ELISA as previously described [12] with sensitivity of 21 pg/mL.

PKC Activity

Protein kinase C (PKC) isoform activity from murine tracheal epithelial cells following in vivo stimulation with ODE and saline was conducted. Whole tracheas were snap frozen in cell lysis buffer and stored at –80°C until later PKCε and PKCα activity assays could be performed as previously described [11]. Prior to assay, the tracheae are thawed and the epithelial cells are extracted using a sterile cell scraper. The collected epithelial cell fraction in lysis buffer is then sonicated and assayed for kinase activity. PKC activity was expressed in relation to the total amount of cellular protein assayed as picomoles of phosphate incorporated per minutes per milligram. PKC activity is reported as fold-increase from baseline: dust-induced kinase activity divided by saline-alone kinase activity.

Serum Vitamin D

Whole blood was collected from mice at time of sacrifice, and serum 25(OH) vitamin D levels were quantified by the institutions' core clinical standard high-performance liquid chromatography-tandem mass spectrometry methods.

Real-Time Quantitative RT-PCR

Human peripheral blood monocytes were stimulated with 1% ODE or saline for 24 h whereupon RNA was extracted from cell pellets using the Magmax 96 kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. For lung tissue, after BALF was removed, the right ventricle was perfused with 10 mL of sterile PBS with heparin to remove blood from the pulmonary vasculature. Whole lung tissues were harvested and stored in RNA later (Applied Biosystems) until RNA extraction could be performed by using TRIzol reagent (Invitrogen). The RNA concentration and purity was determined by NanoDrop spectrophotometer, and samples had A260/A280 ratio of 1.9–2.0. cDNA was synthesized as previously described [24]. Real-time PCR reactions were prepared in triplicate using 1× TaqMan master mix (Applied Biosystems) and primers and probed for TLR2 (Applied Biosystems; human: Hs00152932_m1; mouse: Mm00442346_m1) and TLR4 (human: HS00152939_m1; mouse: Mm00442346_m1). Ribosomal (18s) RNA was used as an endogenous control. PCR was performed using an ABI PRISM 7700 sequence detection system (Applied Biosystems). Threshold values were normalized to the expression of ribosomal RNA. Real-time-PCR results are either expressed as the percent fold-increase in induction (normalized copy number of stimulated cells divided by normalized copy number of unstimulated cells × 100) or as values normalized to expression of ribosomal RNA.

Flow Cytometry

Human peripheral blood monocytes were evaluated for cell-surface molecule expression by means of flow cytometry for TLR2 and TLR4 after incubation with and without vitamin D (100 nM) for 24 and 48 h. Cells (5×10^5) were stained in a standard procedure with antibodies against TLR2, TLR4 (BioLegend, San Diego, CA), and irrelevant isotype control antibodies (to account for nonspecific binding) in PBS containing 0.1% bovine serum albumin. Flow cytometry analyses were performed with the FACSCalibur dual-laser cytometers available in the UNMC Flow Cytometry core (Becton-Dicksinson, Lincoln Park, NJ).

Statistical Analysis

Data are presented as the mean ± standard error of mean (SEM). Statistics were performed using a two-tailed, nonpaired *t* test to determine significant differences between treatment groups. One-way analysis of variance with Tukey multicomparison posttest was employed to compare differences among three or more treatment groups. In all analyses, GraphPad (version 5.01) software was used and p values less than 0.05 were considered statistically significant.

RESULTS

Vitamin D Treatment Reduces Monocyte TLR2 and TLR4 Expression in a Time-Dependent Manner

Because our group and others have demonstrated an important role for TLR2 and TLR4 receptor signaling pathways in mediating ODE-induced airway disease [14, 15], we first sought to confirm recent reports that vitamin D reduces TLR2 and TLR4 monocyte cell surface expression [19]. Treatment with vitamin D reduces monocyte TLR2 and TLR4 mRNA expression as assessed by quantitative real-time PCR and cell surface protein expression as assessed by flow cytometry (Figures 1A and 1B). This was not an immediate response because significant suppression of monocyte mRNA and protein was not observed until 24 h following treatment with vitamin D (data not shown and Figure 1), suggesting that prolonged pretreatment with vitamin D would be required to determine chemokine responses.

Vitamin D Reduces Organic Dust–Induced Epithelial Cell, Monocyte, and Lung Tissue Neutrophil Chemokine Release

We next sought to determine whether vitamin D could downregulate ODE-induced human neutrophil chemoattractant, IL-8, release. ODE-induced IL-8 production was reduced with vitamin D pretreatment (18 h) in both human epithelial cells and monocytes (Figures 2A and 2B). Consistent with single cell culture studies, vitamin D pretreatment resulted in reduced ODE-stimulated CXCL1 and CXCL2 (murine neutrophil chemokine homologs) production from lung slices, a representation of tissue-structured multicellular lung responses.

Dust-Induced Airway Cellular Inflammation and Chemokine Release Is Reduced in Mice Fed a Relatively High Vitamin D Supplemented Diet

Consistent with previous studies [11], acute ODE intranasal inhalation resulted in significant ($p < 0.05$) increases in neutrophil influx and release of murine neutrophil chemoattractants, CXCL1 and CXCL2, in

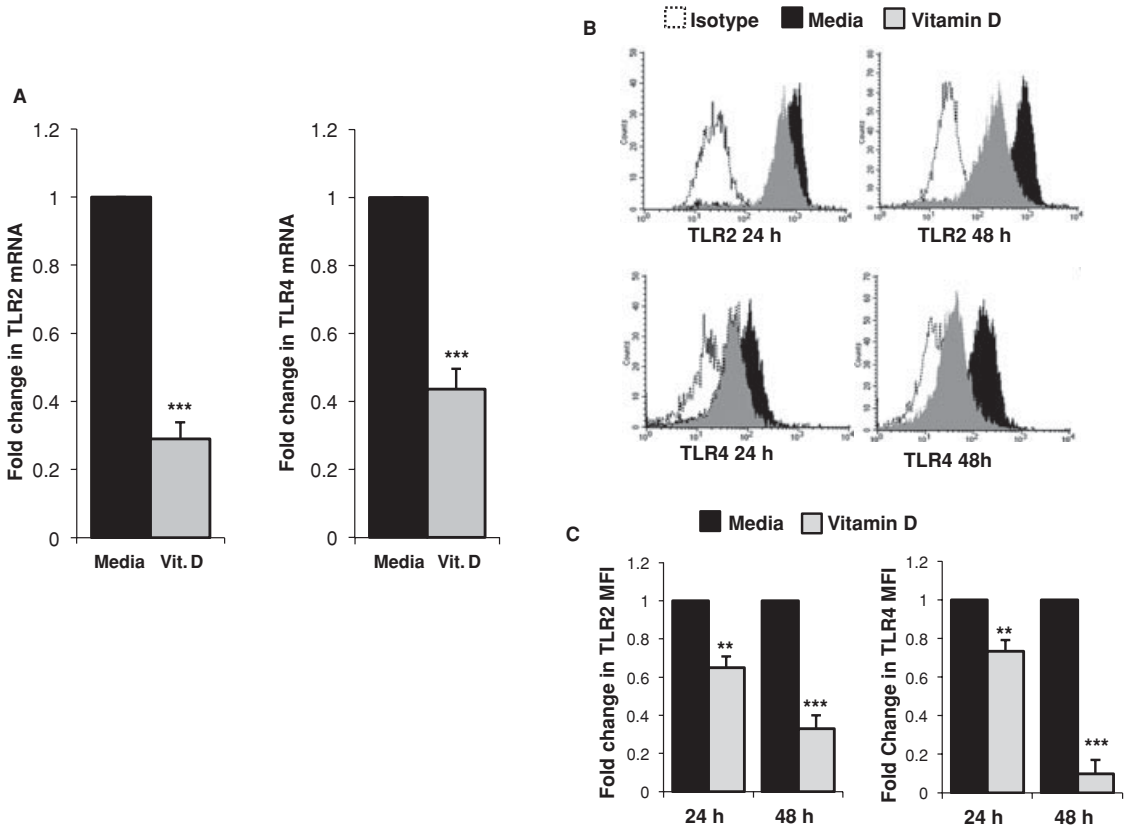

FIGURE 1. Vitamin D treatment decreased TLR2 and TLR4 expression in human peripheral blood monocytes. Vitamin D (100 nmol) decreased TLR2 and TLR4 mRNA at 24 h in human peripheral blood monocytes (A). Results represent mean (± SEM) of TLR2 and TLR4 mRNA normalized to endogenous ribosomal control ($N = 3$ independent experiments). Cell surface protein expression of TLR2 and TLR4 is decreased at 24 and 48 h by flow cytometry in monocytes. (B) Representative histogram of one of four independent experiments and (C) mean ± SEM of the mean fluorescence intensity (MFI) ($n = 4$). Asterisks denote statistical significance as compared to respective media control (*$p < 0.05$, **$p < 0.01$) and ##$p < 0.05$ denotes statistical significance versus ODE alone.

BALF at 5 h postexposure (Figures 3A and 3B). However, the increase in total leukocytes and CXCL1 and CXCL2 release following in vivo ODE challenge was significantly reduced ($p < 0.05$) in mice fed a relatively high vitamin D supplemented diet as compared to mice fed a relatively low vitamin D diet (Figures 3A and 3B). Moreover, serum 25(OH) vitamin D levels were significantly reduced ($p < 0.05$) by approximately 25% in mice given the low vitamin D diet (mean ± SEM: 16.6 ng/mL ± 1.231) as compared to high vitamin D diet (20.5 ng/mL ± 1.009).

Vitamin D Supplementation Reduces Organic Dust–Induced PKCα and PKCε Activity

In our previous work, we reported that PKCα and PKCε activity was important in mediating human bronchial epithelial cell ODE-induced IL-8 release [12] and that tracheal epithelial cell PKCα and PKCε were activated in vivo following organic dust exposure [11]. In this study, there was activation of tracheal epithelial cell PKCα and PKCε in the low vitamin D treatment group following in vivo ODE challenge, which was nearly absent in the high vitamin D + ODE treatment group (Figure 4).

Dietary Vitamin D Supplementation Modulated Whole Lung TLR2 and TLR4 mRNA Response To ODE

To determine whether dietary vitamin D supplementation was important in lung TLR2 and TLR4 expression following ODE exposure, whole lung mRNA expression of TLR2 and TLR4 was investigated following ODE exposure in mice fed a low versus high vitamin D diet for 5 weeks. Mice fed a low vitamin D diet had significantly increased TLR2 and TLR4 expression following ODE exposure as compared to saline exposure (Figure 5). Interestingly, this ODE-induced upregulation in TLR2 and TLR4 mRNA was not observed in mice fed a relatively high vitamin D diet (Figure 5).

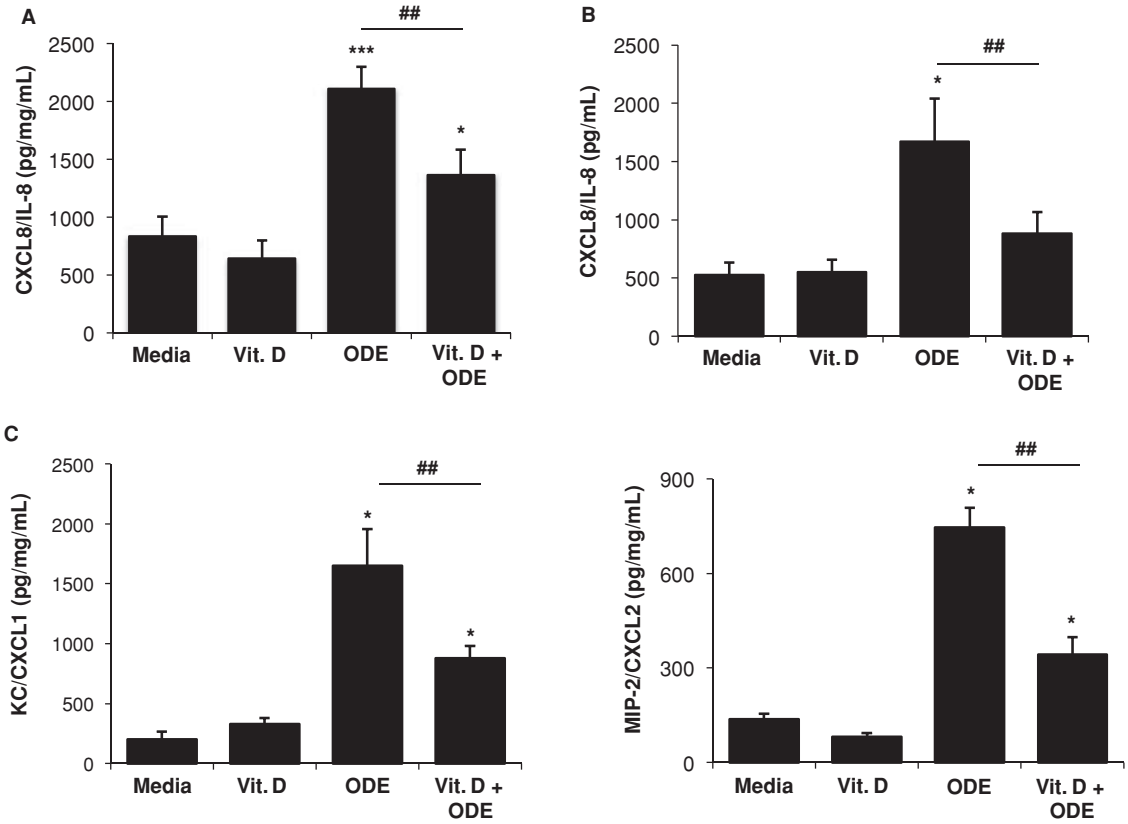

FIGURE 2. Vitamin D pretreatment reduces ODE-induced neutrophil chemoattractant production in bronchial epithelial cells, monocytes, and lung tissue. (A) Human bronchial epithelial cells (BEAS-2B), (B) human peripheral blood monocytes, and (C) murine lung slices were pretreated with vitamin D (100 nmol) or saline for 18 h and subsequently restimulated with or without ODE for 24 h. Results represent mean ± SEM ($N = 7$) of cell-free supernatant levels of neutrophil chemoattractant(s) (human CXCL8/IL-8 and murine CXCL1 and CXCL2) as determined by ELISA. Asterisks denote statistical significance as compared to respective media control (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$) and hatchtexts (#$p < 0.05$) denote statistical significance versus ODE alone.

DISCUSSION

These studies provide evidence that vitamin D may be an important immunomodulator in organic dust–induced airway responses. Vitamin D treatment led to reductions in neutrophil chemoattractant release from ex vivo ODE-stimulated monocytes, epithelial cells, and lung tissues. Furthermore, high dietary intake of vitamin D resulted reduced neutrophil influx and neutrophil chemoattractants release in mice, which was associated with blunted tracheal epithelial cell PKCα and PKCε activity and modulated whole lung TLR2 and TLR4 expression.

Dietary manipulation of vitamin D began immediately following the weaning period (at weeks 3–4), which allowed for normal murine lung development (by week 2) [25]. This distinction in approach is important because deficiencies in vitamin D during early development have been associated with decreased lung volume and lung function, but not alterations in lung architecture [25,26]. Although conversion between human and mouse vitamin D intake cannot be precisely made, the approximated difference between the low versus high vitamin D diet represented a difference of 1500 IU per day for a human. Despite this modest difference, airway inflammatory outcomes differed, suggesting that small increases in vitamin D could have an impact in airway diseases.

Monocyte cell surface TLR2 and TLR4 expression was downregulated with vitamin D treatment, which is consistent with other studies [19], and, moreover, our data support that vitamin D plays a role in modulating lung TLR2 and TLR4 expression following in vivo ODE exposure. Organic dusts within large animal feeding operations are complex and contain diverse mixtures of microbial components [3, 27, 28]. Endotoxins from Gram-negative bacteria are recognized by TLR4, and TLR2 can recognize Gram-positive peptidoglycans, lipoteichoic acids, lipoarabinomannan, zymosan, and other lipoproteins [29]. Mice deficient in TLR2 and TLR4 demonstrate reduced airway inflammatory consequences following acute organic dust challenges

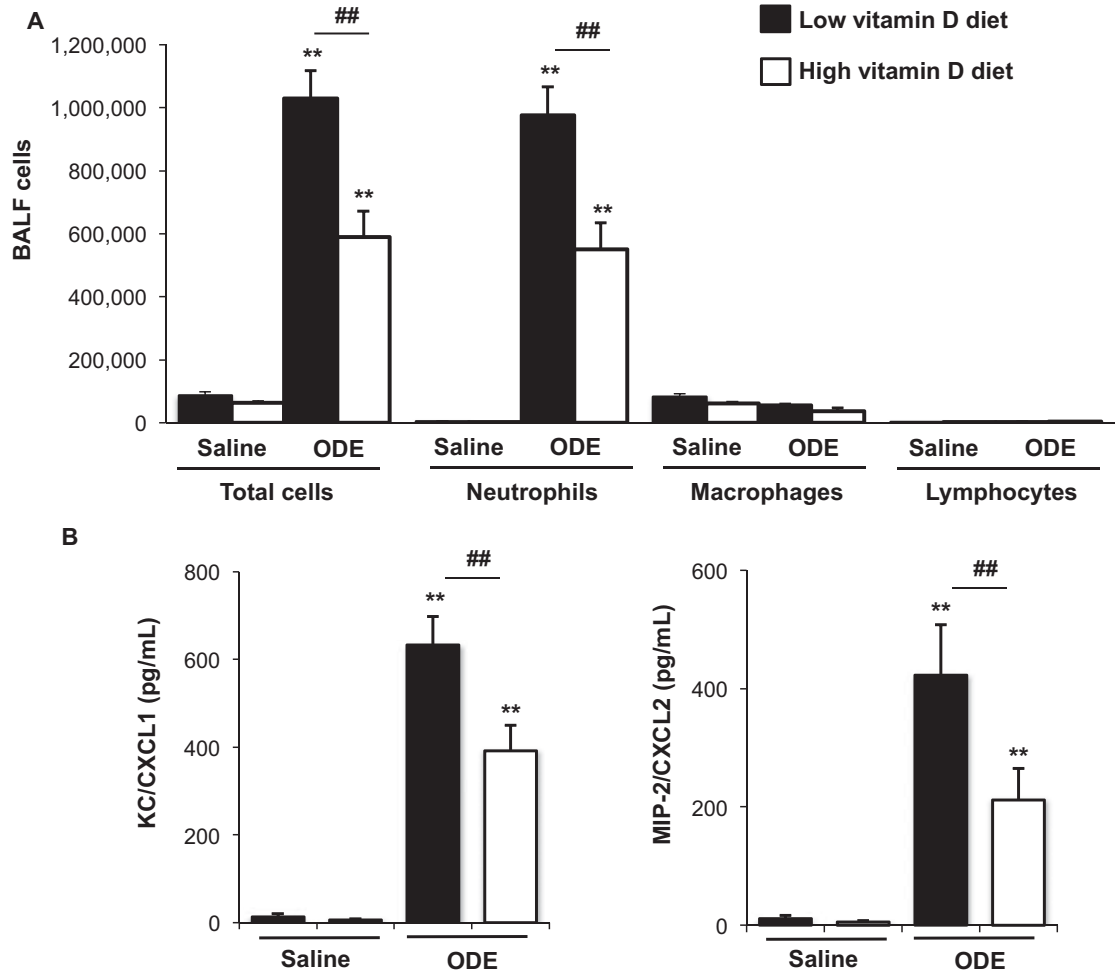

FIGURE 3. (A)—(B): High vitamin D diet resulted in decreased ODE-induced neutrophil influx and neutrophil chemoattractant production in mice. C57BL/6 mice were fed relatively low (2.04 IUD/g) versus high (5.11 IU D/g) vitamin D supplemented rodent chow diet for 6 weeks initiated immediately after weaning (age 3–4 weeks). Mice were treated with ODE or sterile saline via intranasal inhalation. Results represent (mean ± SEM) of whole lung lavage total cell counts and differential (A) and levels of neutrophil chemokines, CXCL1 and CXCL2 (B) at 5 h postexposure. Asterisks (**$p < 0.01$) denote statistical significance compared to matched saline control. Hatchtexts (##$p < 0.01$) denote statistical significance comparing high versus low vitamin D diet. Experimental study with $N = 4$ mice per group, repeated twice with representative sample shown.

[14, 15]. Therefore, vitamin D supplementation may represent a clinically relevant translational approach to intervene in agriculture workers to reduce airway disease burden through its impact on TLR signaling pathways.

Several immunomodulatory properties have been ascribed to vitamin D on monocytes including decreased cytokine production through activation of NF-κB following bacterial challenges as well as enhancement of macrophage function [19,30,31]. We found that vitamin D reduced the release of neutrophil chemoattractants from ODE-stimulated monocytes and also epithelial cells. This observation was extended to an animal model with the functional consequence of reduced neutrophil recruitment following ODE exposure. This later finding is consistent with other studies showing a role for 1,25-$(OH)_2D_3$ as a negative regulator for neutrophil activation among differing cell types [16, 32]. Finally, previous studies have linked PKCα/PKCε activity with ODE-induced neutrophil recruitment [11] and our data suggest that vitamin D decreases neutrophil recruitment through its action on epithelial cells PKCα/PKCε activity. It is possible that the reduction in PKC activity was secondary to diminished TLR pathway activation via downregulation of TLR expression by vitamin D. Others have reported that MyD88 is an important scaffolding protein coupling TLRs to PKC epsilon [33]. Lung cell specific roles need to be further defined.

Complex organic dust mixtures from industrialized livestock farming environments elicit respiratory disease in exposed workers; yet considerable

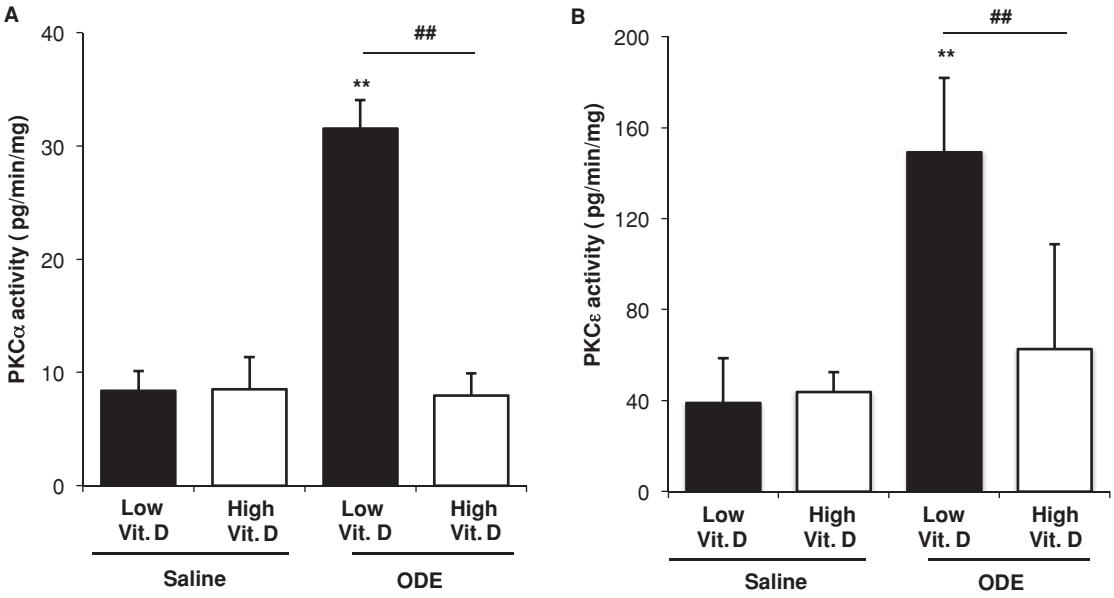

FIGURE 4. ODE-induced murine trachea epithelial cell PKCα and PKCε activity is significantly reduced in mice fed a relatively high vitamin D diet. C57BL/6 mice were fed relatively low (2.04 IUD/g) versus high (5.11 IU D/g) vitamin D supplemented rodent chow diet for 5 weeks initiated immediately after weaning (age 3–4 weeks). Results represent mean ($\pm$ SEM) of PKCα and PKCε activity from isolated tracheal epithelial cells at 5 h following in vivo ODE or sterile saline treatment. Asterisks (**$p < 0.01$) denote statistical significance compared to matched saline control. Hatchtexts (#$p < 0.05$, ##$p < 0.01$) denote statistical significance comparing high versus low vitamin D diet.

variability in respiratory disease outcomes occurs among workers. As agriculture work becomes more specialized and indoors, the likelihood that workers will be exposed to decreasing amounts of natural sunlight exists and vitamin D may be a potential confounder. It is not known whether workers are deficient in vitamin D levels, but our findings suggest that future investigations are warranted because dietary vitamin

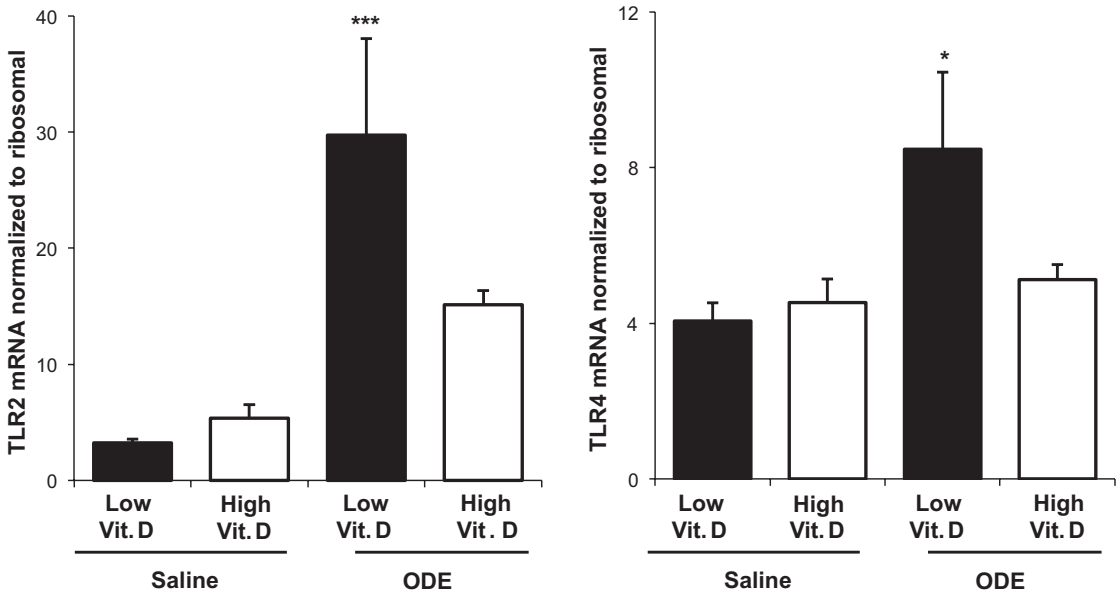

FIGURE 5. High dietary Vitamin D is associated with reduced TLR2 and TLR4 mRNA expression in mice following ODE exposure. C57BL/6 mice were fed relatively low (2.04 IUD/g) versus high (5.11 IU D/g) vitamin D supplemented rodent chow diet for 6 weeks initiated immediately after weaning (age 3–4 weeks). Whole lung tissue TLR2 and TLR4 mRNA expression was increased in mice that consumed a relatively low vitamin D diet and exposed to ODE as compared to sterile saline treated mice. ODE-induced TLR2 and TLR4 mRNA expression in lung tissue was not observed in mice fed a high vitamin D diet ($N = 7$–8 mice/group). Asterisks denote statistical significance as compared to respective media control (*$p < 0.05$, **$p < 0.01$) denotes statistical significance versus ODE alone.

D supplementation may be an important therapeutic option.

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