Original Article

Swine barn dust stimulates CCL9 expression in mouse monocytes through protein kinase C-delta activation

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Abstract

Objective: Exposure to organic barn dusts has been shown to cause numerous lung problems to chronically exposed animal barn workers. Bacterial components in these dusts trigger innate immunity in the lungs. In characterizing these responses, we examined the expression of a lesser examined chemokine believed to be a lower avidity signal for neutrophil migration, CCL9, and how its expression is controlled by dust exposure in a monocyte/macrophage cell line.

Materials and Methods: CCL9 expression was assessed in the RAW267.4 macrophage cell line exposed to organic hog barn dust extracts (HDEs) or components of this dust such as lipopolysaccharide (LPS) and peptidoglycan. CCL9 expression was assessed as well as response of CXCL1 (keratinocyte-derived chemokine [KC]). Protein kinase C (PKC)-α, δ, and ζ were inhibited to assess CCL9 expression.

Results: HDE was able to induce significant increase of CCL9 expression in RAW264.7 cells. The ability of HDE to induce CCL9 relied upon LPS present in HDE samples. Addition of CCL9 to RAW264.7 cells stimulated with organic dust reduced KC expression. Further, CCL9 expression was particularly sensitive to PKC-δ inhibition by chemical or siRNA.

Conclusion: CCL9 is an inducible chemokine present in mouse monocytes exposed to HDE. HDE-induced production of CCL9 appears primarily mediated by LPS in HDE samples. This induction appears to require PKC signaling, with emphasis on PKC-δ expression.

Keywords: Agricultural dust, CCL9, inflammation, lung, monocyte, protein kinase C

INTRODUCTION

Workers in high intensity livestock facilities are exposed to high quantities of organic dust and microorganisms. These exposures can lead to problems such as increased wheeze, chronic bronchitis, asthma and chronic obstructive pulmonary disease. Aside from these problems, workers can also show a general decrease in lung function over their career.[1] These responses are attributed to microbial components in workplace organic dusts, such as endotoxin[2-5] and peptidoglycans (PGNs),[6,7] although a large host of components may also play a role in these

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responses.[8,9] These components trigger innate immune responses through receptors such as toll-like receptor 2 (TLR) 2 and TLR4[2,10] and activate protein kinase C (PKC) signaling,[11] inducing a wide array of immunological changes in the lung. While a number of cytokines and chemokines have been shown to be produced in response to organic dusts, others have yet to be studied.[4,6,7]

Monocytes and myeloid cell lines produce large quantities of CCL9,[12] as do dendritic cells[13] and T cells, in particular Th1 type T cells.[12] Expression of CCL9 also occurs across a broad range of tissues.[13] CCL9 is expressed constitutively in levels as high as 1 mg/ml in the blood of mice.[13] The chemokine binds to the CCR1 receptor[13,14] and while not the most avid binder of CCR1, it is estimated to bind as much as 70% of available CCR1.[13] CCL9 has been shown to induce chemotaxis of and calcium release in neutrophils.[13] Beyond this, little seems to be known of the effects of CCL9 or the signaling leading to its production.

Lung lavages show levels of CCL9 in the lungs that are several hundred times lower than what is present in serum. Upon stimulation with injected lipopolysaccharide (LPS), CCL9 levels were shown to greatly increase,[13] specifically in the lungs, suggesting that CCL9 lung expression is more limited and inducible. Indeed, a mouse silicosis model, suggests a possible role for this chemokine in inflammation, lung injury, and response to particulate insult.[15] In addition, in a recent study we show that CCL9 was specifically increased in response to hog barn dust extract (HDE) exposure, and that low level increases of CO_2 were capable of greatly increasing CCL9 expression both at the protein and mRNA levels in lung.[16] Given that CCL9 may be dynamically controlled in barn environment exposures by factors other than LPS, we sought to investigate the mechanism of CCL9 regulation and control in mouse cells exposed to hog barn dusts. In particular, we were interested in observing any effect of CCL9 on keratinocyte-derived chemokine (KC) production given the shared neutrophil activation capabilities of both. We established the kinetics of exposure to HDE and examined which microbial products may be responsible for the CCL9 response to these dusts. We further show that the expression of CCL9 in response to HDE is regulated by PKC-delta.

MATERIALS AND METHODS

Hog confinement dust extract

Extracts of hog barn dusts were derived from samples of settled dust combined from two swine confinement facilities as has been previously described.[17] Briefly, 1 g of dust was suspended in 10 ml of phosphate buffered saline (PBS) (Dulbecco's PBS, pH 7.4, Grand Island, NY, USA) without calcium at room temperature for 1 h. This was centrifuged 10 min before sterile filtration of the supernatant, for a final concentration of approximately 0.1 g dust/ml dust extract. These extracts have been previously characterized for endotoxin (22.5–48.75 EU/ml), muramic acid (400 pg/ml), and protein (1–2 mg/ml) in a 5% extract.[18] Characterization of bacterial sources of these components has also been previously determined.[19] Extracts were used in cell cultures at a concentration of 1% v/v of culture well (15 μl), or about 0.0016 g/ml. Heat inactivated samples were boiled for 1 h at 100°C. Scrubbing of endotoxin was done via polymixin B column (Pierce, Rockford, IL, USA).[4]

Cell culture and treatments

RAW264.7 mouse macrophage cell line (ATCC, Rockville, MD, USA) was used for most experiments. Cells were cultured as per ATCC recommendations in DMEM + 5% fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA) plus 1% penicillin/streptomycin (Gibco). Frozen cell line aliquots (8 passages) were used and cultured for no more than 4 passages. The same passage (third) was used for all cultures to minimize any possibility of genetic drift. Cells were grown in 6-well tissue culture plates (ThermoFisher, Waltham, MA, USA) at a concentration of 0.5×10^5 cells/well in 1.5 ml media. Cultures were carried out for 24 h unless otherwise specified. Cells were treated with either 1% v/v HDE,[18] 1% v/v heat inactivated HDE, 100 EU LPS/well (*Escherichia coli* O55:B5, Sigma Aldrich, St. Louis MO), 10 ng/ml Pam3CSK4 (Sigma), or 100 ng/ml (or 150 ng/well) PGN (*Staphylococcus aureus* PGN, Sigma).

For PKC inhibition studies, cells were treated with PKC inhibitors 1 h prior to exposure to HDE. Inhibitors for PKC-α (Gö 6976, EMD Millipore, Bedford, MA, USA), and PKC-ζ (Biomedical Research Laboratories, San Diego, CA, USA) were used at a concentration of 1 μM. PKC-δ inhibitor (Rottlerin; EMD Millipore) was used at 20 μM. These inhibitor doses have been used previously by our group and were determined to have no significant effect on cell viability at the concentrations used, and shown to inhibit PKC in RAW264.7 cells.[6,20,21] All inhibitors were soluble in aqueous media.

Cytokine/chemokine enzyme linked immunosorbant assay

Expression of CCL9 in cell culture media was quantified by a CCL9 enzyme linked immunosorbant assay kit (RandD Systems, Minneapolis, MN, USA) according to manufacturer's instructions.

siRNA inhibition

siRNA to the PKC-δ sequence (SMARTpool ON-TARGETplus, L-040147-00) and ON-TARGETplus Nontargeting Control was obtained from Dharmacon (Lafayette, CO). LA4 cells (ATCC, Rockville, MD), a mouse alveolar epithelial line, were used for transfection of siRNA due to an inability to successfully transfect RAW264.7 cells in several trials using this or another vector. Similar to RAW264.7 cells, frozen LA4 aliquots (passage 3) were used and cultured for no more than four passages to minimize the potential of genetic drift. Second passage of LA4 aliquot was used in each case.

Earlier work in our lab revealed problems with siRNA inhibition in RAW cells so transfection was done in a similar LA4 line. LA4 cells were grown identical to RAW264.7 cells and transfected as per the recommended DharmaFECT siRNA transfection protocol (GE Bioscience). Cells were plated in 6-well tissue culture plates at 0.5 × 10^6 cells/well in 1.5 ml Optimem + L-glutamine (ThermoFisher) +10% FBS antibiotic-free media overnight. The next day, lipofectamine RNAiMAX (ThermoFisher) was added at 6 μl to 244 μl serum-free Optimem media and mixed with siRNA at 100 nM concentration in a similar volume and incubated for 15 min at room temperature. The resulting mixture was added to wells that had been washed 1X with PBS and 1.5 ml of serum-free Optimem added back, for a final total volume of 2.0 ml media/well. Cells were incubated for 24 h to bind and transfect. Cells were then washed briefly with PBS and treated with 1% HDE or media alone in serum-free Optimem. Cell media was harvested and tested for CCL9 production.

Statistical analysis

Data were analyzed using GraphPad Prism (GraphPad Software, San Diego, CA, USA). Graph bars represent mean ± standard error. Statistical significance was determined using ANOVA, with 95% confidence interval being considered significant and *post hoc* Bonferroni tests, with $P \leq 0.05$ confidence interval being considered significant.

RESULTS

CCL9 is constitutively produced and induced by hog barn dust extract in RAW264.7 cells

Initially, we tested the effect of HDE at levels of 1% v/v on RAW cells, similar to our previously optimized HDE concentration for monocytes.[22,23] We started with a time course [Figure 1], from 1 to 24 h. As there is a level of constitutive CCL9 expression in these cells, we did not continue the time course beyond 24 h due to excessive buildup of chemokine in culture media. We show that

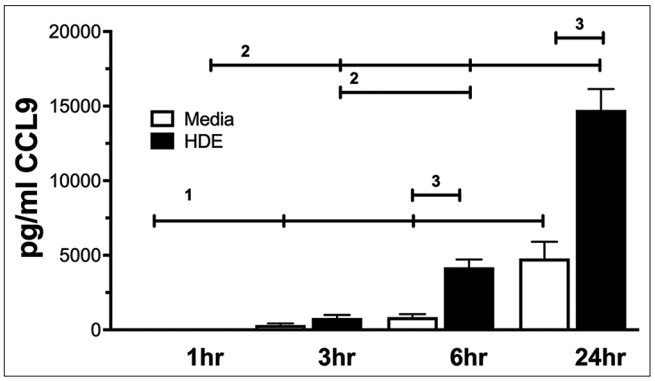

Figure 1: Hog barn dust extract increases CCL9 expression in RAW264.7. Cells were treated for 1, 3, 6, or 24 h with either media or hog barn dust extract at 1% v/v and measured for CCL9. Bars represent average of 3 replicates performed 3 times. Error bars represent standard error mean. 1 = significant between media treatments, $P < 0.05$ or lower; 2 = significant between hog barn dust extract treatments, $P < 0.05$ or lower; 3 = significant between media and hog barn dust extract treatments, $P < 0.05$ or lower

CCL9 was significantly elevated by HDE by 6 h over control, and increased in relation to media concentrations up to 24 h. There was a significant accumulation of CCL9 in cultures over time in media control and HDE treated cells.

CCL9 is produced in response to lipopolysaccharide and peptidoglycan

To further test which components of HDE are responsible for CCL9 production, we tested LPS and PGN, both present in HDE[7] for their ability to induce CCL9 from these cells. In agreement with Poltorak *et al*.[13] LPS induced CCL9 in RAW264.7 cells, as did PGN [Figure 2]. We further tried heat inactivation of the HDE by boiling samples to inactivate both components via thermal degradation. This effectively eliminated most CCL9 expression. Further, endotoxin specific scrubbing of HDE via polymixin B column[7,17] showed a similar removal of CCL9 expression [Figure 2].

RAW264.7 macrophage and LA4 epithelial cells produce CCL9 and protein kinase C-δ is involved in CCL9 induction

To better understand how induction of CCL9 occurs, we investigated PKC signaling in RAW264.7 cells. Previously, we determined that PKC-α, δ, and ζ are all expressed in monocytes.[6] Using inhibitors to PKC-α (Gö 6976), δ (rottlerin), and ζ (myr-PKC ζ inhibitor peptide) isoforms, we showed that PKC-δ was capable of almost totally eliminating CCL9 expression in RAW cells stimulated with HDE [Figure 3]. Background CCL9 levels were also reduced with rottlerin treatment, but not significantly. Interestingly, we also saw what appeared to be a significant enhancement of CCL9 with PKC-ζ treatment. Due to potential lack of specificity with the rottlerin/PKC-δ chemical inhibitor, we confirmed these results by treating LA4 cells for 24 h with siRNA against PKC-δ before treatment. We show that LA4 epithelial cells

can produce CCL9 and that siRNA to PKC-δ was able to significantly inhibit CCL9 production to HDE administration [Figure 4]. Similar to rottlerin treatment, background CCL9 production was not significantly affected by siRNA. This suggests that rottlerin is inhibiting upregulation of CCL9 to HDE rather than generally depressing CCL9 levels. Levels of CCL9 were higher in LA4 cells in general due to the use of a different cell line and longer culture times required for the siRNA inhibition protocol.

CCL9 inhibits keratinocyte-derived chemokine production in RAW cells

As no specific function of CCL9 in the lungs of mice is known, we examined the impact of CCL9 on other cytokines as a possible method of action. Because KC plays a strong role in neutrophil recruitment to lungs in response to HDE,[17] we examined its expression when CCL9 was administered in addition to HDE treatment. We show that CCL9 was able to significantly inhibit the expression of HDE-stimulated KC in RAW264.7 cells at levels comparable to what are seen in circulation [Figure 5].[13,24]

DISCUSSION

CCL9/MIP-1 γ was discovered over 20 years ago, partially due to the very high levels that exist in mouse serum.[13] Since then, very little has been added to our knowledge of this chemokine. Given that CCL9 is constitutively produced in such high quantities in serum, yet inducible in

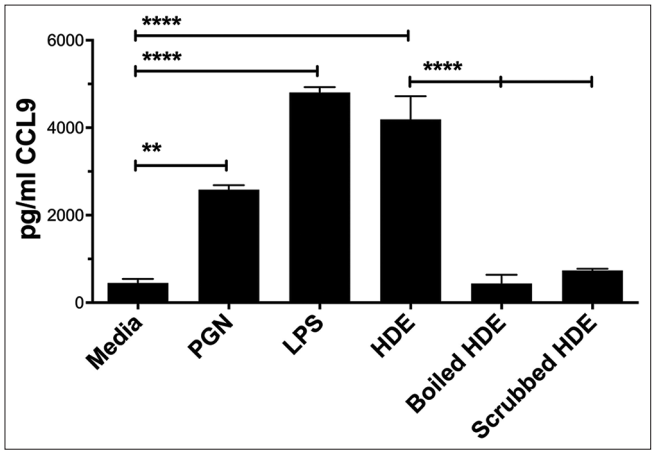

Figure 2: CCL9 is produced in response to lipopolysaccharide and peptidoglycan. RAW264.7 cells were treated 6 h with either media, lipopolysaccharide, peptidoglycan, hog barn dust extract (1% v/v), or hog barn dust extract that was boiled for 1 h or scrubbed with polymyxin B. Bars represent CCL9 protein average of 3 replicates performed 3 times. Error bars represent standard error mean. $^{**}P < 0.01$, $^{****}P < 0.0001$

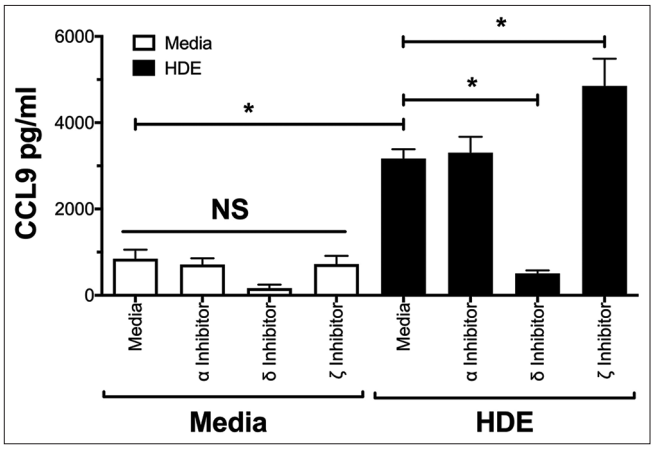

Figure 3: Protein kinase C-δ inhibitor is involved in production of CCL9. RAW264.7 cells were treated 1 h prior to administration of hog barn dust extract with inhibitors to protein kinase C-α, δ, and ζ. hog barn dust extract was then administered and cells incubated an additional 6 h with inhibitors still present. Bars represent average of 3 replicates performed 3 times. Error bars represent standard error mean. $^{*}P < 0.01$. NS = No significance

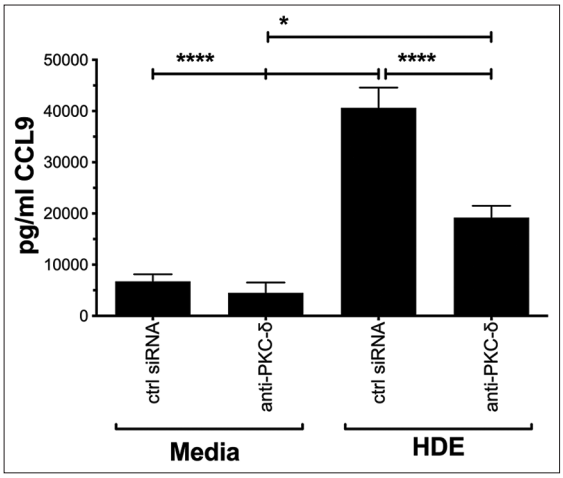

Figure 4: Protein kinase C-δ siRNA blocks CCL9 protein expression. siRNA containing either a nontargeting control (null) or protein kinase C-δ inhibiting sequence (protein kinase C -δ) was transfected into LA4 cells for 24 h prior to treatment of cells with either media or hog barn dust extract for 6 h. Release of CCL9 was measured. Bars represent average of 3 replicates performed 3 times. Error bars represent standard error mean. $^{*}P < 0.05$, $^{****}P < 0.0001$

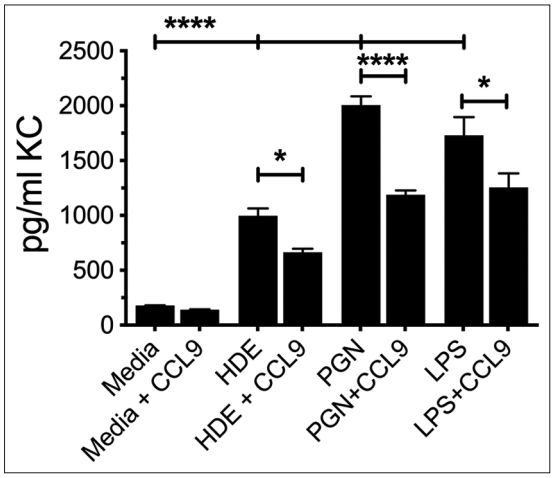

Figure 5: CCL9 inhibits stimulated keratinocyte-derived chemokine protein expression. Purified CCL9 was administered (20 ng/mL) to RAW264.7 cells concurrent with lipopolysaccharide, peptidoglycan, or hog barn dust extract treatment and incubated for 6 h. Bars represent average of 3 replicates performed 3 times. Error bars represent standard error mean. $^{*}P < 0.05$; $^{****}P < 0.0001$

several anatomical compartments such as bone, skin, gut, or lung,[13,25-28] it suggests a potentially complex role for this chemokine that may rely on the context of the cells involved. While one of the first CCL9 papers noted that systemic LPS administration could drive up CCL9 specifically in the heart and lungs,[13] this result appears not to have been followed up in much detail. One of the few reports to note specific changes in CCL9 was in a lung silicosis model.[15] Here, increased CCL9 was found to be higher in female mice compared to males, which correlated with less fibrosis in females, but increased inflammation, primarily by macrophages. While not definitive in causing increased inflammatory cell influx into BAL, this function did agree with earlier reports that suggest a chemotactic role for CCL9.[13,29]

In the bone, CCL9 is produced at the highest tissue levels, and is critical to osteoclast versus osteoblast differentiation of macrophages.[25,26] There are also indications of a timed-specific induction of CCL9 in skin wound healing[27] and follicle-associated epithelium of the gut.[28] Beyond this, little seems to be known of the effects of CCL9 or the signaling leading to its production.

Given that HDE is a particulate exposure that causes significant inflammation in the lungs,[1] we thought to examine its ability to induce CCL9 in monocytes, showing it could induce significant increase in CCL9 by 6 h [Figure 1]. This appears to agree with data from skin wounding studies.[27] Production of increased CCL9 is also early enough to potentially impact other key lung cytokines such as KC, though possibly less so with earlier response cytokines such as tumor necrosis factor-α and interleukin-6.[11]

Earlier work in this cell line shows that LPS can induce CCL9.[13] The active form of both LPS and PGN were able to induce CCL9 [Figure 2]. Both are considered important mediators of barn dust-induced inflammation.[1] As expected, scrubbing dust to remove LPS or boiling to remove LPS and PGN activity reduced CCL9 levels, showing the importance of endotoxin in CCL9 production. This work does suggest that both LPS and PGN activity may be major causes for increased CCL9 production in HDE exposures. This may correspond with a TLR2 or TLR4 mechanism. While LPS appears to be more important in the production of CCL9, we cannot rule out synergism with PGN or other factors in our dust sample, or shifting of peak cytokine expression periods with or without such synergism. We do note that given the complexity of HDE samples that boiling may allow some unspecified components of the dust to adhere to storage tubes and reduce their levels in the final treatment.

Very little is known about the induction path of CCL9. One paper shows a role for retinoic acid receptor-α[30] and another for TLR9.[31] In studying CpG induction of CCL9 in microglial cells, it was found that PI3K, p38, MAKP and ERK inhibitors were all capable of reducing CCL9 expression.[31] Subsequent studies confirm a role for p38 in CCL9 control in myeloid cells.[32] As we have previously shown a role for PKC in immune responses to HDE,[11] we examined the role for this protein kinase family in CCL9 induction [Figure 3]. Of the three PKC isoform inhibitors tested, PKC-δ was very effective at inhibiting induced CCL9 expression. Interestingly, we also observed a slight, but significant increase, in CCL9 with inhibition of PKC-ζ, suggesting different roles for different PKCs on CCL9 expression. We also demonstrate in the process that mouse lung epithelial cell line LA4 is capable of CCL9 production as well.

Chemotaxis and calcium release in neutrophils[13,29] and an association with increased inflammatory conditions[15] or myeloid tumor cell survival via increasing phosphor-AKT and BCL-2[32] are a few of the functions attributed to CCL9 outside the bone environment. We show that KC expression was significantly reduced by administration of purified CCL9 to RAW cells treated with HDE [Figure 5], suggesting a possible moderating or inhibiting role for CCL9. While there is evidence to suggest CCL9 is an inducible chemotactic signal[13,15,29] this would not preclude it from altering cytokine/chemokine expression of the cells that migrate to the site of production. Alternately, as CCL9 is a less stimulatory ligand for CCR1, increased CCL9 may competitively exclude more inflammatory chemokines from binding the receptor, moderating an otherwise strong inflammatory milieu. Alternatively, ligation may result in induction of CCR1 inhibitory mechanisms that may be triggered as a result of CCL9 ligation of CCR1, inducing earlier inhibition. Given that in circulation there are normally very high levels of CCL9,[13] CCL9 may work to establish or maintain homeostasis. This however is much less clear in a location like the lung where lavage levels appear to be approximately ×20 less than in circulation and where CCR1 should subsequently be less saturated with CCL09.[15]

Another possibility is that CCL9 exerts some subtle as yet to be defined effect on innate immunity. We know that it is induced by a variety of insults such as injury,[27] TLRs,[31] and certain particulates.[15,33] The effect may be pro-inflammatory, or transformational on one or more cell types, if results in other tissues can be translated to other tissues such as the lung. In our work, blood levels of CCL9 appeared unresponsive to lung instillation with HDE or

CO_2 inhalation in a mouse model, while being significantly induced in lung.[16]

As with any cell culture study, our results are limited in that the cell culture work may not perfectly reflect what is in whole organisms such as mice. Similarly, some of these results are generated from two tumor cell lines (RAW264.7 and LA-4), so genetic abnormalities in these lines may create other irregularities in comparison to primary cell lines. We also note that care should be taken in interpretation of results between RAW264.7 and LA-4 cells. Both comprise different cell types that exist within the lung. For example, work by others has shown a role for PKC δ in switching of ribosomal protein S3 (RPS3) to aiding in DNA repair,[34] with a subsequent report looking at the role of RPS3 in production of a range of proteins involved in lung immune responses including RAW264.7 and LA-4 cells.[35] While CCL9 was not examined, and there was some similarity between responses of RAW and LA-4 cells, there may still be a possibility of difference between both cell populations. Still, we do again note that inhibiting PKC δ resulted in significantly inhibiting CCL9 in both cell types.

In conclusion, we show that HDE is capable of induction of CCL9 likely through stimulation by LPS and PGN in HDE, and that these increases of CCL9 rely upon HDE-induced PKC-δ signaling. We show a possible role for CCL9 in inhibition of KC production in RAW cells. This could be important in an exposure such as HDE, which is usually accompanied by neutrophil influx.[1,36,37] As CCL9 is constitutively found in great abundance in circulation, however, its role may be less about chemotaxis to the lung. Given the role CCL9 plays in bone osteoclast/osteoblast differentiation, a logical next step is to examine if CCL9 plays a similar role in differentiation of macrophage populations in the lung.

Given its abundance upon HDE stimulation, CCL9 may also serve as a useful marker of exposure to such organic dusts.

CONCLUSION

CCL9 is produced in mouse monocytes to HDE exposure. This production appears to rely heavily on the presence of LPS in these dusts. CCL9 production further appears to rely on PKC-δ expression in these cells. CCL9 can inhibit expression of KC in mouse monocytes.

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Conflicts of interest

There are no conflicts of interest.

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