HHS Public Access

Author manuscript

Environ Dis. Author manuscript; available in PMC 2024 August 23.

Published in final edited form as:

Environ Dis. 2020 ; 5(4): 93–99. doi:10.4103/ed.ed_16_20.

Swine barn dust stimulates CCL9 expression in mouse monocytes through PKC-delta activation

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Abstract

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 that we are still trying to fully characterize.

CCL9/MIP-1γ is constitutively expressed in high quantities in the mouse circulation, but at much lower levels in the lungs where it is inducible under certain circumstances. We show here that extracts from hog barn dusts (HDE) are capable of inducing significant increases of CCL9 mRNA and protein in RAW267.4 monocytic cells as well as in mouse lungs. We further show that incubation of CCL9 with HDE results in cleavage of CCL9, which others have shown to increase chemotactic signaling potential. Endotoxin and proteoglycan were determined to be the likely causes of this increase. We additionally present evidence for a role of PKC-delta in this activation. Addition of purified CCL9 protein to HDE treated cell culture resulted in a small, but significant reduction in KC production, suggesting a possible regulatory role for the chemokine.

Keywords

swine; agricultural dust; CCL9; PKC; lung; inflammation; monocyte

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 COPD (Schiffman et al 2005; Vogelzang et al 2000; Von Essen and Romberger 2003; May et al 2012). Aside from these problems workers will also

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show a general decrease in lung function over their career (Poole and Romberger 2012). These responses are attributed to microbial components in workplace organic dusts such as endotoxin (LPS) (Charavaryamath et al 2008; Donham et al 2000; Kirychuk et al 2006; Zejda et al 1994) and proteoglycans (PGN) (Poole et al 2007; Poole et al 2008), though a larger host of such components may also play a role (Bauer et al 2013; Schneberger et al 2016). These components trigger innate immune responses through receptors such as TLR2 and TLR4 (Charavaryamath et al 2008; Bailey et al 2008) and protein kinase C (Wyatt 2010), inducing a wide array of immunological changes in the lung. While a number of cytokines and chemokines have been characterized, others have yet to be studied.

((((poole 2008: Repetitive organic dust exposure in vitro impairs macrophage differentiation and function>>>>> 2007: Repeat organic dust exposure-induced monocyte inflammation is associated with protein kinase C activity

Zejda: Respiratory health status in swine producers relates to endotoxin exposure in the presence of low dust levels.)))))

CCL9 is expressed constitutively in levels as high as 1 mg/ml in the blood of mice (Poltorak et al. 1995). The role of CCL9 in circulation however is as yet unknown. Monocytes and myeloid cell lines produce large quantities of CCL9 (Hara et al. 1995), as do dendritic cells (Mohamadzadeh et al. 1996) and T cells, in particular Th1 type T cells (Yang and Mossmann 2004). Expression of CCL9 in fact occurs across a broad range of tissues (Poltorak et al. 1995). The chemokine binds to the CCR1 receptor (Hara et al. 1995; Poltorak et al. 1995), and while not the most avid binder of CCR1, it is estimated to bind as much as 70% of available CCR1 (Poltorak et al. 1995). CCL9 has been shown to induce chemotaxis of and calcium release in neutrophils (Poltorak et al. 1995). Beyond this, little seems to be known of the effects of CCL9 or the signaling leading to its production.

Despite high baseline levels in circulation, it has become apparent that concentrations of CCL9 vary greatly in specific tissues with profound effects on health. For example, in the bone, CCL9 is produced at even higher levels, and is critical to osteoclast versus osteoblast differentiation of macrophages (Yang et al. 2006; Hoshino et al. 2010). There are also indications of a timed specific induction of CCL9 in skin wound healing (Kagawa et al. 2009) and follicle-associated epithelium of the gut (Zhao et al. 2003).

Lung lavages show the levels of CCL9 are several hundred times lower than what is present in serum. Upon stimulation with injected LPS, CCL9 levels were shown to greatly increase (Poltorak et al. 1995), specifically in the lungs, suggesting that CCL9 lung expression is more limited and inducible. Indeed, in a mouse silicosis model, survival was correlated with higher CCL9 exposure and inflammatory cells (primarily macrophages), suggesting a possible role for this chemokine in inflammation, lung injury, and response to particulate insult (Brass et al. 2010). In a recent study, we showed that CCL9 was specifically increased in response to 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 (Schneberger et al. 2017). For these reasons, we investigated the mechanism of CCL9 regulation and control in mouse cells exposed to hog barn dusts. 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 protein kinase C delta (PKC-δ).

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 (Romberger et al., 2002). Briefly, 10 g of dust was suspended in 10 ml of PBS (Dulbecco's phosphate buffered saline, pH 7.4, Grand Island, NY) without calcium at room temperature for 1 hr. This was centrifuged 10 min before sterile filtration of the supernatant, for a final concentration of approximately 0.105 g/ml dust. 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 (Poole et al. 2010). Characterization of bacterial sources of these components has also been previously determined (Boissy et al. 2014, e95578). 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 hr at 100°C.

Cell Culture and Treatments

RAW264.7 mouse macrophage cell line (ATCC, Rockville, MD) was used for most experiments. Cells were cultured as per ATCC recommendations in DMEM + 5% fetal bovine serum (Gibco, Grand Island, NY) plus 1% penicillin/streptomycin (Gibco). Cells were grown in 6-well tissue culture plates (Thermo Fisher, Waltham, MA) at a concentration of 0.5 X 10^5 cells/well in 1.5 ml media. Cultures were carried out for 24 hr unless otherwise specified. Cells were treated with either 1% v/v HDE (defined in Poole et al. 2010), 1% v/v heat inactivated HDE, 100 EU LPS (Escherichia coli O55:B5, Sigma Aldrich, St. Louis MO), 10 ng/ml Pam3CSK4 (Sigma), or 1% v/v PGN (*Staphylococcus aureus* PGN, Sigma).

For PKC inhibition studies, cells were treated with PKC inhibitors 1 hr prior to exposure to HDE. Inhibitors for PKC-α (Gö 6976, EMD Millipore, Bedford, MA), and PKC-z (Biomedical Research Laboratories, San Diego, CA) were used at a concentration of 1 μM. PKC-δ inhibitor (Rottlerin; EMD Millipore) was used at 20 μM.

Cytokine/Chemokine ELISA

Expression of CCL9 in cell culture media was quantified by a CCL9 enzyme linked immunoabsorbant assay (ELISA) kit (R&D Systems, Minneapolis, MN) according to manufacturer's instructions.

Western Blot

CCL9 (10 μg; Peprotech, Rocky Hill, NJ) was diluted in PBS and incubated for 20 hr at 37°C with either PBS alone, 10 μl HDE, HDE + 10 μg CCL9 + 4 μl protease inhibitor cocktail (P8340, Sigma), HDE + 10 μg CCL9 + 0.5 μg MMP-9 protease inhibitor (sc-311437, Santa Cruz Biotech, Dallas, TX). HDE and CCL9 were also individually evaluated as controls. Contents of each tube were loaded to a 4–20% polyacrylamide gel

(Bio-Rad, Hercules, CA) for electrophoresis and transfer to PVDF membrane (Bio-Rad). The membrane was blocked with 5% milk in tris-buffered saline + 0.1% Tween20 (TBST) (0.1% Tween), followed by overnight incubation with 1:1000 dilution of rabbit anti-mouse CCL9 (500-P117, Peprotech, Rocky Hill, NJ) at 4°C. After washing, 1:2000 dilution of horseradish peroxide-conjugated goat anti-rabbit antibody (926–80011, LI-COR, Lincoln, NE) was added in 5% milk and incubated for 1 hr at room temperature before washing with TBST and development using SuperSignal West Pico Chemiluminescent Substrate (ThermoFisher). Results were visualized using a C-DiGit blot scanner (LI-COR).

siRNA Inhibition

siRNA to the PKC-δ sequence (SMARTpool ON-TARGETplus, L-040147–00) 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 RAW264.7cells in several trials using this or another vector.

LA4 cells were grown identical to RAW264.7 cells. Cells were plated in 6-well tissue culture plates at 0.5 X 10^6 cells/well in 1.5 ml Optimem + L-glutamine (Thermo Fisher) media. Lipofectamine RNAiMAX (ThermoFisher) was added at 6 μl to 244 μl 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 for a final volume of 2.0 ml media/well. Cells were incubated for 24 hr to bind and transfect prior to treatment with media or 1% HDE. Cell media was harvested and tested for CCL9 production.

Statistical Analysis

Data was analyzed using GraphPad Prism (GraphPad Software, San Diego, CA). Graph bars represent mean +/− SE. 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 HDE 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 (Wyatt et al 2007; Poole et al, 2009). We started with a time course (Figure 1), stopping at 24 hr. As there is a level of constitutive CCL9 expression in these cells, we did not continue the time course beyond 24 hours due to excessive buildup of chemokine in culture media. We show that CCL9 was significantly elevated by HDE by 6 hr, and increased in relation to media concentrations up to 24 hr. There was a significant accumulation of CCL9 in cultures over time.

CCL9 is Produced in Response to LPS and Peptidoglycan

To further test which components of HDE are responsible for CCL9 production, we tested LPS and peptidoglycan, both present in HDE (Poole et al., 2008), for their ability to induce CCL9 from these cells. In agreement with Poltorak et al. (1995), LPS induced CCL9 in RAW264.7 cells, but so too did peptidoglycan (Figure 2). We further tried heat inactivation

of the HDE by boiling samples to remove both components via thermal degradation. In both cases this eliminated most CCL9 expression (Figure 2).

Mouse Lung Macrophage and Epithelial cells produce CCL9 and PKC-δ is Involved in CCL9 Induction

To better understand how induction of CCL9 occurs, we investigated PKC signaling in RAW264.7 cells. Using inhibitors to PKC-α (Gö 6976), δ (rottlerin), and ζ (myr-PKCζ 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. Due to potential lack of specificity with the rottlerin/PKC-δ chemical inhibitor, we confirmed these results by treating LA4 cells for 24 hours 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. This suggested rottlerin is inhibiting up-regulation of CCL9 to HDE rather than generally depressing CCL9 levels. Levels of CCL9 were higher in general due to the use of a different cell line and longer culture times required for siRNA inhibition.

CCL9 Inhibits KC 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. As KC plays a strong role in neutrophil recruitment to lungs in response to HDE (PM Murphy 1997), 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).

(((Murphy PM. Neutrophil receptors for interleukin-8 and related CXC chemokines. 1997. Semen Hematol. 34(4): 311–8.)))

CCL9 is cleaved by HDE

CCL9 can be readily cleaved by some proteases into a form that is more strongly chemotactic than the un-cleaved form (Berahovich et al. 2005). As HDE has protease activity of its own (Romberger et al. 2015), we tested to see if combining HDE with CCL9 could cleave CCL9 to this shorter more bioactive form. We tested this by incubating CCL9 for 20 hr at 37°C with or without 1% HDE and 10 μl SPIC protease inhibitor. Another protease, MMP-9, common in the lung, was also similarly tested. Both HDE and MMP-9 were capable of cleaving CCL9 to a shorter form consistent in size to that seen by others (Berahovich et al. 2005).

Discussion

CCL9/MIP-1γ was discovered over 20 years ago, partially due to the very high levels that exist in mouse serum (Poltorak et al. 1995). Since then very little has been added to our knowledge of this chemokine. This may be due to the fact that few functions have been

easily attributable to its action, or that, by being a lower affinity binder of CCR1, it may be of lesser importance in response to injury or disease. However, given that CCL9 is constitutively produced in such high quantities in serum, yet inducible in several anatomical compartments such as bone, skin, gut, or lung (Kagawa et al 2009; Zhao et al 2003; Yang et al 2006; Hoshino et al 2010, Poltorak et al 1995), it suggests a potentially complex role for this chemokine that may rely on the context of the cells involved. Much of the new information on CCL9 in recent years has been generated with regards to its effects in bone, and the belief that it is instrumental in determining differentiation to either osteoclasts or osteoblasts, with CCL9 driving the differentiation of monocytes to osteoclasts (Yang et al 2006, Hoshino et al 2010).

Less is known of CCL9 in the lung. While one of the first CCL9 papers noted that systemic LPS administration could drive up CCL9 specifically in the heart and lungs (Poltorak et al. 1995), 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 (Brass et al. 2010). Here, increased CCL9 was found to be higher in female mice compared to males, which correlated with less fibrosis in female mice, 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 suggested a chemotactic role for CCL9 (Poltorak et al. 1995; Berahovich et al. 2005). Given that HDE is a particulate exposure that causes significant inflammation in the lungs (Poole and Romberger 2012) we thought to examine its ability to induce CCL9.

First, we looked at basic kinetics of production in RAW cells, similar to those used in the first CCL9 studies. While there was a general increase in CCL9 in cell media over time, those cells that received HDE showed significantly greater increases (Figure 1). The difference between treated and untreated cells was significant by 6 hr, though by 24 hr the ratio of control to treated CCL9 was very significant. Thus CCL9 is produced constitutively by RAW cell macrophages, but HDE stimulation induces clearly increased expression by 6 hr, with production intensifying and stretching out beyond 24 hr. This appears to agree with data from skin wounding studies (Kagawa et al. 2009). 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 TNFα and IL-6 (Wyatt et al 2010).

The next question was what in the dust may be responsible for stimulating CCL9 production. Earlier work in this cell line had shown LPS can induce CCL9 (Poltorak et al. 1995). We tested both LPS and peptidoglycan to see if both could induce CCL9 as both are considered important mediators of barn dust induced inflammation (Poole et al. 2012). As we show (Figure 2), both bacterial cell wall components appear equally capable of inducing CCL9 in RAW cells. As would be expected, baking dust to remove LPS and peptidoglycan activity reduced CCL9 levels to background levels, showing a heat labile product(s) was responsible for CCL9 production. This work suggests that both LPS and peptidoglycan are major causes for increased CCL9 production in organic dust exposures.

Very little is known about the induction path of CCL9. One paper has shown a role for retinoic acid receptor-α (Nunez et al., 2010), and another for TLR9 (Ravindran et al. 2010).

In studying CpG induction of CCL9 in microglial cells, it was found that PI3K, p38, MAKP and ERK inhibitors were all capable of reducing expression of CCL9 (Ravindran et al. 2010). Subsequent studies have confirmed a role for p38 in CCL9 control in myeloid cells (Yan et al. 2015).

As we have previously shown a role for PKC in immune responses to HDE, we looked at the role for this protein kinase family in CCL9 induction (Figure 3). Of the three PKC isoform inhibitors used, rottlerin (PKC-δ inhibitor) was found to be very effective at inhibiting induced CCL9 expression, though interestingly, it had a minor but not significant impact on basal expression levels. This observation was confirmed using siRNA inhibition of PKC-δ in mouse lung epithelial LA4 cells, which also significantly inhibited CCL9. Earlier work in our lab had shown problems with siRNA inhibition in RAW cells so this substitution was made. Thus we show that PKC-δ plays a critical role in the production of HDE-induced CCL9 production. We also show that mouse lung epithelial cell line LA4 is capable of CCL9 production.

There are few attributed functions to CCL9 outside the bone other than chemotaxis and calcium release in neutrophils (Berahovich et al. 2005; Poltorak et al. 1995) and an association with increased inflammatory conditions (Brass 2010) or myeloid tumor cell survival via increasing phosphor-AKT and BCL-2 (Yan et al. 2015). We were curious if CCL9 could modify either IL-6 or KC, two common markers for murine lung inflammation. While an IL-6 pilot test failed to reveal any differences, KC expression was significantly reduced by administration of purified CCL9 to RAW cells treated with HDE (Figure 5). This result may suggest a moderating or inhibiting role for CCL9. While there is evidence to suggest CCL9 is an inducible chemotactic signal (Brass 2010, Poltorak 1995, Berahovich et al. 2005), 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. Given that in circulation there are normally very high levels of CCL9, which might bind as much as 70% of all CCR1 receptors (Poltorak et al. 1995), there is some possibility that 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 20X less than in circulation and where CCR1 should subsequently be less saturated with CCL9.

Another possible scenario 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 (Kagawa et al. 2009), TLRs (Ravindran et al. 2010), and certain particulates (Erdely et al. 2013, Brass et al. 2010). The effect may be pro-inflammatory, or transformational on one or more cell types within the lung, if results in other tissues can be translated to the lung. It is likely that the local tissue effects differ from those of the systemic circulation. 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 (Schneberger et al 2017).

Finally, while chemotaxis to CCL9 isn't as great as with other chemokines, Berahovich et al. (2005) discovered that CCL9 (among other chemokines) was easily cleaved by a number

of proteases to a form that was much more able to induce chemotaxis of myeloid cells. As our group has recently discovered that barn dusts have protease activities (Romberger et al. 2015), we decided to see if a dust extract could cleave CCL9 to a similar sized molecule. As shown in Figure 5, prolonged incubation of CCL9 with HDE was indeed able to cleave CCL9 to a smaller form similar to what has been observed by others (Berahovich et al. 2005) and this action was readily blocked by a protease inhibitor. Interestingly, in testing another proteolytic lung enzyme (MMP-9), we found that it too was capable of cutting CCL9 to a similar size, further expanding the number of known proteases capable of cleaving this chemokine. We did not test for the ability of HDE-cleaved CCL9 to induce chemotaxis, as it would be difficult to separate out modified CCL9 from the multiple components in HDE that may have similar chemotactic properties.

In conclusion we show that HDE is capable of induction of CCL9 likely through stimulation by LPS and peptidoglycan in HDE, and that these increases of CCL9 rely upon HDE induced PKC-δ signaling. We show a possible effector role for CCL9 in inhibition of KC production in RAW cells. This could be very important in an exposure such as HDE which is usually accompanied by neutrophil influx (Schneberger et al 2015; Poole et al 2015; Romberger et al. 2015). Further, exposure of CCL9 protein to barn dust extracts results in its cleavage to what appears to be the more active form showing another possible role for innate organic dust proteolytic activity in signaling the immune system. More work will have to be done to confirm that CCL9 is more active after dust extract cleavage, and how the resulting cleaved protein acts in regards being able to induce cellular chemotaxis. 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 would be to look at 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. This work does further show that the response of the lung to HDE is complex, and involves the action of multiple induced cytokines and chemokines.

Acknowledgements

This work was supported by National Institute of Occupational Safety and Health (R01OH008539) to DJR, and (R01OH010162) to TAW. This work was supported by the Central States Center for Agricultural Safety and Health (CS-CASH; NIOSH U54OH010162). TAW is the recipient of a Research Career Scientist Award (IK6 BX003781) from the Department of Veterans Affairs.

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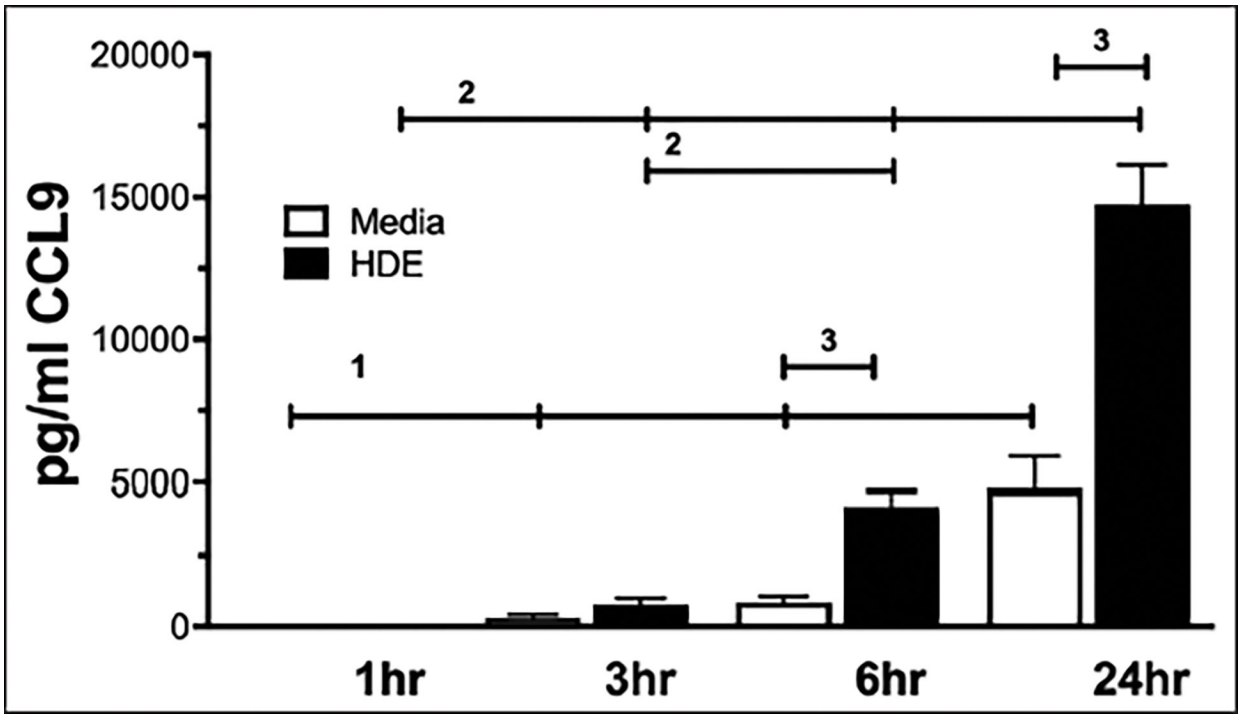

Figure 1:

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

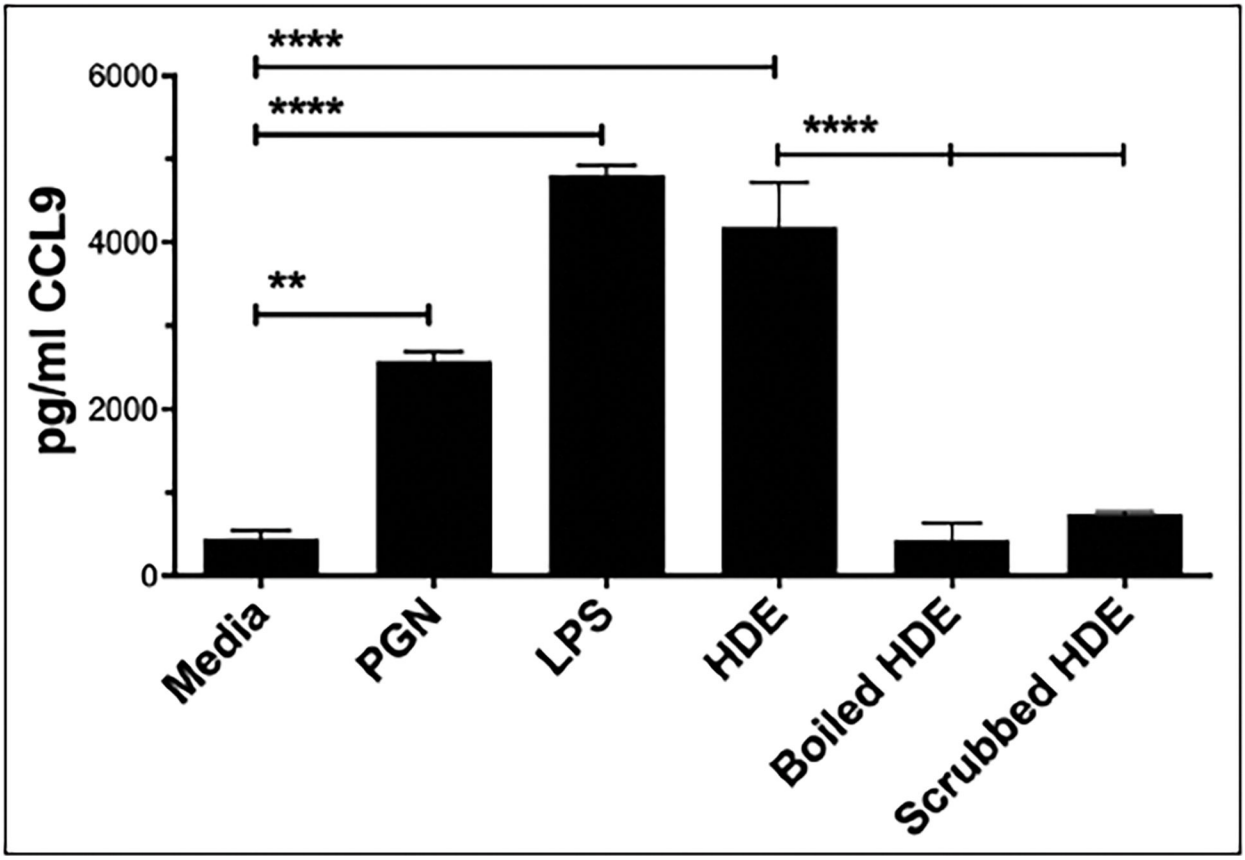

Figure 2:

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. $^{**}P < 0.01$, $^{****}P < 0.0001$

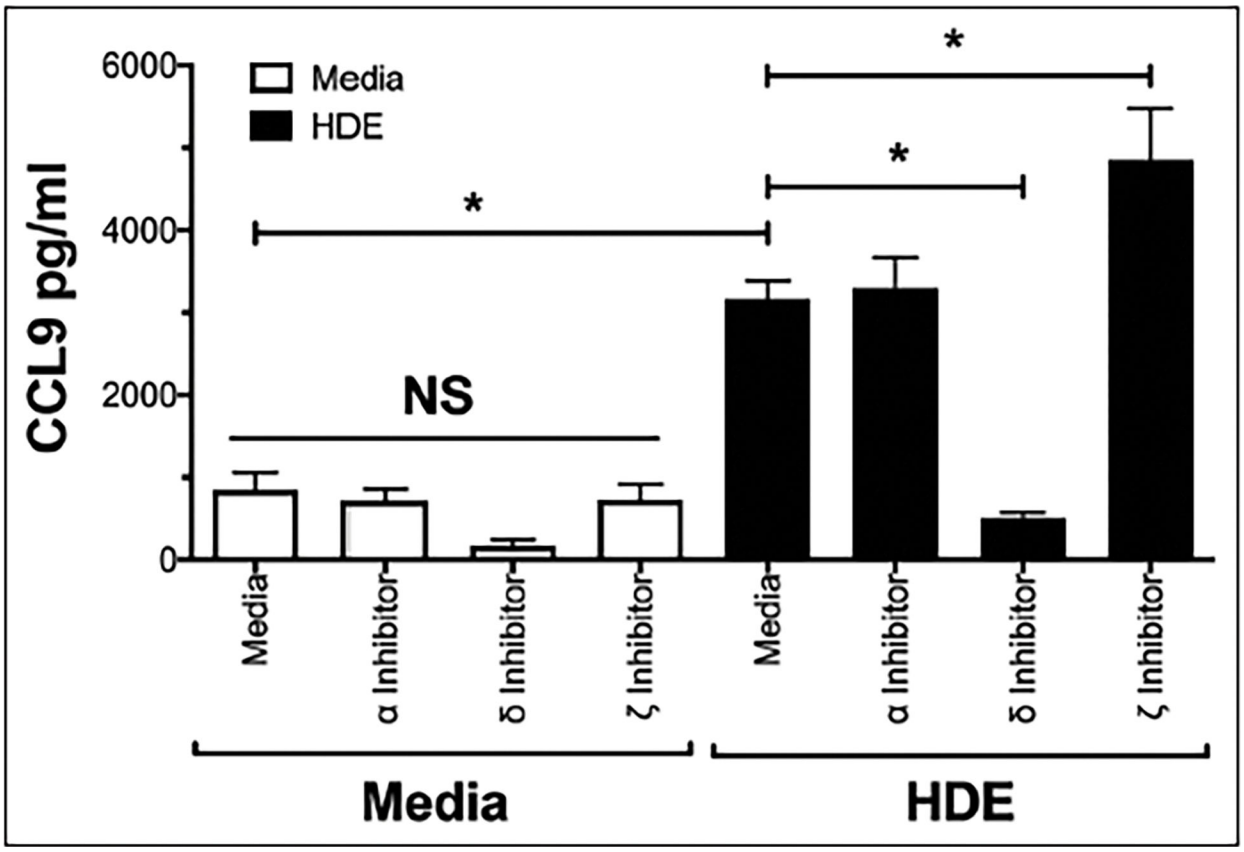

Figure 3:

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

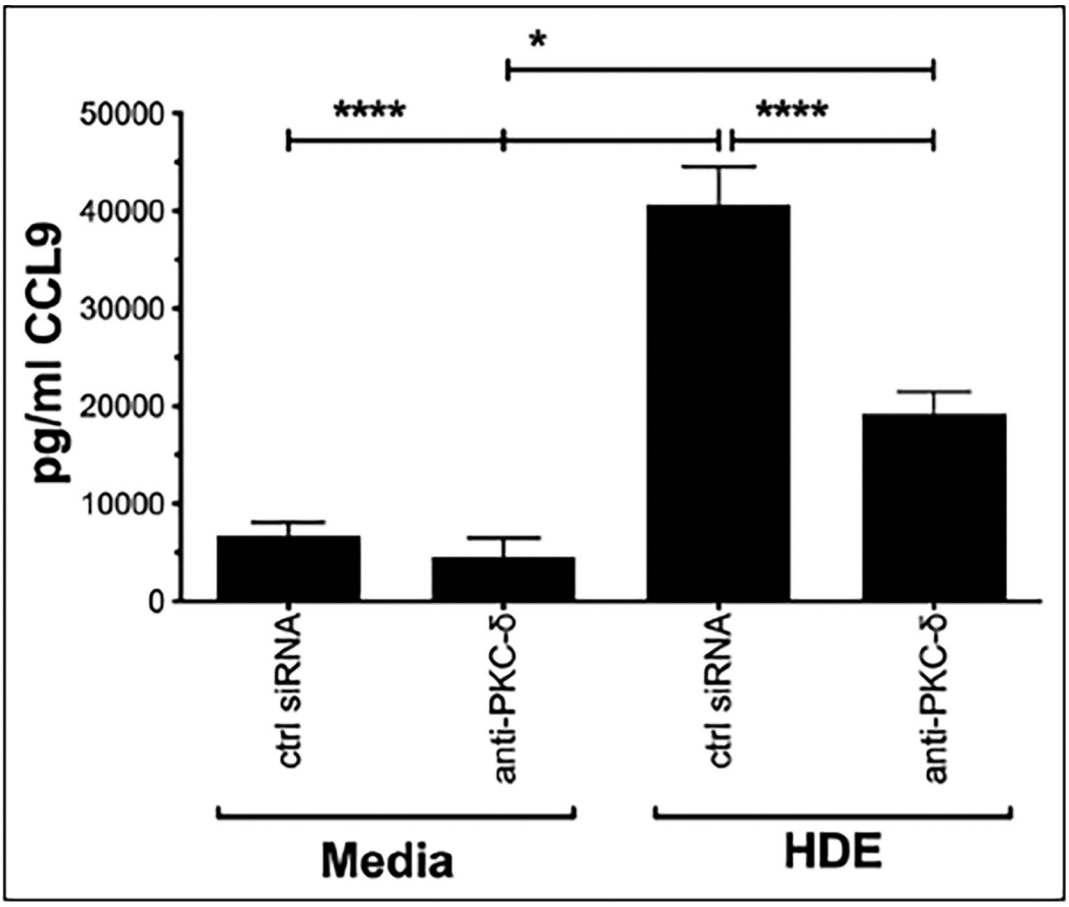

Figure 4:

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. *$P < 0.05$, ****$P < 0.0001$

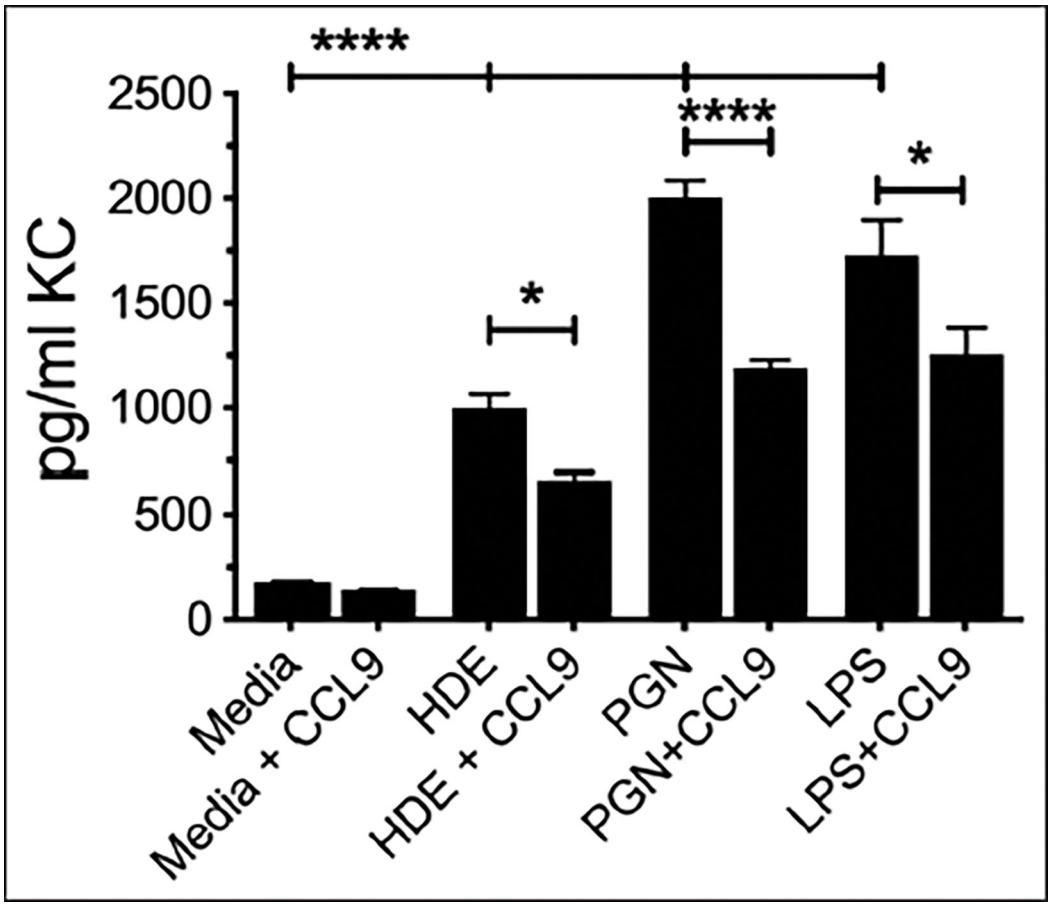

Figure 5:

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; ****$P < 0.0001$