EXPOSURE TO SWINE HOUSING DUST MODULATES MACROPHAGE MORPHOLOGY AND FUNCTION

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ABSTRACT

Swine Confinement Facility (SCF) dust consists of a complex mixture of feed grain particles, bacterial components, organic particulates and gases. When these particles are inhaled they deposit along the respiratory tract and mediate respiratory symptoms and disease in swine farmers and facility workers. Macrophages ingest and eliminate microbes and debris under chronic conditions; however, the role of macrophages in agricultural-related respiratory disease has not been fully elucidated. The goal was to evaluate the hypothesis that chronic exposure to SCF dust causes inflammation by modulating pulmonary protein levels and macrophage function. Balb/c mice were exposed to 5, 12.5 and 25% SCF Dust Extract (DE) via nebulization 30 min/day five days a week, for eight weeks with weekends excluded. Bronchoalveolar Lavage Fluid (BALF) was collected and analyzed for protein concentration, leukocyte distribution and macrophage morphology. For comparison, THP-1 monocytic cells were exposed to 0.1-10% DE overnight and evaluated for phagocytosis and reactive oxygen species production. Repeated exposure to DE via nebulizer caused a significant increase in protein concentration and inflammatory cell number, namely macrophages, in a dose-dependent manner within the lung as compared to controls. Macrophages with pseudopods and vacuoles were the most abundant leukocytes within BALF of mice exposed to DE. Similarly, *in vitro* studies with 10% DE treated THP-1 cells revealed enhanced phagocytosis (p<0.05), pseudopodia and vacuolization following exposure to compared to control cells. In addition, there were time- and dose-dependent increases of intracellular ROS production by THP-1 cells exposed to 5 and 10% DE compared to control (p<0.01). These findings indicate repeated, long-term inhalation of swine confinement facility dust may mediate chronic airway and lung inflammation through modulation of protein concentration and macrophage function. The aerosolized dust-mouse inhalation model presented here may offer a good tool for studying particle mediated chronic inflammation of the tracheobronchial tree and lungs.

Keywords: Nebulizer, Swine Housing Dust, Macrophage, Phagocytosis, Reactive Oxygen Species

1. INTRODUCTION

In an effort to meet growing consumer demand, animal production has shifted from pasture-based approached to large Concentrated Animal Feeding Operations (CAFOs). A myriad of respiratory complications arise from acute or chronic inhalation of organic dusts from CAFOs including non-allergic asthma, rhinitis, sinusitis, chronic bronchitis, Chronic Obstructive Pulmonary Disease (COPD) and organic dust toxic syndrome as reviewed by May *et al.* (2012). Swine facility workers may experience occupationalhazards from inhalation of high levels of organic dusts which consist of feed particles, fecal material, endotoxins, ammonia odors, organic dust, gases and other dried windborne material from animals, microbes and fungi (Donham *et al.*, 2006; Rylander, 2010). Inhalation of endotoxins and carbon dioxide at levels above the recommended health threshold limits (McDonnell *et al.*, 2008) can lead to acute and chronic

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airway inflammation can occur (Schierl *et al.*, 2007) which may lead to loss of lung function, as measured by the decrease in forced expiratory air in one second or less (Costa *et al.*, 2007). After frequent exposure to Swine Confinement Facility (SCF) dust, physiological effects can develop including pulmonary inflammation, severe asthma, chronic respiratory diseases and bronchoconstriction (Demanche *et al.*, 2009). Inhalation of endotoxins and carbon dioxide at levels above the recommended health threshold limits (McDonnell *et al.*, 2008) chronic airway inflammation can occur (Schierl *et al.*, 2007) which may lead to loss of lung function, as measured by the decrease in forced expiratory air in one second or less (Costa *et al.*, 2007). After frequent exposure to Swine Confinement Facility (SCF) dust, physiological effects can develop, including pulmonary inflammation, severe asthma, chronic respiratory diseases and bronchoconstriction (Demanche *et al.*, 2009).

Macrophages are mature myeloid cells that originate from differentiated monocytes and circulate through vasculature or reside within tissues. Although lung tissue macrophages reside in gas-exchange regions of the lung and modulate inflammation therein; tissue inflammatory responses in general signal recruitment of more macrophages to the site of injury (Rodero and Khosrotehrani, 2010). Macrophages and other specialized cells remove pathogens, dead cell remnants and other debris via phagocytosis, a well-orchestrated process involving encapsulation and internalization of large particles (Underhill and Goodridge, 2012). However, the role of macrophages in chronic lung disease as seen in agricultural occupational settings is not fully understood.

A great deal of attention has been devoted to elucidating acute inflammation responses that follow single or short-term exposure to CAFO-style organic dust; however, fewer models describe the effects of chronic or long-term exposures which may require extended exposure durations (e.g., several hours) and frequency (e.g., 8 weeks or several months). The purpose of the present study was to characterize the effect of long-term or chronic organic dust exposure on airway inflammation in mice that were exposed to SCF Dust Extract (DE), 30 min a day, five days per week for eight weeks via nebulizer. After the exposure was completed, mice were euthanized and bronchoalveolar washing was performed to evaluate protein concentrations, leukocyte numbers and cell morphology to characterize inflammation of the lungs. Cell culture comparisons with THP-1 cells were conducted to evaluate DE mediated modulation of macrophage function *in vitro*. We report here that long-term repeated inhalation of aerosolized SCF dust enhances bronchoalveolar fluid protein concentration

and formation of pseudopodia and vacuolation by airway macrophages *in vivo*. Correspondingly, increases in pseudopodia and vacuolation, enhanced phagocytosis and elevated ROS production was observed from THP-1 following DE exposure *in vitro*.

2. MATERIALS AND METHODS

2.1. Reagents

Penicillin-Streptomycin solution, 2-Mercapto-Ethanol And Dimethyl Sulfoxide (DMSO) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Heat-inactivated Fetal Bovine Serum (FBS) was obtained from Atlanta Biologicals (Atlanta, GA, USA). Lipopolysaccharide (LPS) from *Escherichia coli* 0111: B4, RPMI 1640 and HEPES were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Phorbol 12-Myristate 13-Acetate (PMA) was obtained from Tocris (Ellisville, MO, USA). 5-(and-6)-chloromethyl -2 '7'-dichloro dyhydrogluoresceindiacetate, acetyl ester (DCF-DA) was purchased from Invitrogen Molecular Probes (Eugene, OR, USA). Black well, clear bottom tissue culture plates were from Corning Life Sciences (Tewksbury, MA, USA). The THP-1 cell line was obtained from the American Type Cell Collection (Rockville, MD, USA).

2.2. Dust collection and Extract Preparation for *in Vivo* and *in Vitro* Studies

The dust extract was prepared using a protocol similar to Poole *et al.* (2009b). Briefly, several grams of settled dust from raised surfaces were collected from the gestation and farrowing rooms at North Carolina A and T State University's Swine Research Unit and brushed into a zip top bag. A 1:10 (w/v) ratio of dust and Phosphate Buffer Saline (PBS) without calcium and magnesium or Hank's Balanced Salt Solution (HBSS) was prepared. The mixture was vortexed for approximately 1 m and incubated at room temperature for 1 h. The mixture was centrifuged in an Eppendorf 5810 R (Hauppauge, NY) centrifuge for 10 min, at $948 \times g$ at room temperature and the supernatant was transferred to a new tube. The mixture was centrifuged again to remove any leftover dust particles from the first centrifugtion. The mixture was sterilized by filtration (0.22 µm) and the dust extract was used immediately.

2.3. Mice and Nebulizer Dust Extract Exposure

Female Balb/c mice of ages 6 to 7 weeks (Harlan Laboratories, Indianapolis, IN) were maintained at the North Carolina A and T State University (NCAT) Laboratory Animal Resource Unit under standard housing conditions. Mice received water and standard

rodent chow *ad libitum* for the entire course of the study and were weighed weekly. Mice were randomly placed into 1 of 4 treatment groups (n = 6 per groups): Group 1, aerosolization exposure to PBS; and Groups 2, 3 and 4, aerosolization exposure to dust extract (5, 12.5 and 25%), respectively. Our nebulizer SCF dust exposure model is a modified version of a grain dust exposure model (George *et al.*, 2001). In the present study, mice were placed into an exposure chamber connected to a treatment solution-filled nebulizer, which itself was connected to an air pump. To simulated, repeat occupational exposure to swine dust, mice were exposed to DE or PBS for 30 min per day, 5 days a week for 8 weeks with weekends excluded according to their respective treatment groups described above. This procedure was performed twice with three mice per group in each trial for a total of six mice per group. All experimental protocols were reviewed and approved by the North Carolina A and T State University Institutional Animal Care and Use Committee and all protocols conformed to the Guide for the Care and Use of Laboratory Animals (NRC, 2011).

2.4. Bronchoalveolar Lavage Collection and Differential Cell Counting

After the 8 week treatment period, mice were euthanized by xylazine injection (100 mg mL^{-1}) and death was assured by auscultation of the lungs. Bronchoalveolar Lavage (BAL) was collected via the trachea by washing lungs a total of three times with 1 ml-of ice cold PBS; a method similar to Minor *et al.* (2012). Supernatant collected from the first lavage was used for protein assays. Cells from all three lavage washings were pooled. Cell concentration and viability was determined for BAL fluid from pooled samples collected from each mouse by trypan blue dye exclusion. Briefly, in a microcentrifuge tube, 10 µL of trypan blue was added to 10 µL of cells and the cells were allowed to sit for two minutes. Cells (~2,000 in 100 µL) were collected on slides via cytocentrifugation at 55×g for 5 min using a CytoSpin 4 (Thermo Fisher Scientific). Hema 3 System (Fisher Scientific Company, Suwanee, GA) was used to stain cells according to manufacturer's protocol. The stained leukocytes were counted and differentiated based on color and morphology using a Leica Model DME Microscope with an oil emersion lens (Leica Microsystems, Shanghai, China) counting a total of 100 cells.

2.5. Cell Culture and Differentiation

The promonocytic THP-1 cell line (ATCC) was maintained at a density of 2-8×10^5 cells/mL in THP-1 complete media which consisted of RPMI-1640

supplemented with 1% Penicillin (10,000 U)-Streptomycin (10 mg), 0.5 mg mL^{-1} gentamicin, 200 mM L-glutamine, 10 mM HEPES, 50 mM 2-mercaptoethanol and 10% heat inactivated Fetal Calf Serum (FCS), at 37°C in humidified air mixed with 5% CO$_2$. For differentiation, THP-1 cells were stimulated with 160 nM PMA for 24 or 48 h. Non-adherent cells were removed; cell surfaces were washed with PBS and fresh media with or without treatment components was added as described below. For THP-1 cell imaging, cells were seeded onto Millicell EZ glass slides (EMD Millipore, Billerica, MA) at a density of 10^4 cells/cm^2 and exposed to DE as described. To end the experiment media was removed and adherent cells were washed with PBS, slide chambers were aspirated, chambers were then detached and slides were stained with Hema 3 Diff Quick (Thermo Fisher Scientific) according to manufacturer's instructions. Cells were visualized using a Zeiss Axio Imager m2m Optical Microscope (Carl Zeiss Microscopy GmbH, Jena, Germany) at 1000X magnification.

2.6. Measurement of Phagocytosis

Phagocytosis was assessed using the phagocytosis assay kit (FITC) (Cayman Chemical, Ann Arbor, MI). Briefly, THP-1 cells, plated in triplicate at a density of 2×10^5 cells mL^{-1} in the absence or presence of PMA [160 nM], LPS [1µg mL^{-1}], or DE [0.1, 1.0 and 10%] with and without latex beads coated with FITC-labeled rabbit IgG. After 24 h in culture at 37°C, the uptake of the beads into cells was detected using an Accuri C6 flow cytometer (BD Biosciences, Franklin Lakes, NJ.). Data were analyzed using FCS express (DeNovo Software, Los Angeles, CA).

2.7. Measurement of Reactive Oxygen Species Production

Intracellular hydrogen peroxide generation was measured using DCF-DA (Molecular Probes). Briefly, 7.5×0^4 THP-1 cells/mL were plated in black well-clear bottom 96-well plates (Corning Life Sciences, Corning, NY) overnight in the absence or PMA [160 nM], LPS [1µg mL^{-1}], or DE [0.1, 1.0, 5 and 10%]. Cells were loaded with DCF-DA [10 µM] for 1 h at 37°C and fluorescence was measured using a SpectroMax M5 microplate reader (Molecular Devices, Sunnyvale, CA) every 20 min for 2 h at 485 nm excitation and 538 nm emission. Fluorescence intensity results were expressed as arbitrary units.
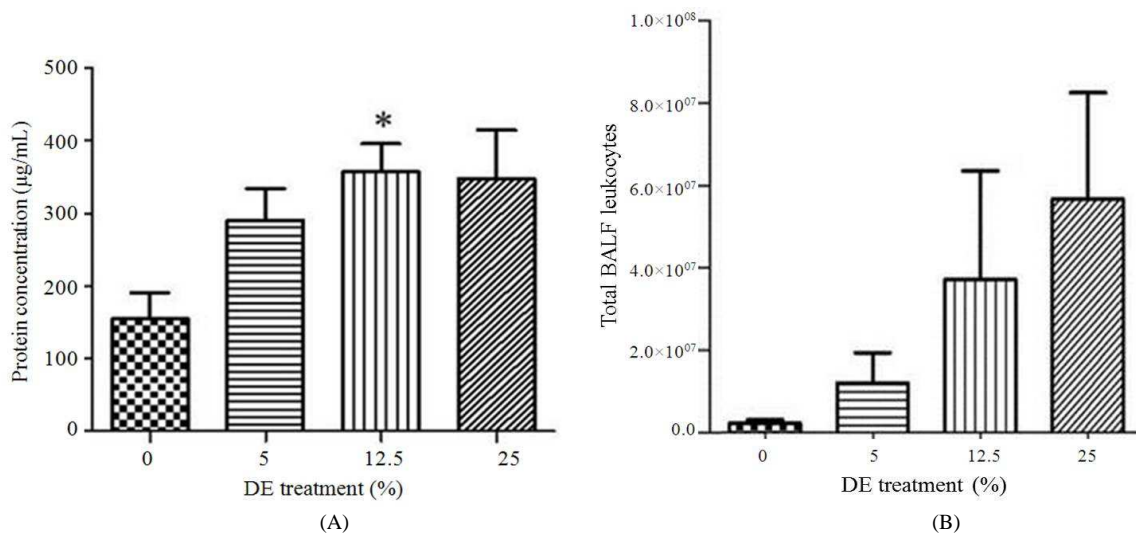
2.8. Statistical Analysis

Two to three biological replicates, with at least three to six technical replicates were performed for each

experiment. Data were analyzed for significance by one-way or repeated measures two-way Analysis Of Variance (ANOVA) with a threshold set at a p-value of 0.05 or below using Prism version 5.0 (GraphPad, La Jolla, CA) followed by Bonferroni post-test corrections, or as described. Data are shown as mean±Standard Error Measure (SEM), mean ± Standard Deviation (SD) or as described.

3. RESULTS

3.1. Inhalation of aerosolized DE Increases Protein Concentration and the Number of Macrophages with Pseudopods and Vacuoles in Mouse BALF

Previous studies have shown intranasal instillation of DE [1-25%] once daily for 2 weeks induces lung inflammation (Poole *et al.*, 2009a). To examine the effect of long-term DE exposure, a mouse model was developed using a nebulizer to aerosolize PBS [0%] or DE [5, 12.5 or 25%] for exposures. Mice inhaled aerosolized PBS or DE for 30 min a day for eight weeks with weekends excluded. At the end of the 8 week period, airways and lungs were washed to collect BALF. A Bradford assay was performed to determine the concentration of protein in BALF. There was a significant increase in BALF protein concentration in 12.5% DE exposed mice compared to PBS-control (**Fig. 1A**).

There was a trend for higher cell numbers in DE-exposed mice (**Fig. 1B**); however, no significant differences were observed when treatment groups were compared to control by one-way analysis of variance (p>0.05). **Figure 2 A-C** shows leukocytes (lymphocytes, monocytes, neutrophils) found within BALF extracted from the lungs after exposure to various DE. The levels of these cells were low and there were no significant changes in the levels of these cells found. In contrast abundant macrophages within the BALF were detected (**Fig. 3A**). Although there was no difference in the percentage of macrophages between the treatment groups we identified macrophages with different morphologies; macrophages with pseudopods, macrophages with large vacuoles. **Figure 3B** depicts a representative image of macrophages from PBS-control mice. We observed higher levels of macrophages with pseudopods and vacuoles in DE-exposed mice as compared to control. There was a significant increase in macrophages with pseudopods (p<0.001) in mice that inhaled aerosolized DE compared to PBS-control (**Fig. 4A**).

Figure 4B is a representative phase contrast image of observed macrophages with pseudopods. There was also a dose-dependent increase in macrophages withvacuoles (p<0.01) in BALF of mice exposed to 12.5 and 25% DE compared to control mice (**Fig. 5A**). A representative image of vacuolated macrophages observed in DE exposed mice is shown in **Fig. 5B**.
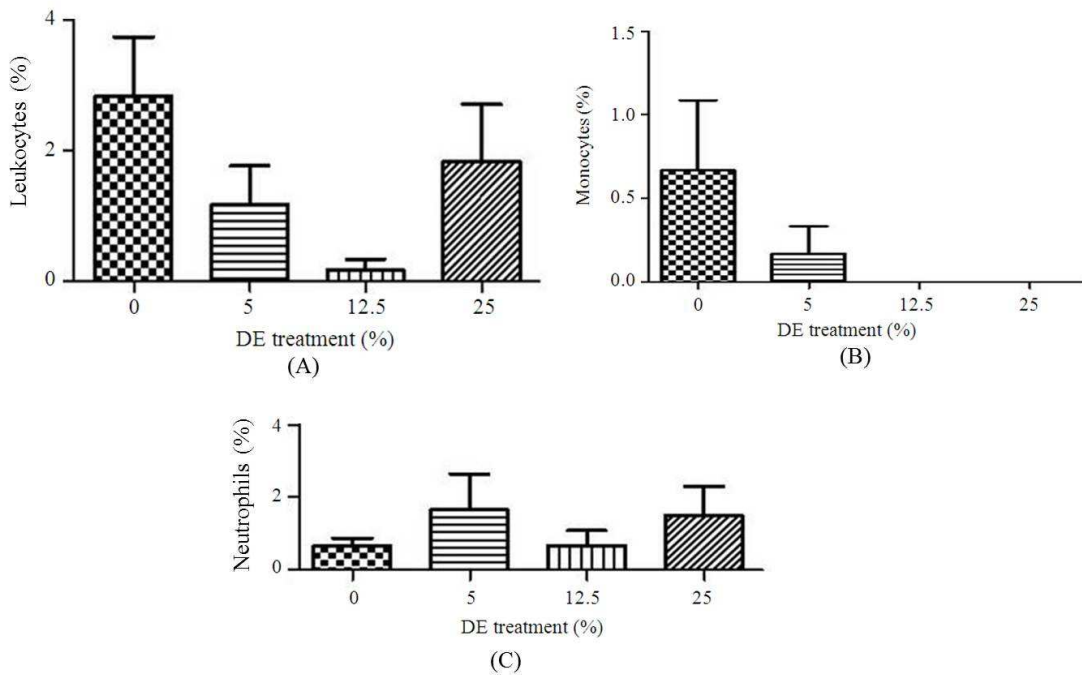

Fig. 1. Bronchoalveolar Lavage Fluid (BALF) protein concentration (A) and total leukocyte number (B) in lungs of mice delivered PBS or DE via nebulizer. Mice were exposed to PBS [0], 5, 12.5 or 25% DE (n = 6 each) via nebulization for 8 weeks. After the 8-week exposure period had ended, BALF was collected by washing lungs three times, cells were affixed to slides and differential cell counts were performed. Protein concentration was determined by Bradford assay. *p<0.05 for 12.5% DE exposed vs. control (0%) mice as compared by one-way analysis of variance paired with Bonferroni posttest corrections. Protein concentration data are expressed as mean ± SEM and cell data are shown as mean ± SD. N = 6 each

Fig. 2. Differential BALF leukocyte count. Mice were exposed to aerosolized PBS [0%] or DE [5, 12.5 and 25%] for 8 weeks. After 8-weeks, lungs were washed three times to collect BALF, cells were affixed to slides and differential cell counts were performed. (A) Lymphocytes; (B) Monocytes; and (C) Neutrophils were the predominate types of leukocytes present in BALF of mice. There were 6 mice per group. Data are shown as mean ± SD
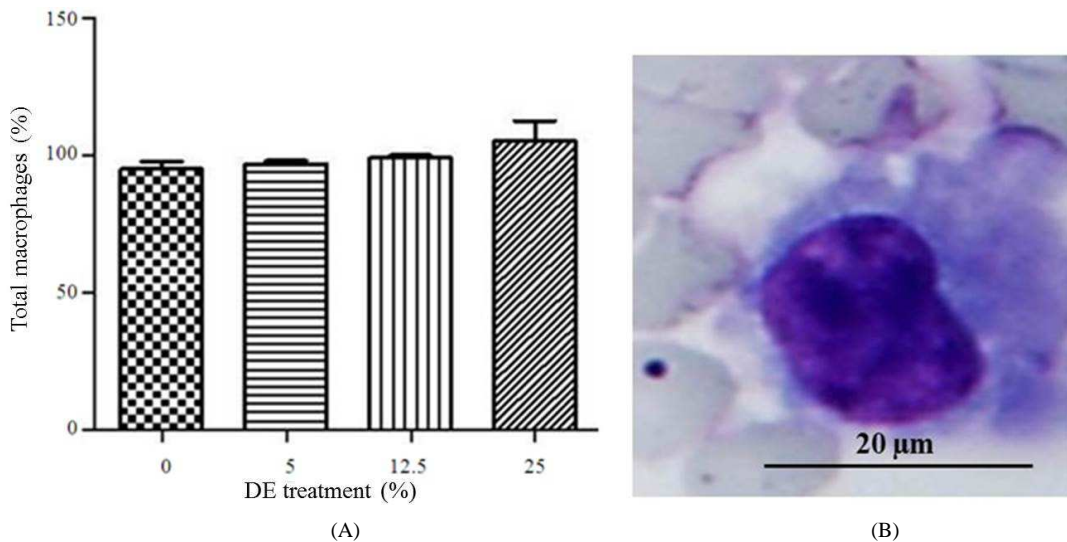
Fig. 3. Total macrophages present in BALF of DE-exposed mice. Mice were exposed to PBS [0%] or DE [5, 12.5 and 25%] via inhalation for 8 weeks. After the 8-week period, lungs were washed three times to collect BALF; cells were attached to slides, stained and differentially counted for macrophages. (A) Total macrophages in BALF. (B) Representative micrograph of macrophages from BALF of mice. There were 6 animals per group. Data are presented as mean ± SD. Magnification, 1000X; Scale bar = 20 µm

(A)

(B)
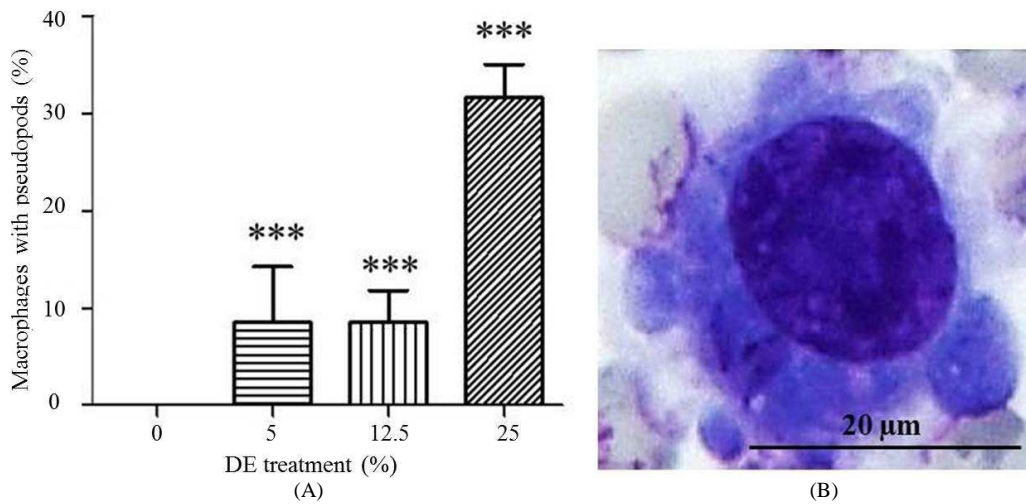
Fig. 4. Macrophages with pseudopodia present in BALF of DE-exposed Balb/c mice. A nebulizer was used to deliver aerosolized PBS [0%] or DE [5, 12.5, 25%] to mice (n = 6 per group) for 2 months. After the 2 month period, BALF was obtained by washing lungs three times; cells were affixed to slides, stained and macrophages with pseudopods were counted. (A) Macrophages with pseudopods in BALF. (B) Representative micrograph of macrophages with pseudopodia in BALF of mice. ***, $p < 0.001$ for DE exposed vs. control (0%) mice when compared by one-way analysis of variance paired with Bonferroni posttest corrections. Data are expressed as mean ± SD. Magnification, 1000X; Scale bar = 20 µm

(A)

(B)
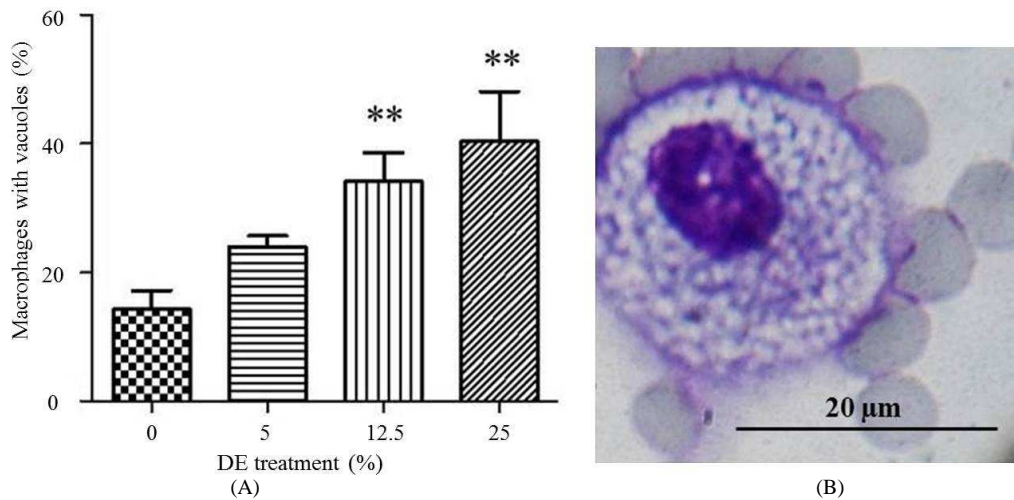
Fig. 5. Dust extract mediated-induction of macrophages with vacuoles in mouse BALF. Aerosolized PBS [0%] or DE [5, 12.5, 25%] was delivered to mice (n = 6 per group) for 8 weeks. After the end of 8 weeks, BALF was obtained from three lung washes; BALF cells were affixed to slides, stained and macrophages with vacuoles were counted. (A) Macrophages with vacuoles in BALF. (B) Representative phase contrast image of macrophages with vacuoles present in BALF of mice. **, $p < 0.01$ for 12.5 and 25% DE exposed vs.control (0%) mice when compared by one-way analysis of variance paired with Bonferroni posttest corrections. Data are expressed as mean ± SD. Magnification, 1000X; Scale bar = 20 µm

3.2. Organic DE Enhances Phagocytosis and ROS Production by THP-1 cells

To evaluate effects of organic DE on macrophage function, THP-1 cells were incubated with or without PMA, LPS and escalating concentrations of DE for 24-48 h. Visual inspection of THP-1 cells exposed to culture media or DE [0.1, 1 and 10%] for 48 h revealed a morphological trend similar to observations of *in vivo* alveolar macrophages (**Fig. 4 and 5**) in that there

appeared to be enhanced pseudopod and vacuole formation in DE-exposed cultures (**Fig. 6**). As shown in **Fig. 6**, panels A-D depict representative images of THP-1 cells cultured in the presence of culture media only (**Fig. 6A**), or media contain 0.1% DE (**Fig. 6B**), 1% DE (**Fig. 6C**) or 10% DE (**Fig. 6D**).

Exposure to occupations dust has been shown to modulate macrophage function and phagocytosis (Meo *et al.*, 2008; Poole *et al.*, 2009a), therefore we assessed phagocytosis of THP-1 cells cultured in the presence of various concentrations of DE. **Fig. 7** shows enhanced phagocytosis by THP-1 cells following exposure to dust suggesting that dust extracts promoted phagocytosis in a dose-dependent manner (**Fig. 7A and B**). It is well accepted that phagocytes generate large amounts of superoxide and hydrogen peroxide when stimulated (Robinson, 2008) and here we demonstrate time and concentration dependent increases in intracellular hydrogen peroxide production by THP-1 cells following exposure to SCF DE (**Fig. 8**).

4. DISCUSSION

Swine confinement facilities and Concentrated Animal Feeding Operations (CAFOs) harbor high levels of dust consisting largely of feed particles, fecal matter, animal dander microbes and their products including lipopolysaccharide and peptidoglycan (May *et al.*, 2012). CAFO dust containing LPS can cause severe inflammatory response (Hollingsworth *et al.*, 2007). Acute inflammation was observed in Spague-Dawley rats exposed to swine barn dust or ambient air for 8 h as determined by enhanced levels of pulmonary intravascular monocytes/macrophages found in bronchoalveolar lavage fluid (Gamage *et al.*, 2007). Acute lung inflammation obesrved in animal models of single exposure to elements of swine barn air is consistent with effects reported from similar human single exposure studies (Charavaryamath and Singh, 2006). However, farmers and animals of SCF are continually exposed to this environment.
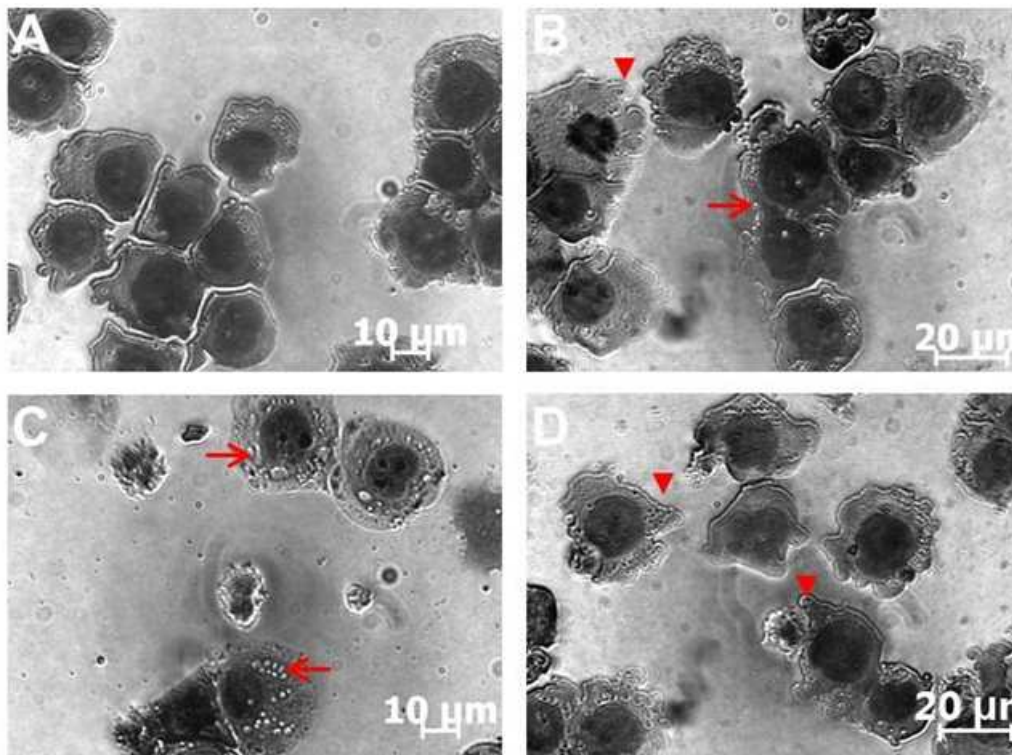
Fig. 6. DE exposure enhanced pseudopod and vacuole formation by THP-1 cells *in vitro*. THP-1 cells were seeded onto Millicell EZ glass slides and exposed to culture media or DE [0.1, 1 or 10%] for 48 h. Cells were stained with Diff Quick and viewed with a Zeiss optical microscope. Phase contrast images of THP-1 cells cultured in the presence of (A) media, (B) 0.1% DE, (C) 1% DE or (D) 10% DE denoting pseudopods (arrowhead) and vacuoles (arrow). Magnification = 1000X
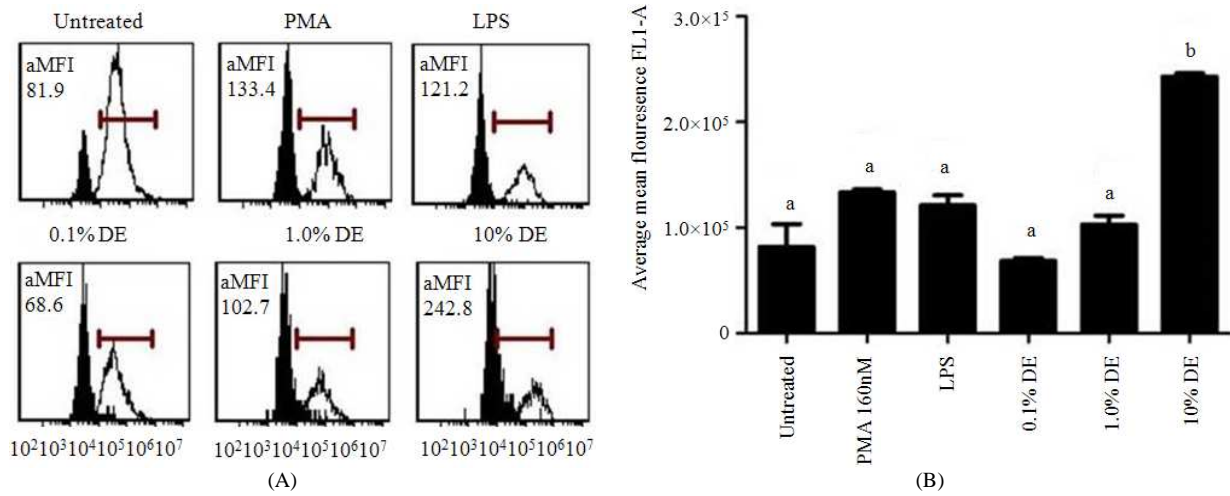
Fig. 7. Phagocytosis is enhanced in the presence of DE. THP-1 cells were left untreated or treated with PMA [160 nM], LPS [1 µg/ml], or DE [0.1, 1.0 and 10%] in the absence or presence of beads coated with FITC-labeled rabbit IgG for 24h. The degree of bead uptake was assessed by flowcytometry where (A) is a representative histogram from each treatment (filled without beads unfilled with beads). aMFI = The average mean flourescence intensity of triplicate wells. Panel (B) is a graph of the aMFI; Significant differences, p<0.05 between 10% DE treated cells (b) and all other treatment conditions (a) as determined by one-way ANOVA with Bonferroni post-test. Data are represented as mean ± SEM
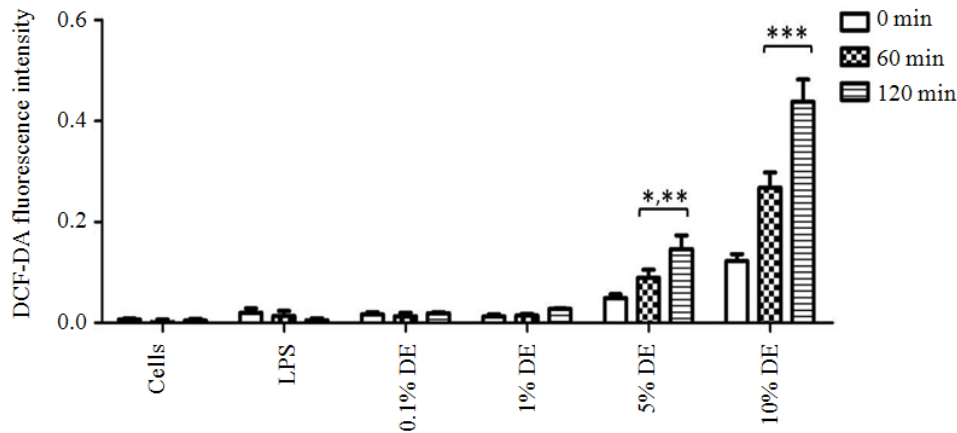
Fig. 8. DE elicits ROS production by macrophages. THP-1 cells were activated by incubation with PMA [160 nM] for 48 h, then left untreated or incubated with 1 µg/mL LPS or DE [0.1, 1, 5 or 10%]. After 24 h following stimulation with LPS or DE, cells were stained with DCF and intracellular ROS production was monitored for an additional 2 h. *, p<0.05 for 5% DE Vs. cells at 60 min; **, p<0.001 5% DE Vs. cells at 120 min; ***, p<0.001 for 10% DE vs. cells at 60 min and 120 min. Data are shown as mean ± SEM

This can lead to chronic lung inflammation, which can lead to diseases, such as, Hypersensitivity Pneumonitis (HP), asthma-like symptoms, dust toxicity syndrome, mucous membrane inflammation syndrome and chronic bronchitis (Charavaryamath and Singh, 2006).

Inhalation of organic dust is particularly harmful to the airway because dust absorbs gaseous substances and airborne microorganisms (Kim *et al.*, 2008). Swine confinement facilities harbor numerous aerosol components including gases and endotoxins may adhere to dust particulates which may have a size range from <0.01 to 100 µm with 40% of those particles being respirable; particles ≤5µm in aerodynamic diameter (Kirkhorn and Garry, 2000). Coarse particulate matter

(PM; with a mean-median airway diameter, MMAD, less than 10 µm), such as present in ambient air, may exacerbate asthma and COPD. Macrophages in induced sputum of healthy volunteers that inhaled between fine PM (2.5-10 µm) for 30 min during three separate visits, displayed significantly enhanced phagocytic activity compared to inhalation of heat inactivated fine PM (2.5-10 µm) (Alexis *et al.*, 2006). Inhalation of 0.4 ppm ozone significantly increased the number and expression of cell surface markers associated with innate immunity (specifically, mCD14, CD11bm CD16) and antigen presentation (CD86, HLA-DR) on airway macrophages in induced sputum in healthy volunteers compared to inhaling filtered air (Lay *et al.*, 2007). Likewise, there was a trend for higher levels of phagocytosis by macrophages in sputum of PM2.5-10 exposed subjects compared to saline control (p = 0.06); however, formal significance (p<0.05) was not observed (Alexis *et al.*, 2006).

In the current study we report that phagocytosis of particles and ROS production by a macrophage cell line was enhanced after *in vitro* exposure to DE from a swine confinement facility. Furthermore, while, the current study did not provide concrete evidence of mediation of macrophage recruitment after mice were exposed to DE; however, there was evidence of phagocytic activity *in vivo*. We report that although total macrophage numbers did not differ from controls there were differences in the levels of macrophages with pseudopodia and vacuoles in lungs of DE exposed mice as compared to those that inhaled PBS. The formation of vacuoles occurs during phagocytosis; after the pathogen (i.e., dust) has been engulfed by macrophages. Within the cells' intestinal lamina, vacuoles or lysosomes are loaded with degradative enzymes to defend the host from any infection. In this present study, the vacuoles of macrophages present in BALF of DE-exposed mice may have assisted in the digestion of inhaled dust particles. Maturation of vacuoles into phagolysosomes for elimination of engulfed particles has been well characterized (Krajcovic *et al.*, 2013). The current study did not provide concrete evidence of mediation of macrophage recruitment after mice were exposed to DE; however, we speculate that the dust caused macrophages within the lungs to become activated and differentiate into macrophages with pseudopods or phagocytic vacuoles. Pseudopods aid in chemotaxis, defined as the movement of pseudopods along a chemical concentration gradient either toward or away from the chemical stimulus. Elevated levels of macrophages with pseudopods may indicate enhanced directional movement in response to inhaled particulates within the DE. This is a possible indication that with the increase in macrophages with pseudopods, more DE particles were potentially captured, engulfed and phagocytosed by macrophages.

Here mice repeatedly exposed to DE displayed elevated levels macrophages and proteins in the BALF. It was found that there was a significant increase in protein concentrations between control and 12.5% DE treatments. There was also a trend of higher BALF protein levels in mice that inhaled 5 and 25% DE than inhalation of PBS, although these values did not reach formal significance (p<0.05). Measurement of BALF components including immune cells, microbial/host protein content, cytokines and chemokines, is the method of choice for elevating airway and lung inflammation (Elizur *et al.*, 2008; Sagel *et al.*, 2009). This is consistent with findings reported by Poole *et al.* (2007) where acute exposure to dust induces an inflammatory response demonstrated by secretion of proinflammatory cytokines; however, re-stimulation resulted in a diminished response. This observation may be explained by phenomena termed chronic inflammation adaptation response (Sundblad *et al.*, 2009; May *et al.*, 2012) in which repetitive dust exposure yields a less severe response in agriculture workers compared to naïve subjects. Sundblad *et al.* (2009) report an adaptive response in farmers (who had been exposed to a pig farm daily in the six months preceding the study) compared to controls and symptom-free cigarette smokers acutely exposed to dust in a pig barn as determined by elevated nitric oxide exhalation, nasal lavage IL-8 and sputum IL-6 in smokers and control subjects compared to farmers.

Farmers may experience occupational hazards, including inhalation of endotoxins and carbon dioxide at levels above the recommended health threshold limit in SCF (McDonnell *et al.*, 2008). After these threshold limits are exceeded, acuteand chronic airway inflammation can occur (Schierl *et al.*, 2007). Airway inflammation can lead to loss of lung function, as measured by the decrease in forced expiratory air in one second or less (Costa *et al.*, 2007). Poole *et al.* (2009b) reported increased cellular inflammation, predominated by neutrophils and neutrophils and macrophages, in mouse BALF following a single and 1/2 week intranasal instillation of DE, respectively. Mice continuously exposed to swine CAFO dust via nebulizer for 4 h developed BALFneutrophilia (Mueller-Anneling *et al.*, 2006). The role of macrophages present in the lungs of mice chronically treated with DE is not known. In our model it is apparent that chronic DE exposure elicits macrophage-mediated cellular inflammation. Taken together, this information suggests that continued improvements of the workplace and provisions for health protection are necessary (McDonnell *et al.*, 2008). Having a better understanding of the mechanisms

involved in the mediation of chronic airway inflammation and disease to organic dust can help lead to preventative or therapeutic agents.

5. CONCLUSION

In conclusion, delivery of aerosolized substances to mice via nebulizer is not novel; however, to our knowledge this is the first report documenting an increase in macrophages with vacuoles and pseudopods following long-term, repeated exposure to aerosolized swine housing dust extract using an *in vivo* mouse model. This mouse model is distinct in that it is uncoupled from sensitization with agents such as LPS, ovalbumin or methacholine prior to the administration of swine housing dust. Furthermore, this research has shown an increase in protein in BALF, likely reflecting an increase in plasma protein exudate; however, this requires further exploration.

One limitation of the present study is that cytokines, which influence disease pathogenesis, were not determined. However, similar studies in organic dust-exposed mice or cultured macrophages have respectively shown that BALF and cell-free supernatants contains cytokines and inflammatory mediators (Poole *et al.*, 2012). The objective of this study was to determine presence of macrophage populations in the nebulizer model of mice exposed to aerosolized SCF DE over an 8-week period in the absence of prior sensitization or tolerization. Our results show that repeated, long-term inhalation of SCF DE was successful in modulating BALF macrophage populations despite the fact that total leukocyte numbers did not vary significantly among control and DE exposed mice. The abundance of pseudopod-bearing and vacuolated macrophages in mice that inhaled DE for 8 weeks was mirrored by an acute DE exposure of THP-1 cells *in vitro*. Notably, significant increases in THP-1 cell phagocytosis were observed in response to incubation with DE in a dose-dependent manner. Thus, we speculate that macrophages within mouse BALF displayed enhanced phagocytosis following inhalation of DE.

Although swine farmers display low-level inflammation following acute exposures to dust, chronic lower respiratory diseases remain an issue. Studies to elucidate the role of occupational exposure to CAFO dust in onset and exacerbation of chronic non-allergic asthma, chronic bronchitis and COPD are needed.

6. ACKNOWLEDGEMENT

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