RESEARCH REPORT
SWINE HEALTH

Title: Effects of Exposure to Organic Dust on Macrophage Function: Implications for Swine Respiratory Health – **NPB #11-064**

Investigator: Dr. Christopher K. Tuggle

Institution: Iowa State University

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Industry Summary

Exposure to the dust in pig barns has been shown to affect short-term measures of inflammation and immune function in swine workers. Models of such exposure that used human or mouse lung immune cells (alveolar macrophages, AM) showed negative effects on both maturation and function of these important cells. Because these effects could be detrimental to swine health, we want to know whether pigs are similarly affected by such dust, or if they have become tolerant to it. As a first step, our objectives in this project were to test whether extracts of swine barn dust (termed organic dust extract, ODE) have the same effects on swine macrophages in culture as seen for human macrophages.

We collected AM from eight healthy pigs, exposed these cells in culture to different amounts of ODE, and found that exposure to ODE activated the swine macrophages to express inflammation signaling proteins, as well as other proteins known to be part of a normal immune response process. Interestingly, one of the proteins is the co-receptor for PRRSV. As AM are the primary cell infected by PRRSV, exposure to dust could potentially be increasing the vulnerability of these lung macrophages to PRRSV infection. Further, ODE exposure decreased important immune function of these cells, including their normal ability to uptake particles in the environment and to kill intracellular bacteria, as has been seen in human cells similarly exposed to ODE. We also found that the ODE-treated pig cells could not fully activate a critical regulator of inflammatory pathways in response to a separate inflammatory signal (endotoxin), providing a mechanism for some of these negative effects. We interpret the results of this in vitro model to indicate that swine macrophages can be affected by exposure to dust, and that experiments to test whether pigs themselves are negatively affected by

such exposure is warranted. If we can demonstrate in a follow-up study that growth or resistance to stress or disease is negatively affected, this would be important information to the industry as it evaluates the utility of dust mitigation. Further, the possible negative impact of dust exposure on PRRSv resistance should be specifically investigated.

Keywords

Swine barn dust, pig, macrophage, airway inflammation, respiratory disease

Scientific Abstract

Respiratory diseases are responsible for a significant amount of animal morbidity and mortality in the swine industry, including the majority of nursery deaths and grower/finisher deaths. Innate immunity and the maintenance of lung macrophage health and function is an important defense mechanism against respiratory pathogens and their associated losses. Chronic exposure of swine industry workers to airborne barn dust results in significant predisposition to airway diseases, yet few studies have examined the impact of dust on the swine immune system. We evaluated cytokine production, cell surface marker expression, and the phagocytic, antibacterial and nuclear translocation capacities of porcine macrophages after in vitro exposure to an organic swine barn dust extract (ODE). To our knowledge, this is the first study to demonstrate the effects of swine barn dust components on pig macrophage activation and function.

Exposure to ODE induced AM secretion of both pro- and anti-inflammatory cytokines, suggesting a complex activation profile. Additionally, ODE induced expression of genes involved in sensing Gram-positive bacteria, a major component of barn dust. ODE also enhanced the expression of several cell surface markers of activation, including a receptor for porcine reproductive and respiratory syndrome virus (PRRSv). Moreover, two of the primary functions of AM, phagocytosis and bacterial killing, were impaired after exposure to ODE. Treatment with ODE for the first 72 h of differentiation also inhibited the ability of monocyte-derived macrophages (MDMs) to translocate NF-κB to the nucleus after stimulation. Taken together, these results indicate that dust exposure negatively affects pig macrophage function and alters immune phenotype, potentially enhancing host susceptibility to a variety of respiratory infections.

Introduction

A significant amount of animal loss is attributed to respiratory disease in the swine industry. The National Animal Health Monitoring System (NAHMS) 2006 report found that respiratory problems accounted for the highest percentage of all nursery deaths (53.7%) and the majority of grower/finisher deaths (60.1%) (NAHMS, 2006). Respiratory diseases also contribute to costly losses in swine production by decreasing feed intake and

average daily gain (Jericho and Harries, 1975; van Reeth and Nauwynck, 2000). Swine barn workers are significantly predisposed to airway diseases, including rhinitis, bronchitis and chronic obstructive pulmonary disease, due to their constant exposure to barn dust (Von Essen and Romberger, 2003).

Swine barn dust is composed of a myriad of components derived from feed, dander, fecal waste, microbial particles and other sources, and AMs are among the first immune cells to respond to these inhaled particles (Poole and Romberger, 2012). Microbial constituents of organic dust are rich in highly conserved pathogen-associated molecular patterns (PAMPs) that are recognized by host pattern recognition receptors (PRRs) in order to activate cellular inflammatory responses (Barton and Medzhitov, 2002; Poole and Romberger, 2012). The non-allergic inflammation elicited by inhaled dust is accompanied by local and systemic production of inflammatory cytokines, such as TNF-α, IL-1β and the chemoattractant CXCL8, resulting in pyrexia, enhanced mucus production and neutrophil influx into the airways (Larsson et al., 1997; Sahlander et al., 2012; Wang et al., 1998). In mice, humans and pigs, dust inhalation has been directly linked to increased airway inflammation and lung pathology (Donham et al., 1995; Poole et al., 2009b; Urbain et al., 1999). Exposure to swine barn ODE in vitro impaired human and murine macrophage function (Poole et al., 2008) and altered human dendritic cell maturation (Poole et al., 2009a).

To date, few studies have examined the impact of chronic barn dust exposure on the swine immune system, and none have attempted to directly test whether dust impairs macrophage phenotype or function. We sought to define the functional alterations in cytokine production, cell surface marker expression and phagocytosis of pig AMs exposed to organic dust extract (ODE) obtained from swine barns. We found that ODE exposure induced both pro- and anti-inflammatory cytokine production, enhanced surface expression of activation markers and enhanced the expression of genes involved in sensing Gram-positive bacteria. We further identified diminished phagocytosis and reduced bacterial killing after ODE treatment. Moreover, we demonstrated that ODE exposure during the early differentiation of monocyte-derived macrophages (MDMs) reduced translocation of nuclear factor kappa B (NF-κB) to the nucleus after stimulation. Together, these data show that swine barn ODE suppresses macrophage function. Given that swine respiratory immunity must be optimal for disease resistance and efficient growth in today's modern production facilities, barn dust exposure may be an underappreciated underlying cause of porcine respiratory disease outbreaks.

Objectives

1. Determine if organic dust components affect pig macrophage activity against swine respiratory bacterial pathogens in vitro, as has been shown for human macrophages.

2. Determine if organic dust components affect pig macrophage activation and cytokine production.

Materials & Methods

Organic dust extract (ODE)

The ODE was a kind gift from J. A. Poole, University of Nebraska Medical Center; it was collected, prepared and analyzed for composition as previously described (Poole et al., 2012; Poole et al., 2007; Romberger et al., 2002). Briefly, settled dust was collected three feet above the floor from a swine confinement facility of 500-700 animals. Dust samples were then placed into solution, vortexed and centrifuged. The supernatant was filter sterilized (0.22 μm) and frozen (-20°C) for shipping. Endotoxin concentration was used as a biomarker of swine barn exposure conditions to calculate relevant dose as previously described (Poole et al., 2008; Poole et al., 2009a). There was no evidence of cell death after incubation with any of the treatments.

Animals, lavage and macrophage culture

Ten pigs between 8-12 weeks of age of either gender were euthanized with an overdose of sodium pentobarbital given intravenously according to Iowa State University Laboratory Animal Resources experimental guidelines. The IACUC at Iowa State University approved all protocols involving animals. Lungs were removed and lavaged twice with 300 mL of cold, sterile PBS and an approximate 200 mL total volume was recovered. Lavage fluid was centrifuged at 500 x g for 15 min and cell pellets were pooled and washed once in 30 mL cold PBS. Erythrocytes were lysed in 3 mL lysis buffer (0.15 M NH4Cl, 10 mM KHCO3, 0.1 mM EDTA). Cells were washed in cold PBS, resuspended in complete culture media (RPMI 1640, 5% heat-inactivated normal swine serum (Sigma-Aldrich, St. Louis, MO), 5 mM HEPES, 1 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin , and 15 μg/ml gentamicin sulfate), plated into 150 x 15-mm cell-culture-treated dishes and allowed to adhere for 2 h at 37°C with 5% CO2. After 2 h, nonadherent cells were removed and discarded. Macrophages were harvested by scraping, washed and counted via trypan-blue exclusion on a hemacytometer. Macrophages were resuspended at a concentration of 5 x 10^5 cells/mL in culture medium for plating. For bacterial phagocytosis and killing assays, complete culture media without antibiotics was used. Medium was added in equal volume to the 1% ODE treatment, endotoxin was added at 10 μg/mL, and ODE was added as a percentage of total culture volume as indicated.

To obtain porcine monocyte-derived macrophages (MDMs), monocytes were isolated from whole blood via density gradient centrifugation. Briefly, peripheral blood was diluted 1:2 (v:v) in sterile PBS, overlayed onto Lymphocyte Separation Media (LSM; Mediatech, Manassas, VA) and centrifuged at 500 x g for 30 min. Peripheral blood mononuclear cells (PBMC) were obtained from the LSM/plasma interface and washed twice with sterile PBS. Monocytes were further enriched to > 95% purity by positive magnetic bead selection as previously described by Bimczok et al. (2007) with some modifications. Monocytes were plated in Dulbecco's

modified eagle's medium containing 4.5 mg/mL glucose, 10% heat-inactivated normal swine serum, 2 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, 25 mM HEPES and 0.05 µM 2-mercaptoethanol) at a density of 5 x 105 cells/mL. Medium was supplemented with 30% conditioned media from confluent cultures of L929 fibroblasts to serve as a source of Colony Stimulating Factor (CSF) to induce monocyte differentiation into macrophages.

RNA isolation and reverse transcription-PCR

Cells were harvested after 5 h in culture with treatments as indicated. Total RNA was isolated and purified using an RNeasy Mini kit and DNase I kit (Qiagen, Valencia, CA), and the quantity and quality of RNA was measured using Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA). Resulting RNA was reverse transcribed into cDNA using 200 ng of total RNA with a SuperScript VILO cDNA synthesis kit (Invitrogen, Carlsbad, CA). Primer designs were obtained from the Porcine Immunology and Nutrition (PIN) database (Dawson et al., 2005). Real-time PCR was performed using a SYBR Green PCR kit (Applied Biosystems, Carlsbad, CA) according to the manufacturer's instructions. Assays were performed in triplicate. Average CT values of duplicate wells were compared to a standard curve for each gene to determine relative expression, and expression was normalized to expression of RPL32 and ACTB genes.

Flow cytometry

Approximately 2.5 x 105 macrophages were placed in each staining tube, washed in PBS containing 0.1% NaN3 and 0.1% BSA, blocked using 10 µL normal swine serum and stained with corresponding mAbs. Irrelevant isotype controls were also used to account for non-specific binding, and primary staining was allowed for 15 min on ice. Cells were then washed and stained with either anti-IgG1 phycoerythrin (PE) or anti-IgG2 fluorescent secondary antibodies for 15 min on ice before fixing. Surface marker expression was reported as mean fluorescence intensity (MFI) minus isotype background MFI. To measure macrophage phagocytosis, 0.2 µM FITC-loaded FluoSpheres (1:1,000 dilution; Invitrogen) were added to cultures of alveolar macrophages and incubated for 1 h at 37°C. Cells were then placed on ice, harvested via scraping, washed to remove any extracellular particles and fixed in 1% paraformaldehyde prior to analysis via flow cytometry.

Cytokine analysis

Supernatants were collected and analyzed via a multiplex cytokine assay as described in Lawson et al. (2010) with some modifications. Briefly, magnetic microspheres (Luminex Corporation, Austin, TX) were covalently coupled to mAbs followed by a streptavidin-PE conjugate (eBioscience). Fluorescence intensity and cytokine concentrations were measured against a standard curve via a Bio-Plex 200 array system (Bio-Rad Laboratories, Hercules, CA).

Bacterial culture, phagocytosis and killing assays

A field isolate of Salmonella enterica serovar Cholerasuis obtained from the respiratory tract of an infected pig was a kind gift from R. W. Griffith, Iowa State University. Bacteria were grown on either 5% blood agar plates or cultured in brain heart infusion (BHI) broth.

Phagocytosis assays were completed as previously described (Poole et al., 2009a) with some modification. Bacterial cultures were opsonized in heat-inactivated normal swine sera for 30 min. Macrophages were then inoculated at an MOI of 100:1 and incubated for 30 min at 37°C with 5% CO2. Cultures were then placed on ice, media was removed, and 500 μL of antibiotic-containing culture media was added. Cells were harvested via scraping and divided into two aliquots noted as t0 and t120. Aliquots for t0 were lysed immediately with 1% saponin on ice for 15 min, and the lysate plated in duplicate in serial dilutions on 5% blood agar plates. Aliquots for t120 were incubated with occasional agitation for 120 min at 37°C with 5% CO2, after which they were lysed and plated as described for t0 aliquots. Phagocytosis was determined by the CFU count from t0 aliquots, and percent killing was calculated as [(cfu at t0 - cfu at t120)/(cfu at t0)] X 100.

Nuclear translocation assay

Monocytes isolated and cultured as described in section 2.2 were also cultured either in the presence or absence of 1% ODE during the first 72 h of differentiation. After a total of 6 d in culture, MDMs were stimulated with 10 μg/mL of Salmonella endotoxin or an equal volume of medium (No Stim) for 1 h at 37°C to induce NF-κB translocation. Cells were then fixed and stained with an anti-NF-κB p65 pAb followed by a secondary stain with an Alexa Fluor 555 goat anti-mouse IgG. Immediately prior to acquisition, DRAQ5 (BioStatus, Leicestershire, UK) was added to visualize the nucleus. Translocation was visualized by multi-spectral imaging flow cytometry (Amnis ImageStreamX, Seattle, WA) and a Similarity Score was calculated by software extrapolation of the log transformed Pearson's Correlation Coefficient of image pixel intensity for NF-κB.

Statistics

Data were analyzed using the GLIMMIX procedure of SAS (Version 9.2, SAS Institute, Cary, NC) with treatment as a fixed effect and pig as the subject of repeated measures. For nuclear translocation data analyses, fixed effects were treatment and replicate. Gaussian distribution of response variables was assumed. Least square means were calculated and treatments were compared using the SLICE and SLICEDIFF procedures. An adjusted p-value was calculated by using Tukey corrections for multiple comparisons among treatments. Differences were considered to be significant if adjusted p-values < 0.05.

Results

Organic dust extract altered AM surface marker expression.

Compared to all other treatments, AM exposed to 1% ODE had increased expression of SWC9 ($p < 0.05$), a pyrophosphatase that is widely used as a marker of macrophage maturity (Ezquerra et al., 2009). The 1% ODE treatment had the same effect on SLA-II expression ($p < 0.05$), the porcine homolog of MHCII responsible for macrophage antigen presentation to lymphocytes. Both the 0.1% ODE and the 1% ODE treatments enhanced expression of CD163 when compared to either medium only ($p < 0.001$) or endotoxin ($p < 0.01$). This marker has been identified as a receptor for PRRSv entry into macrophages, and its expression level has been highly correlated with PRRSv replication (Patton et al., 2009). There was no statistically significant effect of treatment on surface expression of CD14. Taken together, these results may indicate that organic dust exposure enhances the maturation and activation state of AMs as identified by increasing SWC9 and SLA-II expression, while potentially making them more susceptible to PRRSv infection by enhancing expression of CD163.

Organic dust exposure induced both a pro- and anti-inflammatory cytokine response.

To identify the cytokine response from AMs exposed to organic dust extract at 24 h, supernatants were collected, pooled within pig and treatment, and analyzed via multi-plex array to detect multiple cytokines within a single sample. Both IL-1β and TNF-α had a similar pattern of dose-dependent secretion when comparing 0.1% and 1% ODE to media alone ($p < 0.05$, $p < 0.0001$). Similarly, 1% ODE also induced secretion of CXCL8 ($p < 0.05$), a chemokine involved in neutrophil recruitment to sites of inflammation (van Reeth and Nauwynck, 2000). Both 0.1% and 1% ODE stimulated release of the pro-inflammatory mediator IFN-γ ($p < 0.05$, $p < 0.01$), and macrophages exposed to 1% ODE also had a marked increase in secretion of IL-10 ($p < 0.01$), an anti-inflammatory cytokine.

ODE induced the expression of cytokine genes, as well as TLR2, NOD2 and CXCL2.

Expression of IL1B, TNFA, IFNG, IL8 and IL10 was measured to confirm the cytokine patterns, and TLR2, TLR4, NOD2 and CXLC2 expression was measured to identify potential signaling mechanisms involved in the AMs response to dust. After 5 h in culture with treatments, cells were harvested for RNA extraction, reverse transcription and quantitative PCR. All observed cytokine production responses to treatments were confirmed by gene expression with the exception of IFNG, which did not demonstrate any significant difference between any treatment, and IL12B, which was undetectable. CXCL2 expression was increased in response to both endotoxin and 1% ODE ($p < 0.05$). Expression of TLR2 was significantly increased after exposure to 0.1% ODE ($p < 0.05$), however there was no significant differences in TLR4 expression. NOD2 expression was significantly enhanced in response to 1% ODE ($p < 0.01$).

Exposure to organic dust diminished phagocytic ability.

To elucidate the effects of ODE on macrophage function, phagocytic ability of macrophages was measured by uptake of FITC-labeled FluoSpheres. Macrophages exposed to both 0.1% and 1% ODE exhibited significantly decreased phagocytosis of FluoSpheres ($p < 0.05$, $p < 0.01$) as reported by a lower MFI. These data indicate that exposure to ODE impairs phagocytosis, one of the primary functions of AMs in protecting the lung from pathogens and inhaled particles.

Bacterial killing was inhibited after exposure to organic dust extract.

To further identify the effects of ODE on macrophage function, an intracellular viability assay was used to determine the capacity for bacterial killing. The AMs were inoculated with *Salmonella enterica* serovar Cholerasuis after incubation for 24 h with treatments. After 30 min to allow for phagocytosis, macrophages were either lysed immediately or after an additional 120 min to evaluate ability to kill phagocytosed bacteria as enumerated in the Materials and methods section. Treatment with 1% ODE significantly impaired bacterial killing when compared to media alone (87.61% vs. 99.11%, $p < 0.05$). This dampened ability to kill internalized bacteria is further evidence that ODE exposure impairs AM function and may contribute to enhancing respiratory disease susceptibility in pigs.

Differentiation in the presence of organic dust impaired nuclear translocation of NF-κB in MDMs.

After differentiation in the presence of ODE or medium only, MDMs were stimulated with 10 μg/mL of endotoxin or an equal volume of medium (No Stim) for 1 h at 37°C to induce NF-κB translocation. Fixed cells were stained with DRAQ5 DNA-stain (red) to visualize the nucleus, or with a mAb against NF-κB (green) and representative images of each treatment are shown. Treatment with 1% ODE for the first 72h of differentiation inhibited the ability of MDMs to translocate NF-κB to the nucleus after endotoxin stimulation (39.25% vs. 66.49%, $p < 0.05$).

Discussion

Inhalation of organic dust induces an inflammatory response that can result in respiratory diseases in humans. Approximately 60% of all workers in confined animal feeding operations are estimated to develop at least one respiratory disease symptom after only six years in their field (Donham et al., 1989). The pigs housed in these barns are constantly exposed to the same organic dust that elicits these responses in humans, however the effect of dust on pig respiratory health is less understood. Given the critical role of macrophages in responding to inhaled particles and pathogens, we examined the effects of occupational levels of ODE on macrophage phenotype and function. For the first time, we demonstrate the changes in phenotype, activation and depressed function of pig macrophages in response to swine barn dust extract.

As the cause of the most economically important disease in the swine industry, PRRSv and it's utilization of CD163 to infect pig macrophages have been studied in some detail (Neumann et al., 2005). Expression of CD163 is macrophage specific, and the primary target of PRRSv is porcine AMs (Duan et al., 1997). Moreover, non-permissive cells can be made permissive to PRRSv infection by transfection with CD163 cDNA (Welch and Calvert, 2010). CD163 is a haptoglobin-hemoglobin complex scavenger receptor, and it is hypothesized that it removes these complexes to assist in resolving inflammation (Schaer et al., 2002). We observed an increase in surface expression of CD163, as well as an increase in TLR2 gene expression in response to ODE. Others have reported that ligation of TLR2 and TLR5 on human monocytes corresponded with enhanced surface expression of CD163 (Weaver et al., 2007). Chemical analysis of ODE revealed a high concentration of muramic acid, a ligand for TLR2, and human macrophage surface expression of TLR2 increases in response to ODE (Bailey et al., 2008; Poole et al., 2011b). It is possible that the muramic acid present in ODE ligates TLR2, and this in turn enhances surface expression of CD163. Notably, IL-10 treatment has been reported to increase CD163 surface expression, with a corresponding increase in PRRSv infection (Patton et al., 2009; Sulahian et al., 2000). Given our observed increases in IL-10 in response to ODE, it is possible that there is an association between TLR2 ligation, IL-10 production and CD163 surface expression in response to dust. These relationships and their potential impact on PRRSv infection would be interesting to explore in future studies.

Consistent with a pro-inflammatory profile, we also found AMs increased in IL-1β, TNF-a, CXCL8, and IFNγ production in response to ODE. IL-1β and TNF-a are a well-characterized part of the early innate immune response, and in the porcine lung induce bronchoconstriction, enhance mucus secretion and activate endothelial cells, neutrophils, lymphocytes and macrophages (van Reeth and Nauwynck, 2000). We identified an increase in production of CXCL8, as well as in gene expression of CXCL2 in response to ODE. Both chemokines are powerful neutrophil chemoattractants, and this is consistent with the observed neutrophil influx in the airways of swine barn workers (Larsson et al., 1997), and in pigs after exposure to airborne dust contaminants (Jolie et al., 1999). Macrophages also enhanced production of IFN-g in response to ODE. It has been postulated that AMs produce IFN-g to act in an autocrine or paracrine manner, enhancing their bactericidal capabilities (Fenton et al., 1997). Together, these pro-inflammatory cytokines have been widely noted to induce lung inflammation and pathology, largely contributing to respiratory disease in pigs (van Reeth and Nauwynck, 2000). Our observation of increased IL-10 being produced simultaneously with these classically pro-inflammatory cytokines is not surprising, as AMs have been shown to produce both IFN-g and IL-10 simultaneously in disease states as a measure of balancing the immune response (Oltmanns et al., 2003). These results provide further evidence that ODE activates AMs, stimulating production of both pro- and anti-inflammatory cytokines. Although the activation of AMs is designed to resolve infection, chronic activation can lead to increased lung inflammation and disease pathology (Poole et al., 2009b), and this may be a mechanism by which dust exposure predisposes pigs to future respiratory disease.

We examined the phagocytosis and microbicidal activity of porcine AMs after treatment with ODE as key aspects of macrophage function. We are the first to show that fully-differentiated porcine AMs have diminished phagocytic ability after exposure to ODE. We also observed that ODE-treated AMs are less capable of killing a respiratory isolate of a common swine pathogen, Salmonella enterica serovar Choleraesuis. Together, these findings support the hypothesis that swine barn dust exposure reduces several aspects of macrophage functionality, and may make pigs more susceptible to inhaled pathogens.

Although dust exposure elicits inflammation, evidence also exists for an adaptation response. Studies examining repeated dust exposure have demonstrated that the inflammatory response to ODE is less robust upon a second exposure (Poole et al., 2009b). After 3-5h of work in a hog barn, leukocyte numbers in the BALF of naïve subjects was greater than those found in the BALF of swine confinement workers, although both were elevated above basal levels (Larsson et al., 1994). Moreover, human monocytes secreted TNF-α, IL-6, IL-10 and CXCL8 in response to an initial treatment with dust extract; however, only the IL-10 and CXCL8 response remained consistently elevated after a second exposure (Poole et al., 2007). A potential mechanism behind this reduction in the inflammatory response may be the negative regulation demonstrated by the constitutively expressed PRR NOD2. NOD2 is ligated by muramyl dipeptide, a constituent of Gram-positive bacterial cell walls, and induces NF-κB translocation for the synthesis of inflammatory mediators, antimicrobial peptides and additional NOD2 molecules (Franchi et al., 2009; Fritz et al., 2006). We demonstrated that a single exposure of ODE induces the gene expression of NOD2 in porcine macrophages. This has also been reported for human cell lines, in addition to a reduction in NOD2 expression upon inhibition of the NF-κB pathway (Poole et al., 2011a). NOD2 knockout mice demonstrate increases in cytokine expression, enhanced airway inflammation and increased TLR-2-mediated activation of NF-κB when compared to wild-type (Poole et al., 2011a; Watanabe et al., 2004). Researchers demonstrating that NOD2 activation results in the co-localization of TLR2 suggested that NOD2 is activated to control the internalization of TLR2, limiting the effects of PAMP signaling (Muller et al., 2010). Although the mechanism has yet to be defined, these results together indicate that NOD2 is a negative regulator of dust-induced inflammation, especially in the context of TLR2 signaling. It would be interesting to expand these studies and examine the effects of repeated and chronic exposure to swine barn dust on the porcine respiratory system, both in vitro and in vivo.

We also report dampened NF-κB translocation in porcine macrophages differentiated in the presence of ODE, and our demonstration of NF-κB translocation on a single-cell basis further supports a potential adaptation response. Previous studies have reported that human cellular differentiation in the presence of ODE resulted in a less functional and mature phenotype (Poole et al., 2008; Poole et al., 2009a). It is possible that monocyte maturity is correlated with NF-κB responsiveness, however others demonstrated that undifferentiated monocytes are quite capable of rapidly secreting products of NF-κB pathway activation (Poole et al., 2007). Moreover, monocytes treated with ODE for only 18 h showed diminished cytokine production upon re-

stimulation, indicating that undifferentiated monocytes are susceptible to an adaptation response. It is therefore more likely that ODE inhibits MDM NF-κB translocation by a different mechanism than restricting maturity. It has been hypothesized that NOD2 activation restricts nuclear translocation of the RelA subunit of NF-κB, as RelA activation is increased in the absence of NOD2 (Strober et al., 2006). This may be a mechanism by which NOD2 participates in an adaptation response, and negatively regulates the NF-κB translocation we observed.

In conclusion, our results demonstrate that swine barn dust exposure stimulates AMs to secrete cytokines that have been linked to increased lung inflammation and pathology, as well as altering surface marker expression. Moreover, exposure to ODE significantly increased surface expression of a receptor that has been highly correlated with enhanced intracellular PRRSv replication. Exposure to ODE also negatively affects three important components of macrophage function: phagocytosis, intracellular bacterial killing, and NF-κB translocation. The in vitro results described herein indicate that swine barn dust exposure negatively affects macrophages. Maintaining macrophage health is a critical innate defense mechanism, and the inhibition of macrophage function may potentially increase porcine susceptibility to respiratory disease and subsequent costly decreases in growth efficiency and health.

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Figures

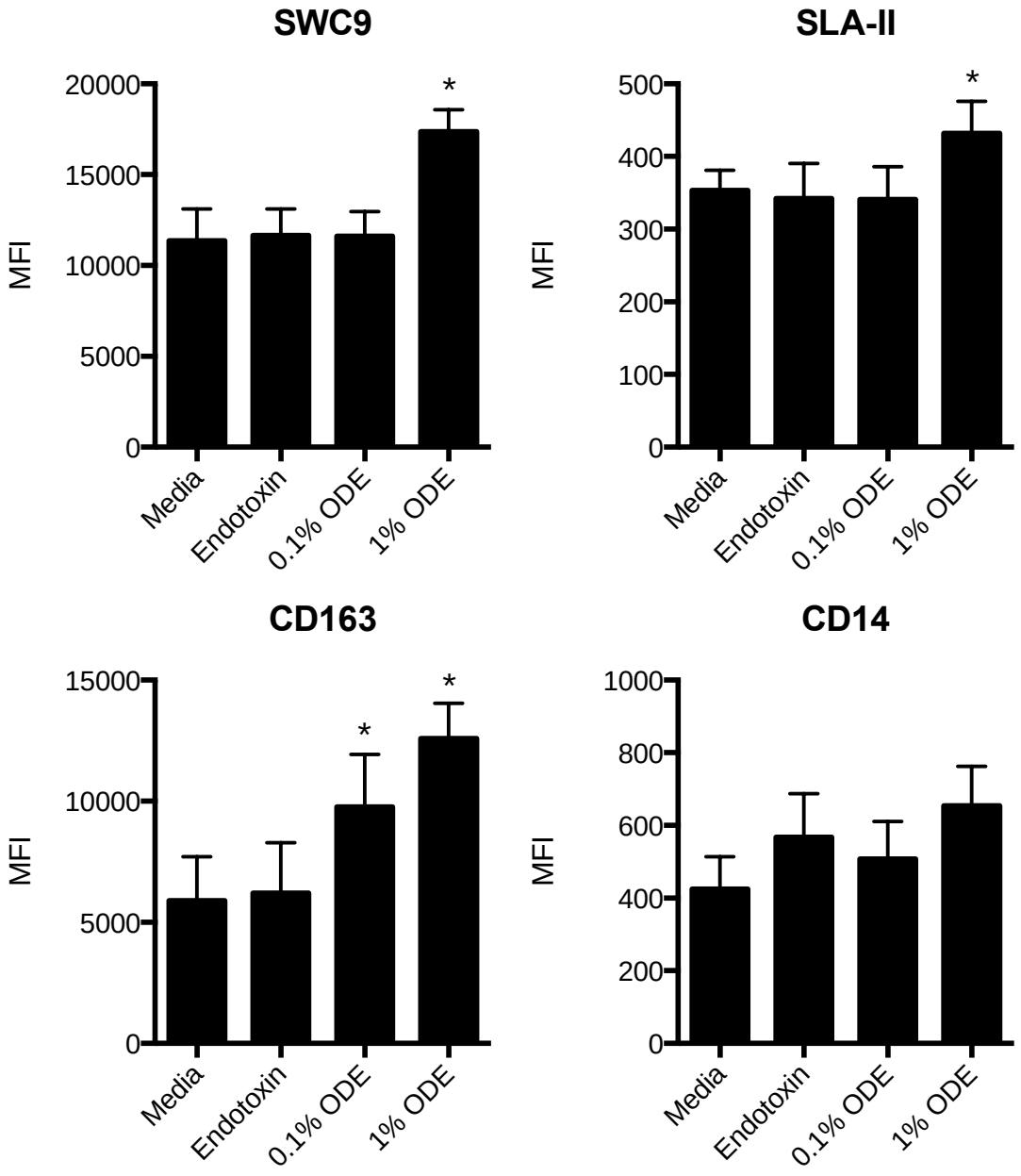
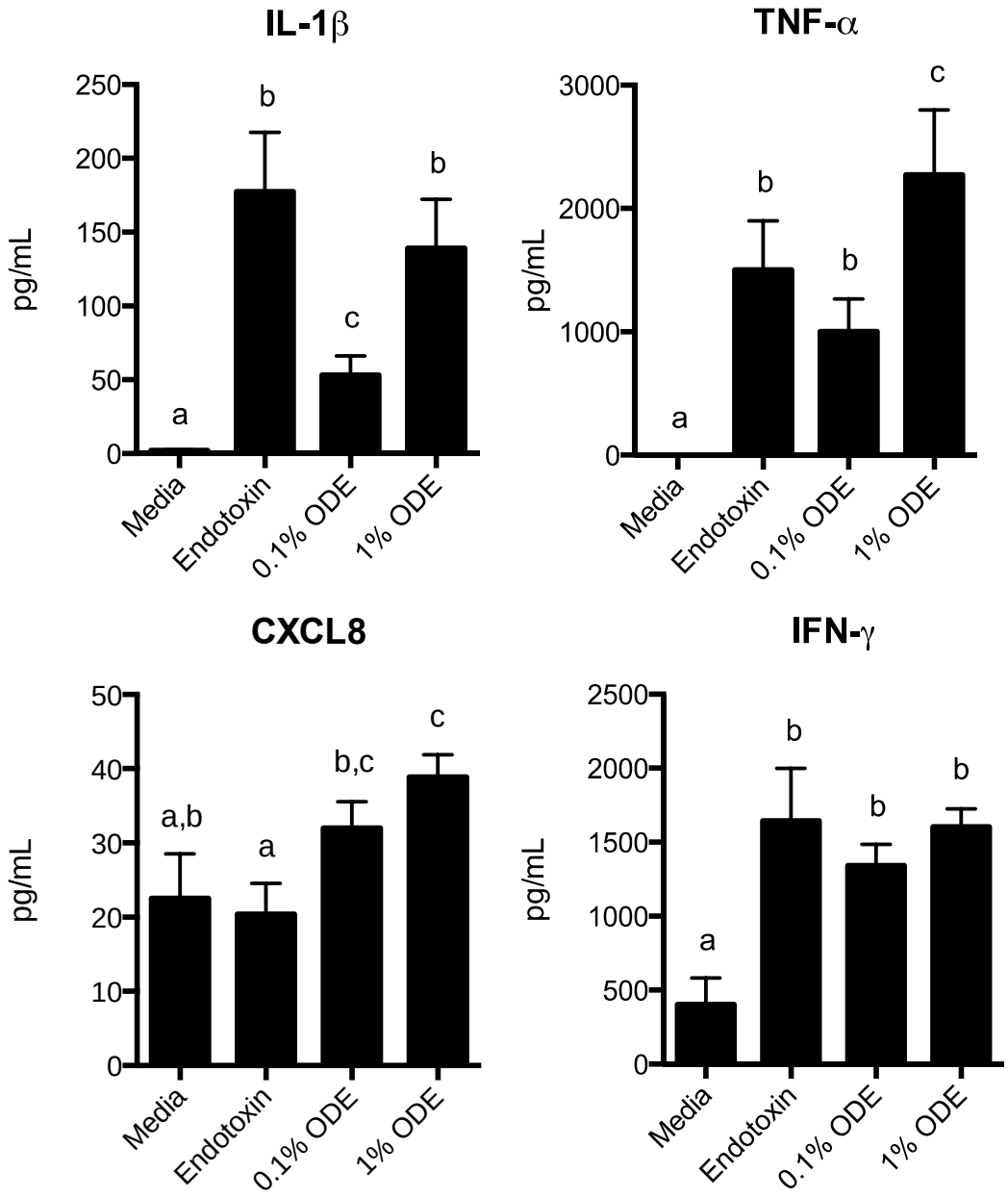


Figure 1. Modulated surface marker expression of AMϕs in response to medium, endotoxin, 0.1% ODE or 1% ODE after 24 h. Data is reported as mean fluorescence intensity (MFI) minus isotype background MFI. Means are represented as ± SEM; asterisks (*) indicate statistically significant difference from unmarked bars at $p < 0.05$.


IL-1β
TNF-α
CXCL8
IFN-γ
pg/mL
Media
Endotoxin
0.1% ODE
1% ODE
a
b
c
a,b
b,c

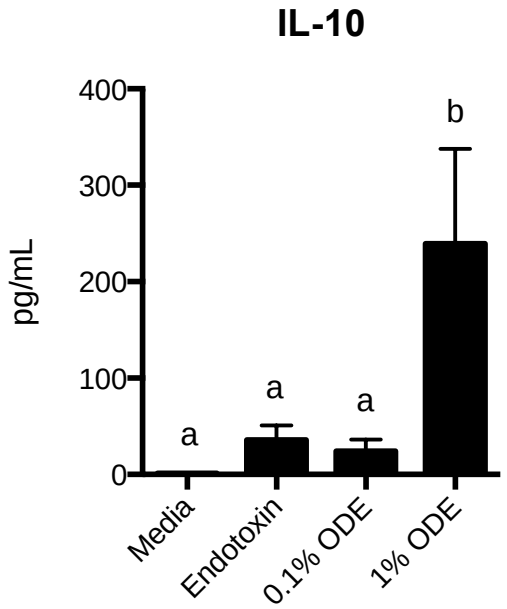
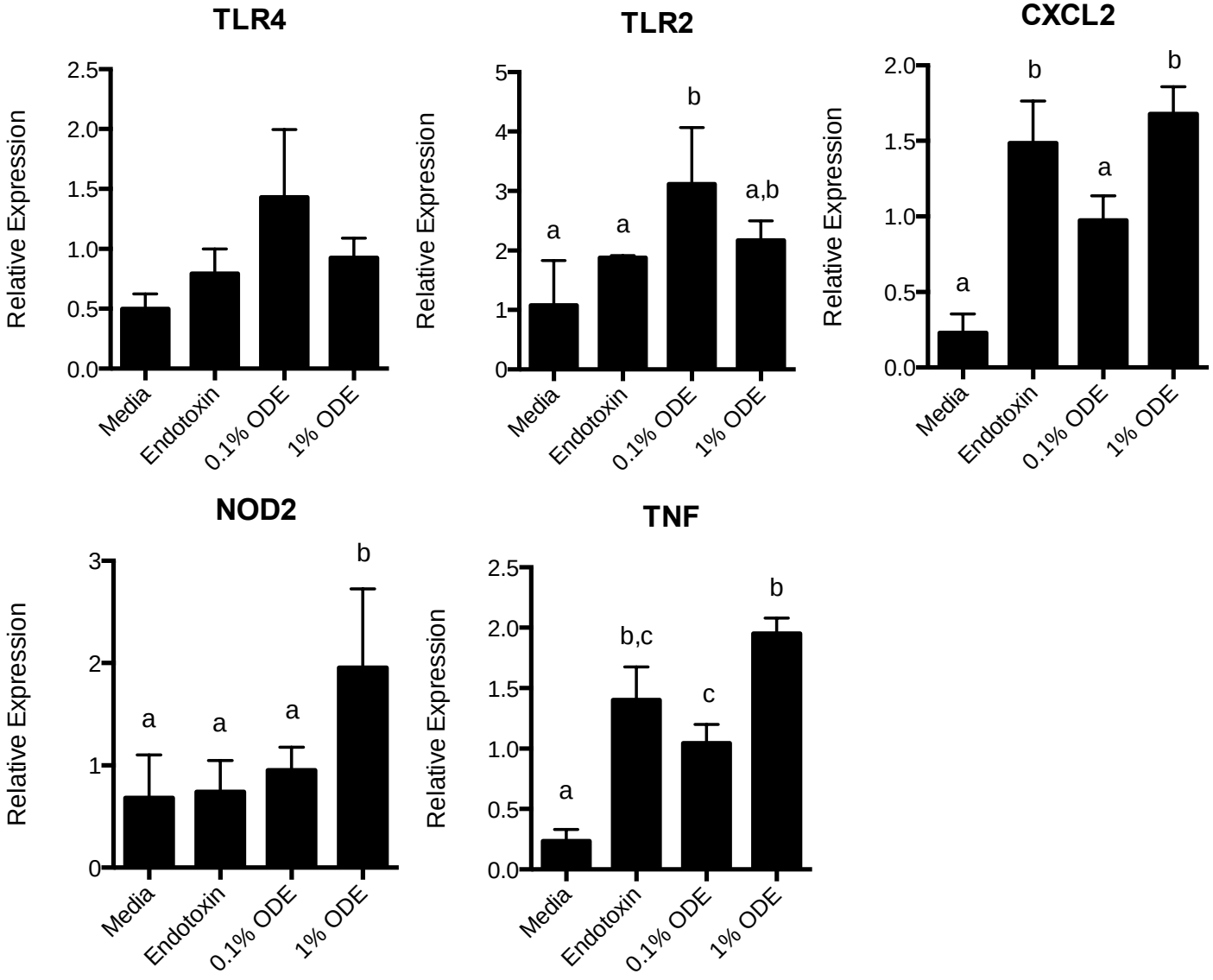


Figure 2. Analysis of cytokine concentrations in cell-free supernatants via multiplex fluorescent bead assay for porcine IL-1β, CXCL8, IL-10, IFN-γ and TNF-α. Means are represented ± SEM; bars without a common letter indicate statistically significant difference at $p < 0.05$.


TLR4
Relative Expression
2.5
2.0
1.5
1.0
0.5
0.0
Media
Endotoxin
0.1% ODE
1% ODE
TLR2
Relative Expression
5
4
3
2
1
0
a
a
b
a,b
Media
Endotoxin
0.1% ODE
1% ODE
CXCL2
Relative Expression
2.0
1.5
1.0
0.5
0.0
a
b
a
b
Media
Endotoxin
0.1% ODE
1% ODE
NOD2
Relative Expression
3
2
1
0
a
a
a
b
Media
Endotoxin
0.1% ODE
1% ODE
TNF
Relative Expression
2.5
2.0
1.5
1.0
0.5
0.0
a
b,c
c
b
Media
Endotoxin
0.1% ODE
1% ODE

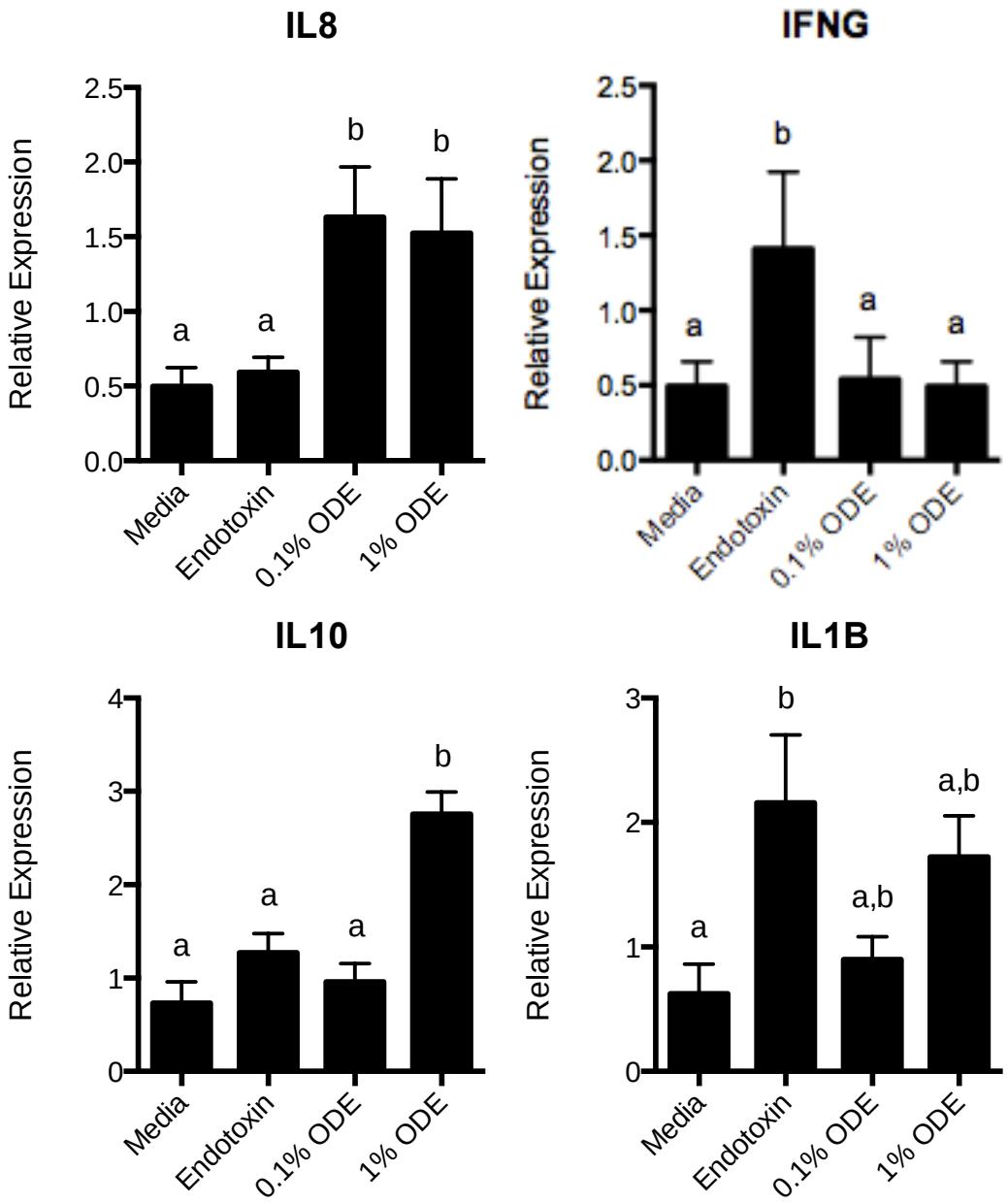
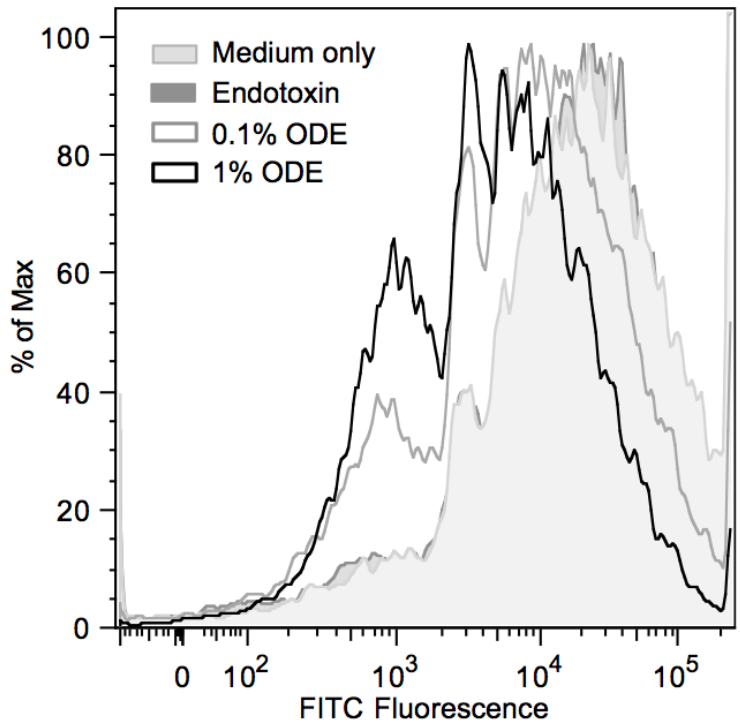


Figure 3. Gene expression of TLR2, NOD2, CXCL2, TLR4 and cytokine genes in response to medium, endotoxin, 0.1% ODE or 1% ODE after 5 h. Average CT values of duplicate wells were compared to a standard curve for each gene to determine relative expression, and expression was normalized to RPL32 and ACTB genes. Treatment means are represented ± SEM; bars without a common letter indicate statistically significant difference at $p < 0.05$.

A



B

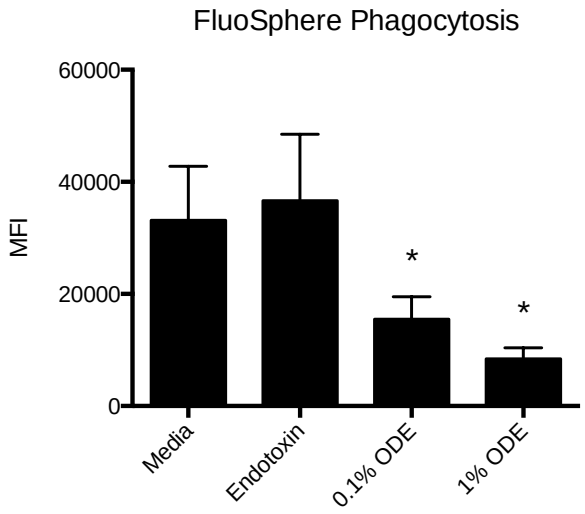


Figure 4. Flow cytometry analysis of AMϕ phagocytosis after treatment with medium, endotoxin, 0.1% ODE and 1% ODE for 24 h. (A) Representative histogram of AMϕ fluorescence intensity; right shift indicates brighter intensities that represent higher levels of phagocytosis. (B) Means are represented ± SEM; asterisks (*) indicate statistically significant difference from media only or endotoxin controls at $p < 0.05$.

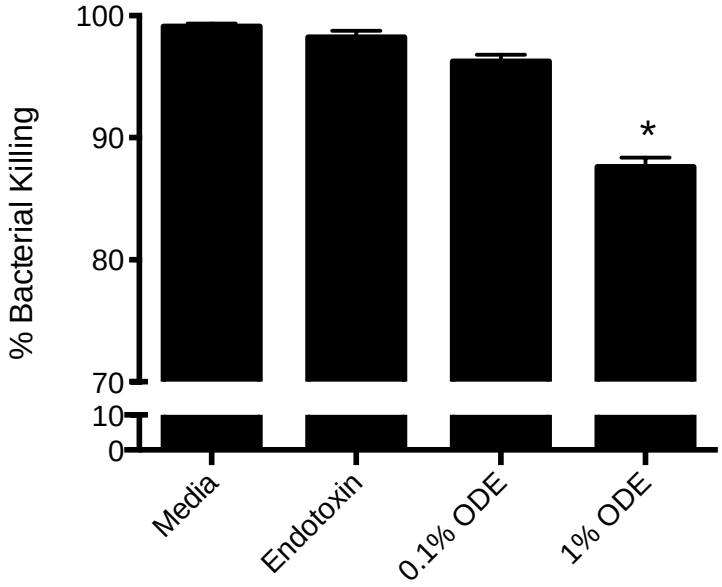
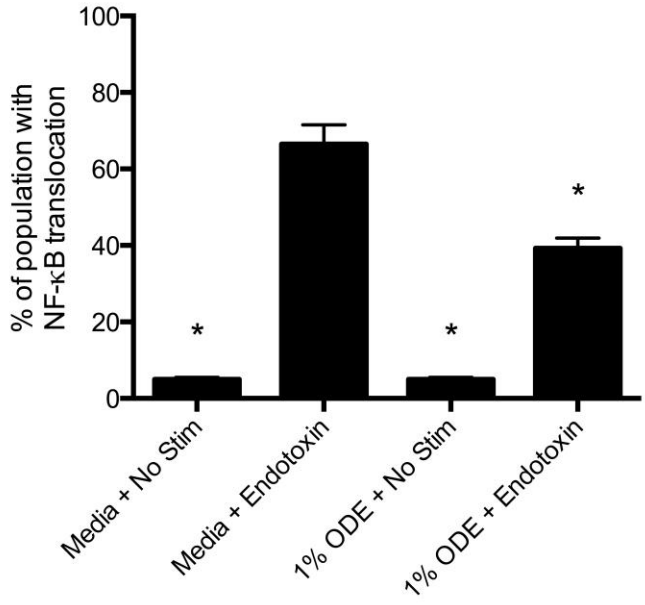


Figure 5. Analysis of intracellular bacterial killing following pretreatment with either medium only, endotoxin, 0.1% ODE or 1% ODE for 24 h. AMϕ were inoculated with *Salmonella enterica* serovar Choleraesuis, allowed time for phagocytosis, followed by lysis of extracellular bacteria. Killing was assessed by plating lysed AMϕ supernatants either immediately (t0) or after 120 min (t120). Number of intracellular bacteria were quantified via plate counts. Percent killing was calculated as [(cfu at t0 - cfu at t120)/(cfu at t0)] X 100. Means are represented ± SEM; asterisk (*) indicates statistically significant difference from all other treatments at $p < 0.05$.

A



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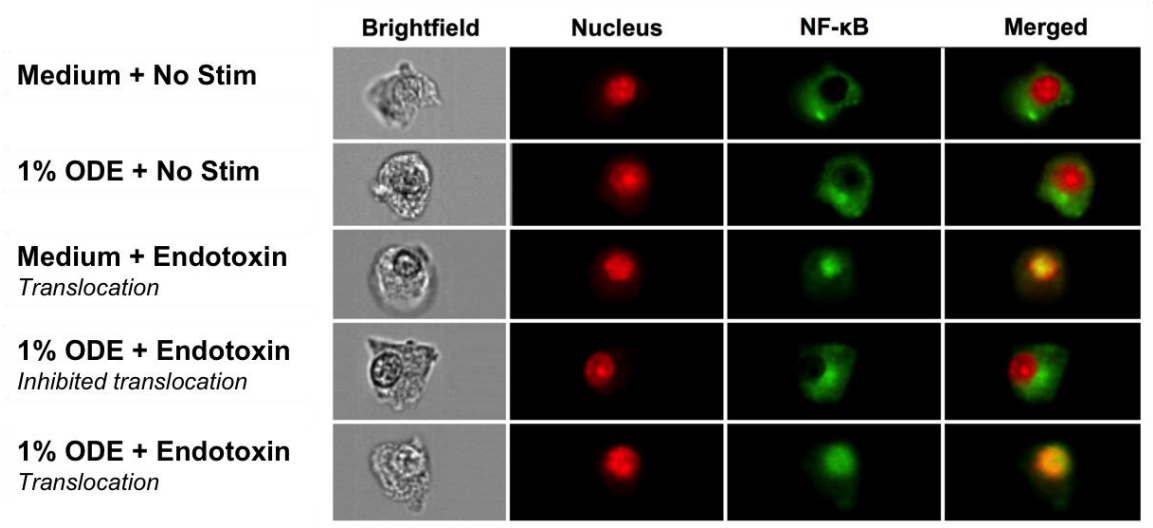

Fig. 6. Nuclear translocation of NF-κB was assessed by multi-spectral imaging flow cytometry to measure NF-κB component images (AF555, green) and nucleus component images (DRAQ5, red). (A) Percent of the population with NF-κB translocation means from three replicated experiments are represented ± SEM; asterisk (*) indicates statistically significant difference from Medium + Endotoxin positive control at $p < 0.05$. (B) Example images of each of the treatment populations.