Repetitive organic dust exposure *in vitro* impairs macrophage differentiation and function

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Background: Organic dust exposure in the agricultural industry results in significant airway disease and lung function decrease. Mononuclear phagocytes are key cells that mediate the inflammatory and innate immune response after dust exposure.
Objective: We sought to investigate the effect of organic dust extract (ODE) from modern swine operations on monocyte-derived macrophage (MDM) phenotype and function.
Methods: Peripheral blood monocytes were obtained by means of elutriation methodology (>99% $CD14^+$) and differentiated into macrophages in the presence of GM-CSF (1 week) with and without ODE (0.1%). At 1 week, cells were analyzed by means of flow cytometry for cell-surface marker expression (HLA-DR, CD80, CD86, Toll-like receptor 2, Toll-like receptor 4, mCD14, and CD16), phagocytosis (IgG-opsonized zymosan particles), and intracellular killing of *Streptococcus pneumoniae*. At 1 week, MDMs were rechallenged with high-dose ODE (1%), LPS, and peptidoglycan (PGN), and cytokine levels (TNF-α, IL-6, IL-10, and CXCL8/IL-8) were measured. Comparisons were made to MDMs conditioned with heat-inactivated dust, endotoxin-depleted dust, LPS, and PGN to elucidate ODE-associated factors.
Results: Expression of HLA-DR, CD80, and CD86; phagocytosis; and intracellular bacterial killing were significantly decreased with ODE-challenged versus control MDMs. Responses were retained after marked depletion of endotoxin. PGN, LPS, and PGN plus LPS significantly reduced MDM surface marker expression and, except for LPS alone, also reduced phagocytosis. ODE-challenged MDMs had significantly diminished cytokine responses (TNF-α, IL-6, and IL-10) after repeat challenge with high-dose ODE. Cross-tolerant cytokine responses were also observed.
Conclusion: Repetitive organic dust exposure significantly decreases markers of antigen presentation and host defense function in MDMs. Bacterial cell components appear to be driving these impaired responses. (J Allergy Clin Immunol 2008;122:375-82.)

Key words: *Monocyte, macrophage, organic dust, phagocytosis, intracellular killing, cell surface molecules, cytokines, inflammation, LPS, peptidoglycan*

In the United States a variety of farming operations, including modern, large-scale, concentrated, closed, animal-feeding operations, can generate significant amounts of dust. Chronic organic dust exposure to workers in this agricultural industry, particularly swine production, results in significant airway diseases, including rhinitis, bronchitis, and obstructive pulmonary disease.[1] Initial exposure to organic dust induces an intense airways inflammatory response that attenuates over time; however, less well examined are persons repetitively exposed to organic dust environments. These individuals are at an increased risk of lung function decrease, persistent inflammation, and respiratory disease but not IgE-mediated disease.[1-4] These observations suggest that chronic organic dust exposure significantly modulates the airways inflammatory response.

Organic dust is a complex mixture containing particulate matter and microbe-associated components that can elicit innate immune responses. Although endotoxin has been well described for its role as a potent inflammatory stimulus, there has not been a consistent association with endotoxin levels and inflammatory outcomes in swine-confinement workers.[5] We and others have also demonstrated that the endotoxin component in swine facility dust does not completely explain the inflammatory response in cultured monocytes, epithelial cells, and whole blood.[6-8] Peptidoglycans (PGNs), cell-wall components most commonly found in gram-positive bacteria but also to a lesser degree in gram-negative bacteria, are also commonly found in this environment and are

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Abbreviations used
COPD: Chronic obstructive pulmonary disease
FITC: Fluorescein isothiocyanate
MDM: Monocyte-derived macrophage
MFI: Mean fluorescence intensity
ODE: Organic dust extract
PE: Phycoerythrin
PGN: Peptidoglycan
TLR: Toll-like receptor

found to correlate with inflammatory outcomes in exposed workers and mediate inflammation in human alveolar macrophages and epithelial cells.[9,10] Thus given the complex nature of organic dust, studies with organic dust samples obtained from these environments are necessary.

Macrophages, which are key innate immune cells that initially respond to and are rapidly activated by exposure to inhaled environmental toxins, such as organic dust, bacterial endotoxin, particulate air pollution, and ozone, have a potential role in the pathogenesis of organic dust–induced respiratory diseases.[11-13] Macrophage-derived inflammatory mediators can induce pyrexia, neutrophil recruitment, and activation of airway epithelial cells.[14] Monocytes are precursor cells that can be recruited to sites of inflammation and, depending on which maturation and differentiation factors are present in the airways milieu, differentiate into macrophages or dendritic cells. It has now been shown that acute lung inflammation triggers a rapid recruitment of monocytes that replace the resident alveolar macrophage population.[11,15] It is also recognized that there are different inflammatory cell patterns and macrophage phenotypes present in the airways of subjects with chronic respiratory diseases, which might be driven by various environmental factors.[16-18]

Despite the finding that macrophages are increased in swine workers,[1] there are few studies investigating the role of repetitive organic dust exposure in monocytes/macrophages. We have previously demonstrated that repetitive organic dust exposure from modern swine facilities modulates inflammatory responses in human monocytes compared with one-time dust exposure.[6] However, the effect of repetitive organic dust exposure on the differentiation of monocytes to macrophages and the subsequent effect on cell phenotype and function have not been investigated. This information will enhance our understanding of how repetitive organic dust exposure modulates the innate immune response.

In this study we hypothesized that repetitive organic dust exposure from modern swine facilities would impair human macrophage differentiation and function. Macrophages derived from peripheral blood monocytes (monocyte-derived macrophages [MDMs]) from healthy individuals were investigated to determine the effect of organic dust exposure on immune cell-surface phenotype, phagocytosis, and intracellular bacterial killing to test this hypothesis. We also evaluated the ability of MDMs cultured in the presence of organic dust, LPS, and PGN to secrete proinflammatory mediators when subsequently rechallenged with high-dose organic dust extract (ODE), LPS, and PGN. Moreover, to determine the potential role dust-associated materials play, we compared ODE-treated MDMs with MDMs derived in the presence of LPS, PGN, LPS plus PGN, ODE depleted of endotoxin, and heat-inactivated ODE (devoid of organic compounds).

METHODS

Preparation of ODE

Organic dust was obtained from settled surface dust from modern swine-confinement, animal-feeding operation facilities housing approximately 500 to 700 animals. For all experimental studies, the organic dust was placed into solution and sterile filtered (ODE) by using a standard published procedure.[6,7] To assess the response of endotoxin-depleted dust extract, the ODE was applied to polymyxin B (binds and inactivates endotoxin) columns (Pierce, Rockford, Ill). The mean endotoxin concentration present after depletion was less than 0.2 ng/mL. In other experiments the organic dust was heat inactivated by heating the dust for 24 hours at 120°C before placing into solution, and this is referred to as heat-inactivated ODE.

Complete analysis of the organic dust can be found in the Methods section of the Online Repository at www.jacionline.org. Briefly, analysis of organic dust before placing it into extract form revealed trace metals, a predominance of gram-positive bacteria (98%), and high muramic acid (marker of PGN/bacterial biomass). The endotoxin equivalent concentration in ODE 0.1% is 4.8 ng/mL (range, 2.6-7.0 ng/mL), as determined by using the Limulus amebocyte lysate gel clot assay (Cambrex, Walkersville, Md).

MDM preparation

Populations of monocytes were obtained from the institution's Elutriation Core Facility. In brief, monocytes were isolated by means of countercurrent centrifugal elutriation of mononuclear leukocyte–rich fractions of blood cells from healthy donors undergoing leukopheresis.[19] Elutriated monocytes were greater than 99% pure, as determined by means of surface antigen expression of mCD14 and measured by means of flow cytometry. Peripheral blood was taken with written informed consent, and studies were approved by the institutional review board.

Monocytes were cultured with and without ODE (0.1%) in complete RPMI in the presence of 1000 U/mL recombinant human GM-CSF (R&D Systems, Minneapolis, Minn) to induce the *in vitro* differentiation of monocytes to macrophages (MDMs). Growth factor–supplemented media was changed every 48 hours for 6 days. Successful macrophage differentiation was confirmed by means of microscopic identification, as well as flow cytometric identification. MDMs were incubated without GM-CSF and ODE for 18 hours before use in experimental assays to eliminate their direct effect. At the end of 1 week, MDMs cultured with GM-CSF alone and MDMs cultured with GM-CSF and ODE (0.1%) were referred to as control and ODE MDMs, respectively. Because others have found that the first 48 hours might be the critical window in monocyte differentiation,[20] monocytes were cultured with ODE (0.1%) for the initial 48 hours only and then maintained in culture with GM-CSF alone for the remainder of the week, and these were referred to as MDMs/early ODE exposed. A schematic of the experimental design is depicted in Fig E1, *A* (ODE-MDMs) and *B* (MDMs/early ODE exposed), which is available in the Online Repository at www.jacionline.org. Macrophages were also derived in the presence of LPS (10 ng/mL, 2× equivalent concentration in ODE 0.1%), PGN (4 μg/mL, approximating protein concentration in ODE 0.1%), combination LPS and PGN, heat-inactivated ODE (0.1%), heat-inactivated LPS plus PGN, endotoxin-depleted dust extract (0.1%), and iron (ferric ammonium citrate; 40 ng/mL, equivalent concentration in ODE 0.1%). Details of all reagents used can be found in the Methods section of the Online Repository.

Flow cytometry

After 1 week, all MDMs were evaluated for cell-surface marker expression by means of flow cytometry for MHC II (HLA-DR), B7 costimulatory molecules (CD80 and CD86), and the pathogen-associated receptors Toll-like receptor (TLR) 2 and TLR4. Cells were also stained for mCD14 (monocyte/macrophage marker) and CD16/FcγRIII (macrophage marker) to ensure appropriate cell differentiation. MDMs (5×10^5 cells) were stained in a standard procedure with antibodies against mCD14, CD16, CD80, CD86, HLA-DR, TLR2, and TLR4 in PBS containing 0.1% bovine albumin. Cells were also incubated with irrelevant isotype control antibodies to account for nonspecific binding. Flow cytometric analyses were performed with the FACSCalibur dual-laser cytometers (Becton-Dickinson, Lincoln Park, NJ).

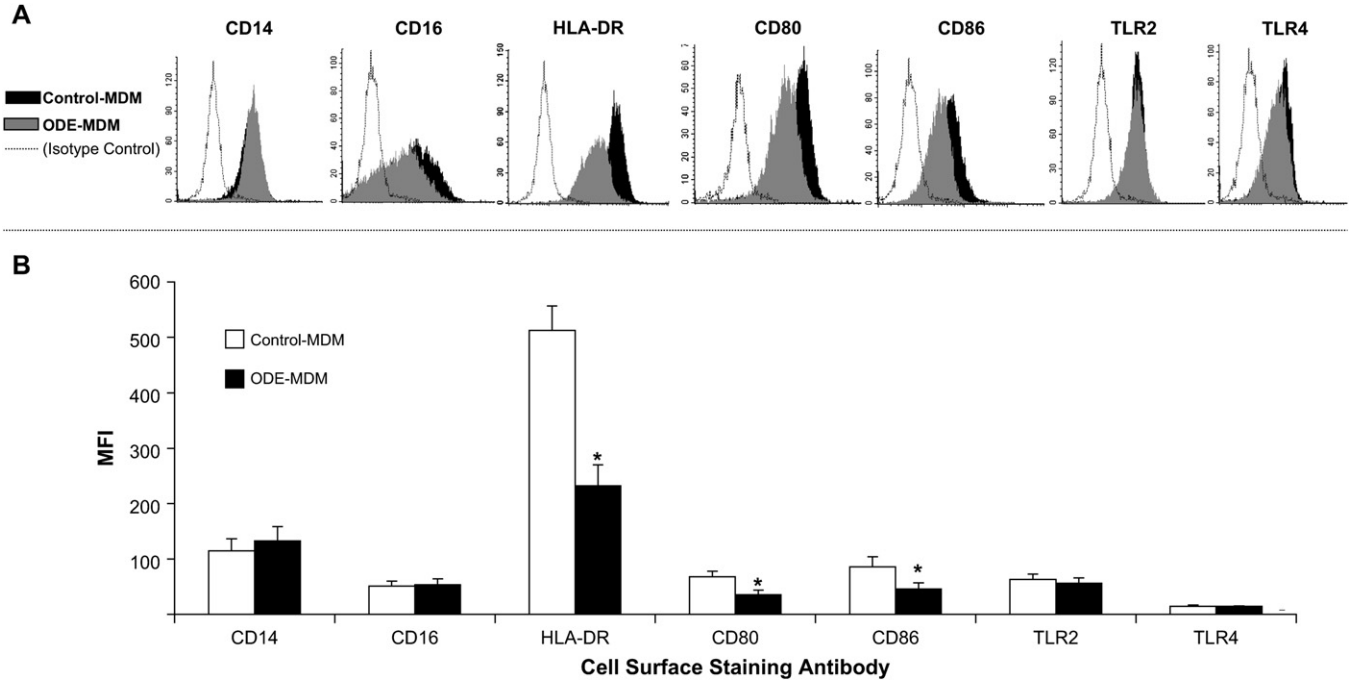

FIG 1. Cell-surface marker expression in MDMs. After 1 week, control and ODE MDMs demonstrated mCD14 and CD16 expression by means of flow cytometry. HLA-DR, CD80, and CD86 expression was diminished with ODE versus control MDMs. **A**, Representative histogram of 4 separate studies. **B**, Mean ± SEM of the MFI (n = 4). *Statistically significant, $P < .05$).

Cell-surface marker expression was reported as mean fluorescence intensity (MFI) minus isotype background MFI. In all experiments control cells from the same donor (cells incubated with GM-CSF alone) were run and compared in side-by-side experiments with conditioned cells.

Phagocytosis assay

The phagocytic ability of MDMs was assessed by means of flow cytometry with previously published methods.[13] Details can be found in the Methods section of the Online Repository. Phagocytosis of the fluorescein isothiocyanate (FITC)–labeled IgG-zymosan particles was determined by assessing the proportion of cells in the zymosan-exposed population at 60 minutes compared with cells exposed for 0 minutes (expressed as fold change in MFI). Cells were also quenched with trypan blue and acid wash to eliminate cell-surface adhesion versus internalization-associated fluorescence.

Intracellular bacterial killing assay

Killing of *Streptococcus pneumoniae* by control and ODE MDMs was determined as previously described, with some modification.[21] Details can be found in the Methods section of the Online Repository. Percentage of killing was calculated as follows:

$$[(cfu\ at\ time\ 0 - cfu\ at\ 60\ \text{min})/(cfu\ at\ time\ 0)] \times 100.$$

Cytokine/chemokine assays

After 1 week, control MDMs and all conditioned MDMs (5×10^5 cells/mL) in duplicate were subsequently challenged with a high concentration of ODE (1%) and media (control) for 5 hours to determine cytokine responsiveness of MDMs. Cell-free supernatant was subsequently harvested and stored at −20°C until assayed for cytokine secretion. At 1 week, ODE, LPS, PGN, and control MDMs (5×10^5 cells/mL) in duplicate were restimulated with high-concentration ODE (1%), LPS (100 ng/mL), PGN (40 μg/mL), and media alone (control) for 5 hours to investigate further whether cross-tolerance occurs. In all experiments cell counts and cell viability after the 5-hour culture condition were assessed by using the trypan blue exclusion method.

Cytokines/chemokines were assayed by means of sandwich ELISA, as previously published.[6] Cytokine secretion is reported as the concentration (in picograms per milliliter) per 5×10^5 viable cells, as determined on completion of the experimental protocol, by means of the trypan blue exclusion method. In cross-tolerant studies mean results are presented as the percentage of ODE-induced cytokine secretion (ODE-induced cytokine secretion in conditioned MDMs divided by ODE-induced cytokine secretion in control MDMs multiplied by 100).

Statistical analysis

Data are presented as the mean ± SEM. Statistics were performed by using 2-tailed nonpaired or paired *t* tests (as appropriate) to determine significant changes among treatment groups.

RESULTS

Organic dust and microbial components modify macrophage cell-surface marker expression

Human monocytes were incubated with and without ODE (0.1%) in the presence of GM-CSF for 1 week, as detailed in the Methods section, to examine the effect of ODE on differentiation and cell-surface phenotype expression of macrophages derived from monocytes. After 1 week, MDMs demonstrated surface marker expression of mCD14, CD16, HLA-DR, CD80, and CD86, which is consistent with a macrophage phenotype. Compared with control MDMs, MHC class II molecules (HLA-DR) and B7 costimulatory molecules (CD80 and CD86) were significantly downregulated with ODE- versus control-treated MDMs (Fig 1). Expression of the pathogen-associated receptors TLR2 and TLR4 was not downregulated. We found that the initial 48-hour exposure to ODE (MDMs/early ODE exposed) was the critical time point to observe the downregulation of the innate immune cell-surface marker expression (Fig 2, *A*).

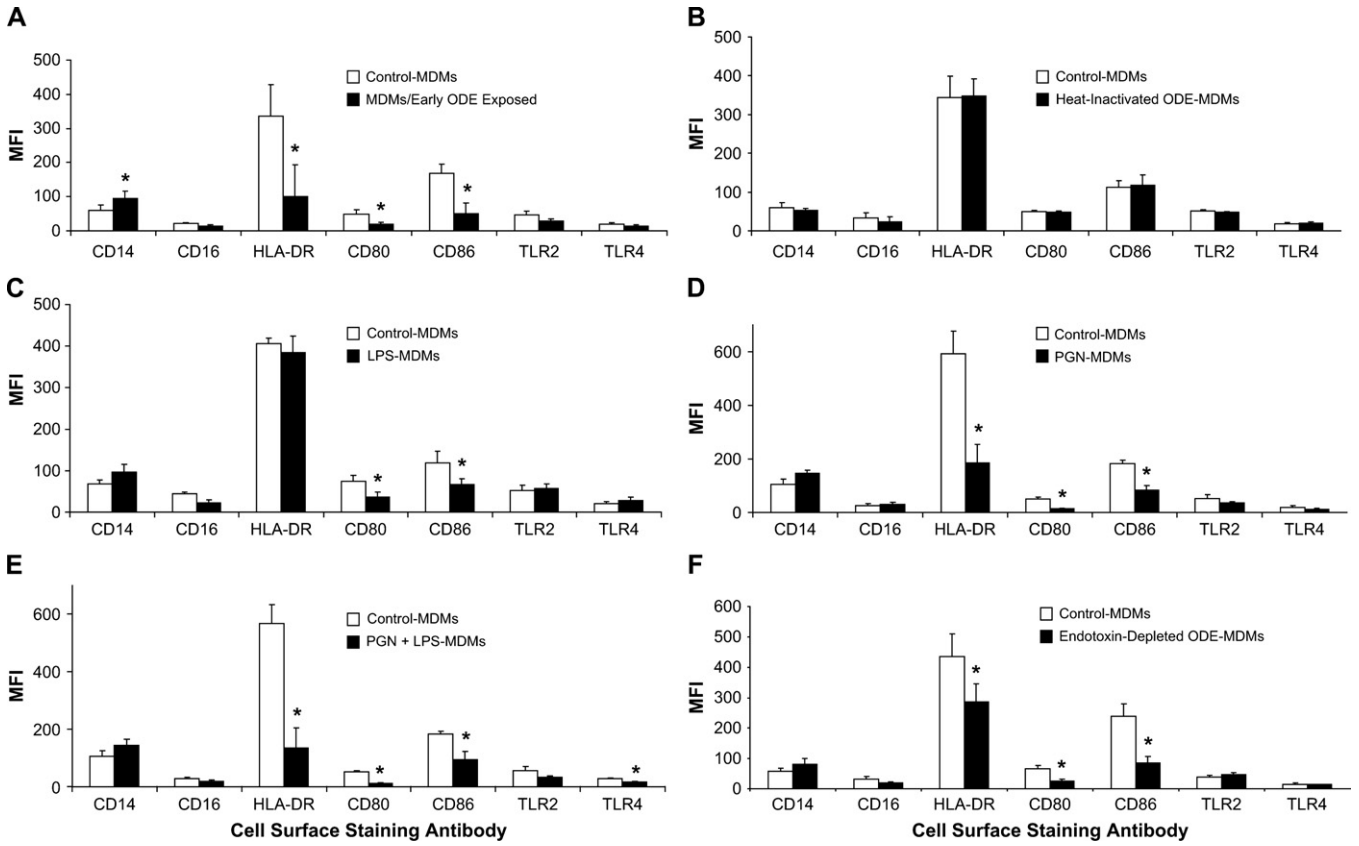

FIG 2. Cell-surface marker expression in MDMs. After 1 week, control MDMs were compared with MDMs/early exposed (**A**; n = 3) and MDMs conditioned with heat-inactivated dust (**B**; n = 3), 10 ng/mL LPS (**C**; n = 4), 4 μg/mL PGN (**D**; n = 4), combination LPS plus PGN (**E**; n = 4), and endotoxin-depleted ODE (**F**; n = 3). Values are expressed as means ± SEMs of the MFI. *Statistically significant, $P < .05$.

Macrophages were also derived from monocytes in the presence of LPS, PGN, LPS plus PGN, and endotoxin-depleted and heat-inactivated dust extract and evaluated for immune cell-surface marker expression to determine possible components driving these observations with ODE. Results of all conditions are shown in Fig 2, *B* through *F*. LPS conditioning significantly reduced CD80 and CD86 expression but did not modify HLA-DR expression compared with control values. Compared with control, PGN conditioning, the combination of LPS and PGN, and endotoxin-depleted ODE significantly diminished HLA-DR, CD80, and CD86 expression. Heat-inactivated dust, iron (data not shown), and heat-inactivated LPS plus PGN (data not shown) did not significantly affect the expression of any of the innate immune cell-surface markers tested. These results suggest that heat-sensitive and nonendotoxin components in ODE are the dominant factors modulating ODE-induced MDM innate immune cell-surface marker expression.

Organic dust and PGN exposure diminishes the phagocytic ability of macrophages

After 1 week, phagocytosis of IgG-opsonized zymosan particles was measured at 0 and 60 minutes in MDMs treated with and without ODE (0.1%) by means of flow cytometry. Phagocytosis was demonstrated by a rightward shift in fluorescence (Fig 3) and reported as the proportion of cells in the zymosan-exposed population at 60 minutes compared with cells exposed for 0 minutes (expressed as fold change in MFI). The 0-minute time point represented particle adhesion, whereas the rightward shift in fluorescence at 60 minutes represented particle internalization, as evidenced by shifts in both side (cell granularity) and forward (cell size) scatter, as well as the lack of fluorescence quenching by means of trypan blue staining or acid washing. Phagocytosis was significantly diminished in ODE MDMs (Fig 3, *A* and *B*) and MDMs/early exposed compared with control MDMs (Fig 4, *A*).

We next investigated the effect of LPS, PGN, LPS plus PGN, endotoxin-depleted ODE, and heat inactivation of the dust on the phagocytic ability of MDMs, with all results shown in Fig 4, *B* through *F*. Compared with control MDMs, there was a slight but nonsignificant decrease in phagocytic ability with the heat-inactivated dust extract conditioning. There was no effect on phagocytic ability with iron and heat-inactivated LPS plus PGN conditioning (data not shown). LPS conditioning did not significantly diminish phagocytic ability. However, PGN conditioning, PGN plus LPS, and endotoxin-depleted ODE MDMs significantly reduced phagocytic ability. These studies suggest that nonendotoxin components in ODE are responsible for impaired phagocytic ability of MDMs.

Organic dust exposure significantly impairs intracellular bacterial killing

The ability of control MDMs versus ODE MDMs to phagocytoze and kill *S pneumoniae* was investigated, as detailed in the

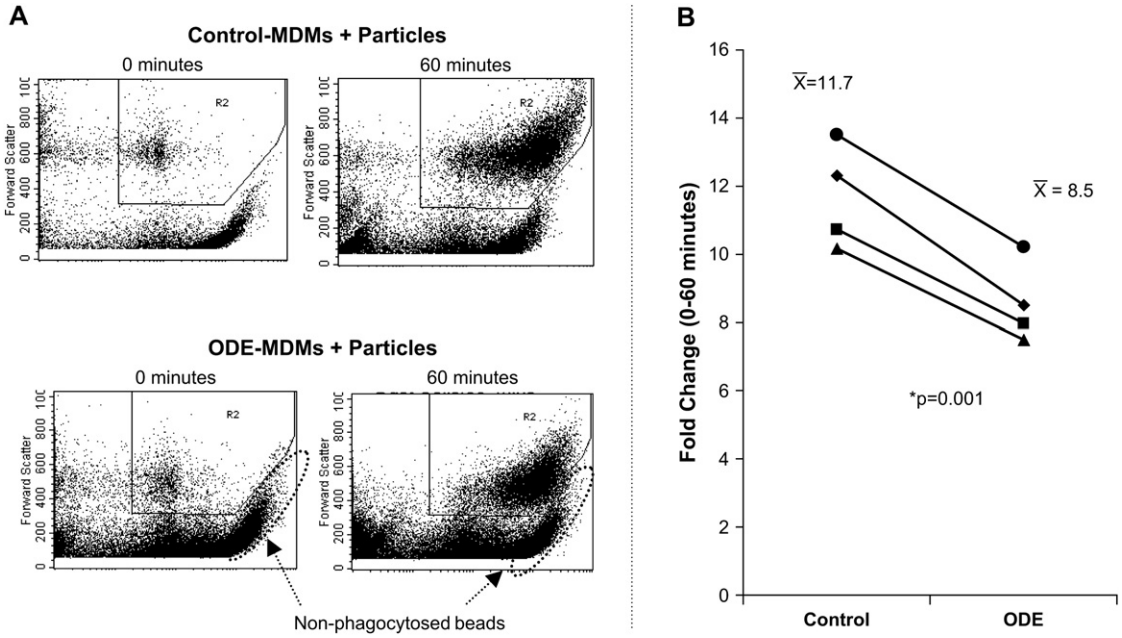

FIG 3. Phagocytosis of IgG-opsonized *Saccharomyces cerevisiae* zymosan bioparticles. Phagocytosis was diminished in ODE-treated MDMs compared with that seen in control MDMs (n = 4, $P = .001$). **A**, Representative dot plot of rightward shift in fluorescence from 4 separate studies. **B**, Fold change in MFI (proportion of cells in the zymosan-exposed population at 60 minutes compared with cells exposed for 0 minutes). *Statistically significant, $P < .05$.

Methods section, to extend the findings regarding phagocytosis in ODE-conditioned MDMs. In these studies we verified that phagocytosis was significantly impaired (70.6% ± 10.5% reduction in phagocytosis of *S pneumoniae* in ODE MDMs compared with control, n = 3) and further demonstrated that intracellular killing of *S pneumoniae* was also consistently significantly impaired (72.0% ± 19.8% reduction in killing in ODE MDMs compared with control, n = 3). These studies suggest that ODE conditioning significantly impairs host defense function marked by both a reduction in phagocytosis and intracellular bacterial killing.

Organic dust exposure affects cytokine responsiveness of MDMs

In these studies the effect of ODE on innate immune inflammatory cytokine responsiveness of MDMs was investigated. As described in the Methods section, after 1 week in culture, control MDMs and ODE MDMs were subsequently rechallenged with ODE (1%) for 5 hours. Control and ODE MDMs secreted TNF-α, IL-6, IL-10, and CXCL8 (IL-8) when restimulated with ODE (1%) compared with those with no restimulation (media control). However, we found that ODE-treated MDMs had significantly reduced secretion of TNF-α, IL-6, and IL-10 on restimulation compared with control MDMs (Fig 5, *A-C*). There was no significant difference in CXCL8 secretion (Fig 5, *D*). There was no difference in cell count or cell viability between treatment conditions to explain these results.

We next investigated the cytokine responsiveness of all conditioned MDMs to restimulation with 1% ODE (cross-tolerant studies). MDMs conditioned with LPS, PGN, LPS plus PGN, endotoxin-depleted ODE, and early ODE exposure (initial 48 hours only), but not heat-inactivated ODE and heat-inactivated LPS plus PGN (data not shown), demonstrated significant impairment in TNF-α and IL-6 secretion (see Fig E2 in the Online Repository at www.jacionline.org). Restimulation with a relatively high concentration of LPS of MDMs conditioned with ODE, LPS, and PGN also demonstrated impairment of TNF-α and IL-6 secretion (see Fig E3, *A* and *B*, in the Online Repository at www.jacionline.org). This cross-tolerant pattern of impaired TNF-α and IL-6 expression was also observed when conditioned MDMs were restimulated with a relatively high concentration of PGN (see Fig E3, *C* and *D*).

DISCUSSION

Organic dust exposure is an important environmental factor that has been implicated in increased morbidity among repetitively exposed subjects.[1] Macrophages are key innate immune cells derived from monocytes that have a potential role in the pathogenesis of organic dust–induced respiratory disease; however, little is known about the effect of repetitive organic dust exposure on macrophage phenotype and function. In this study repetitive *in vitro* organic dust exposure resulted in an overall impairment in macrophage host defense capability. This was demonstrated by a marked reduction in expression of cell-surface molecules associated with antigen presentation, phagocytic and bactericidal ability, and cytokine responsiveness.

Macrophages can orchestrate inflammation and activate T-cell responses through antigen-presenting ability. The first signal in the antigen presentation–T-cell activation process is regulated by MHC class II molecules, and the classic second signal is regulated by the B7 costimulatory molecules CD80 and CD86. Our data demonstrate that repetitive swine facility dust exposure significantly downregulates MHC class II and costimulatory molecule (CD80 and CD86) expression. The reduction in the expression of HLA-DR and costimulatory molecules on macrophages would be consistent with an impaired host defense state.[22] We speculate that this state might favor a decreased ability to induce an

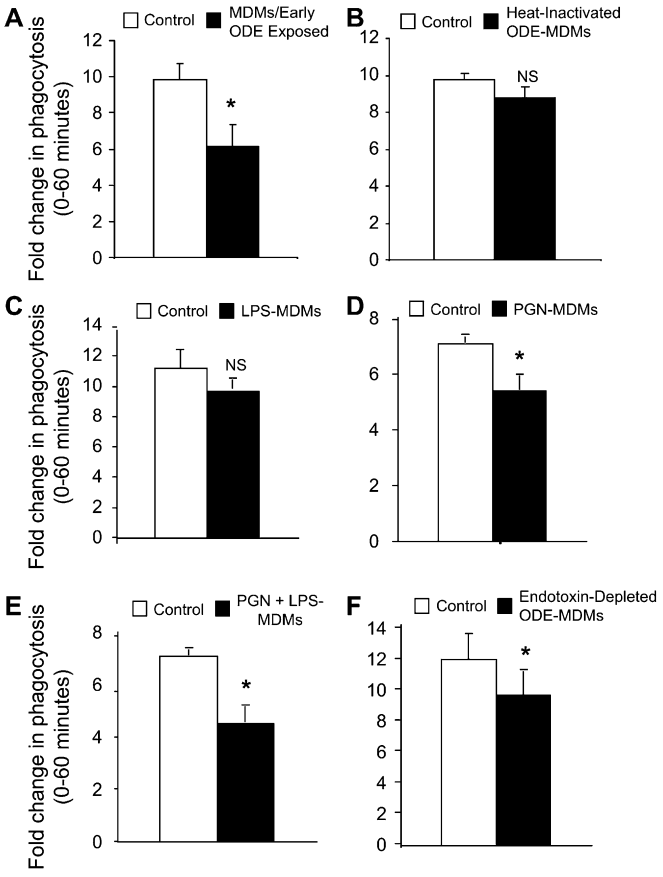

FIG 4. Phagocytosis of IgG-opsonized zymosan bioparticles in MDMs. Fold change in MFI on the *y-axis* (proportion of cells in the zymosan-exposed population at 60 minutes compared with cells exposed for 0 minutes) with MDMs/early ODE exposed (**A**; n = 3) and MDMs conditioned with heat-inactivated dust (**B**; n = 3), LPS (**C**; n = 3), PGN (**D**; n = 3), LPS plus PGN (**E**; n = 3), and endotoxin-depleted ODE (**F**; n = 3) is shown. *Statistically significant, $P < .05$. *NS*, Nonsignificant, $P > .05$).

appropriate acquired immune response to microbial stimuli, which could ultimately perpetuate or promote bacterial colonization within the airways milieu.

Interestingly, our findings with repetitive organic dust exposure are consistent with observations made in subjects with chronic obstructive pulmonary disease (COPD). Alveolar macrophages from subjects with COPD, chronic smokers, or both have lower expression of costimulatory molecules and MHC class II compounds.[16,17] In contrast, macrophages from subjects with allergic asthma appear to overexpress CD80 and CD86 and also MHC class I and II molecules compared with those of healthy control subjects.[18,23] It is well recognized that workers who are routinely exposed to swine barns have chronic bronchitis and progressive lung function decrease as features of their disease, and this is particularly true in young workers aged 25 to 35 years.[3,24] Thus our *in vitro* model is consistent with the phenotype of macrophages from subjects with chronic bronchitis who have, as a central feature of their disease, increased neutrophilic inflammation and bacterial infection.

Another key macrophage function is phagocytosis, a process by which cellular debris and pathogens are removed. In this study we directly measured macrophage phagocytosis by examining ingestion of fluorescently labeled zymosan particles by means of flow cytometry.[13,25] We found that phagocytic ability was significantly impaired in organic dust–treated macrophages compared with that seen in control macrophages and verified this by demonstrating significantly impaired phagocytosis of *S pneumoniae* by plate counting. We then found that intracellular killing of *S pneumoniae* was significantly impaired in organic dust conditioned macrophages. We interpret these findings to also be consistent with a COPD/chronic bronchitis–macrophage phenotype. Others have suggested that alveolar macrophage phagocytic ability might be diminished in subjects with COPD.[16,26]

Macrophages also elicit inflammatory responses through production and secretion of cytokines/chemokines. We found that at 1 week, macrophages differentiated in the presence of organic dust can still release TNF-α, IL-6, IL-10, and CXL8 when restimulated with high-dose ODE, but when compared with non–dust-treated cells (control), the secretion of TNF-α, IL-6, and IL-10 was significantly decreased. The organic dust–conditioned MDMs were also cross-tolerant to other microbial stimuli (LPS and PGN). These observations are also consistent with a COPD/chronic bronchitis phenotype whereby macrophages from subjects with COPD/chronic bronchitis secrete lower amounts of inflammatory cytokines compared with healthy control subjects.[27]

An interesting finding in this study is that the impairment in organic dust–induced MDM host defense functions occurs during the initial culture-exposure time interval. Exposure of fresh monocytes to organic dust for 48 hours, followed by washing and maintaining the cells in culture with GM-CSF for the remaining week, demonstrated similar findings to exposing cells to organic dust for the entire week. Consistent with this observation, others have found that signaling necessary for monocyte survival and development into macrophages might end during the first 48 hour of culture in GM-CSF.[20]

The organic dust used was obtained from modern swine-confinement facilities that are becoming increasingly common, replacing the smaller farming operations. In this study we sought to better characterize the organic dust obtained from these modern facilities in our region (Midwest United States). We found that gram-positive bacteria predominated in the dust, particularly muramic acid, a gram-positive bacterial component of PGN. Finally, several elemental compounds, such as iron, which could elicit inflammatory responses and affect phagocytosis, were found to exist in the dust, although at relatively low levels.

Our interpretation is that metals likely play a minor role in the responses observed here because dust that was heat inactivated at 120°C, a process that inactivates the biologics and leaves the metals intact, did not significantly modify our markers of immune cell function and phenotype or cytokine secretion. Phagocytosis was slightly decreased, but this was not statistically significant. In addition, we found that soluble iron at the comparable low concentrations found in the ODE did not affect macrophage host defense (data not shown). It appears our observations are more in line with studies using ambient air pollution particles, diesel exhaust particles, or both.[13,28] In these studies a slight decrease in phagocytic ability was observed in macrophages, but the major immune effects were found to be secondary to the organic or biologic components in the particles.

Given the inherent complexity with the organic dust samples, we chose to investigate the effects of organic dust depleted of endotoxin, LPS, and PGN individually and LPS and PGN combined to further understand the relative contribution of gram-positive and gram-negative bacterial components in our

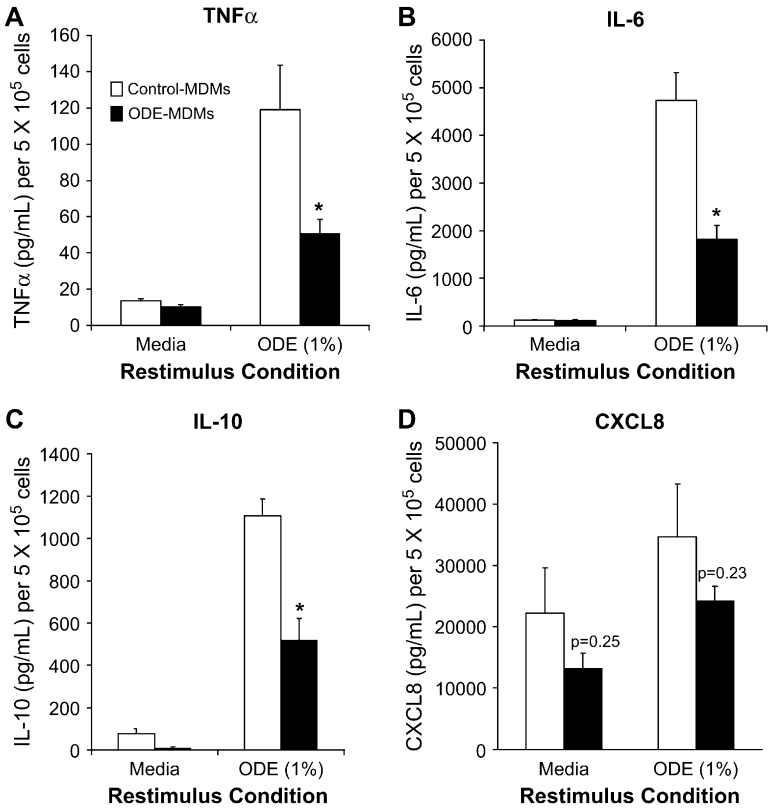

FIG 5. Secretion of cytokines/chemokines in MDMs at 1 week after restimulation with ODE 1% for 5 hours. TNF-α **(A)**, IL-6 **(B)**, and IL-10 **(C)** secretion was dampened in ODE MDMs compared with that seen in control MDMs (n = 5, $P < .05$). CXCL8 **(D)** expression was not significantly altered (n = 5). Mean results are presented per 5×10^5 cells ± SEMs. *Statistically significant, $P < .05$).

in vitro macrophage model. We previously reported that bacterial components, such as LPS on coarse fraction particulate matter ($PM_{2.5-10}$), play an important role in driving innate immune responses (phagocytosis, mCD14, HLA-DR, and TNF-α) on airway macrophages after a single (acute) *in vivo* exposure of healthy volunteers to coarse particulate matter.[13] In this study we found that repetitive exposure to LPS during macrophage differentiation resulted in diminished costimulatory molecule expression (CD80 and CD86) but exerted no change in MHC class II expression. PGN conditioning and organic dust depleted of endotoxin results in similar findings to those seen after ODE conditioning, namely marked reduction in MHC class II, CD80, and CD86 expression. The combination of LPS plus PGN conditioning further reduced innate immune cell-surface marker expression. As anticipated, LPS did not significantly affect phagocytic ability, but PGN conditioning, combination LPS plus PGN, and organic dust depleted of endotoxin significantly reduced phagocytosis. The observation that PGN inhibits phagocytic ability has been supported by others.[29] Our findings in this study are also consistent with other studies and our previous studies demonstrating that the endotoxin component in swine barn dust does not completely explain the immune inflammatory response in cultured airway epithelial cells and monocytes.[6-8]

It appears that gram-positive bacterial components might be the driving component of the organic dust–induced responses observed in our study, but LPS plus PGN most closely mimicked the observations we observed with ODE, suggesting that combinations of biologic agents are important in real-life exposures. However, it would be an oversimplification to state that a TLR2 agonist, TLR4 agonist, or both completely explains the effect observed with organic dust because we did not observe any modulation in TLR2 and TLR4 cell-surface expression with organic dust conditioning. Studies in our laboratory also have failed to show that blocking TLR2 with commercially available antibodies reverses the impaired host defense response in organic dust–conditioned MDMs (data not shown). However, because of the complexity of blocking TLR2 in 1-week cell-culture systems of human cells, we plan to study the role of TLR2 in a murine model of organic dust exposure before making definitive conclusions. In addition to TLR2, other potential receptor targets include several of the PGN recognition molecules, including mCD14, the nucleotide-binding oligomerization domain family, Nod1 and Nod2, and a family of PGN recognition proteins.[30] Our study does add to the growing body of literature that various combinations of bacterial cell-surface components might exert more powerful activities on host defense cells than that of individual components.[31] Organic dust is one such environmental factor whereby the study of various combinations of bacterial cell components will be critical for future therapeutic options.

In conclusion, macrophages differentiated in culture from peripheral blood monocytes from healthy volunteers showed impaired host defense function after exposure to organic dust. Various bacterial cell-wall components, particularly gram-positive PGN, appeared to drive these responses. The *in vitro* model used in this study can next be applied to subjects with chronic airways disease and will also be important when investigating

possible therapeutic interventions aimed at preventing impaired host defense function with organic dust exposure.

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Key messages

- **Repetitive organic dust exposure impairs MDM host defense functions.**
- **Gram-positive bacterial cell components might be driving this impaired response.**
- **Organic dust–induced macrophage dysfunction might be important in respiratory disease development.**

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METHODS

Reagents

Complete RPMI was supplemented with 10% heat-inactivated FBS (Biofluids, Inc, Rockville, Md), 2-mercaptoethanol (5×10^{-5} mol/L), 50 μg/mL streptomycin (Invitrogen, Carlsbad, Calif), and 80 U/mL amphotericin B (Invitrogen). The LPS used was from *Escherichia coli* (O55:B5), and *Staphylococcus aureus* PGNs were from Sigma (St Louis, Mo). To verify previous reports that heating LPS and PGN to greater than 100°C for a minimum of 10 to 20 minutes inactivates their respective activity[E1,E2] and as an important comparison to heat-inactivated ODE, we heat inactivated PGN (4 μg/mL) plus LPS (10 ng/mL) at 120°C for 24 hours, which is referred to as heat-inactivated PGN plus LPS. Ferric ammonium citrate (soluble form of iron) was purchased from Sigma. For flow cytometric studies, the following antibodies were purchased from BD Biosciences PharMingen (San Jose, Calif): mCD14 (FITC conjugated), CD16 (phycoerythrin [PE] conjugated), CD80 (PE-Cy5), CD86 (PE), and HLA-DR (FITC). TLR2 (FITC) and TLR4 (PE) were purchased from eBioscience (San Diego, Calif).

Organic dust and ODE analysis

A semiquantitative elemental analysis by means of inductively coupled plasma-mass spectrometry revealed the presence of metals (100% concentrate of dust: B, 1380 ng/mL; Mg, 144,600 ng/mL; Ti, 1166 ng/mL; Mn, 375.5 ng/mL; Fe, 4226 ng/mL; Co, 59.7 ng/mL; Ni, 371 ng/mL; Cu, 3295.5 ng/mL; Rb, 1076.5 ng/mL; Mo, 132 ng/mL; and Zn, 8797.5 ng/mL; Research Triangle Park, Research Triangle, NC). The organic dust was analyzed for the presence of bacteria before placing in solution (extract form) and before sterile filtration. As identified by mens of colony morphology, approximately 98% of the colonies were gram-positive bacteria (*Staphylococcus*, *Bacillus*, *Streptomycetes*, and *Enterococcus* species), whereas the remaining 2% were gram-negative bacteria. As a corollary, the organic dust was compared with house dust for chemical marker analysis by means of gas chromatography–tandem mass spectrometry for 3-hydroxy fatty acids (markers of LPS), ergosterol (marker of fungal biomass), and muramic acid (marker of PGN/bacterial biomass) by using previously published methods.[E3] Analysis demonstrated that the organic dust had 10 times more muramic acid then house dust (mean, 203.5 vs 25 ng/mg), slightly increased levels of 3-hydroxy fatty acids (0.0723 vs 0.0494 nmol/mg), and no difference in ergosterol (3.53 vs 6.1 ng/mg).

The total protein concentration in the dust extract (100% concentration) was approximately 2 to 4 mg/mL, as determined by means of Bradford protein assay (Bio-Rad, Hercules, Calif). The mean endotoxin concentration in a 100% concentration, as determined by using the Limulus amebocyte lysate gel clot assay (Cambrex, Walkersville, Md), was 0.0048 mg/mL (range, 0.0026-0.0070 mg/mL). The concentration of ODE (0.1%) used in the majority of experimental conditions represents a "low" swine barn dust exposure environment. By using endotoxin as a biomarker of exposure, 0.1% ODE equates to approximately 4.8 ng/mL of endotoxin, and others have defined low endotoxin and dust exposure in a swine barn as a mean endotoxin value of approximately 45.2 ± 6.6 ng/m^3.[E4] Low ODE (0.1%) and high ODE (1%) concentrations do not affect cell viability or proliferation in prolonged cell cultures (48 hours), as evaluated by using the tetrazolium compound 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (R&D Systems, Minneapolis, Minn).

Phagocytosis of IgG-opsonized, FITC-labeled zymosan particles

Saccharomyces cerevisiae zymosan A BioParticles (Molecular Probes, Inc, Eugene, Ore) conjugated to FITC were opsonized with opsonizing reagent (IgG) for 45 minutes and then washed. MDMs at 4×10^6 cells/mL and opsonized, FITC-labeled zymosan at 4×10^7 particles/mL were incubated (1:10 ratio) for 0 and 60 minutes in the presence of 10% human AB serum. Cells were fixed at 1% paraformaldehyde concentration and analyzed on the same day of particle exposure by means of flow cytometry. Particle uptake was identified as a rightward shift in fluorescence on histogram analysis.

Intracellular bacterial killing assay

Killing of *Streptococcus pneumoniae* by control and ODE MDMs was determined as previously described, with some modification.[E5] Briefly, unencapsulated *S pneumoniae* (strain DW 3.8 kindly gifted by David A. Watson) was preopsonized for 30 minutes at 37°C with 20% human serum in HBSS containing 0.1% gelatin. After washing, the opsonized organisms (4×10^6) were combined with control and ODE MDMs (4×10^5 cells) in side-by-side experiments in RPMI supplemented with 10% human AB serum. Phagocytosis was allowed for 45 minutes at 37°C with gentle rocking. Phagocytosis was stopped by the addition of cold PBS, and unassociated organisms were removed by means of differential centrifugation (3 centrifugations at 180*g*). The final MDM pellet was resuspended in RPMI with 10% human AB serum. Aliquots of the suspension were removed immediately (time 0) and after 120 minutes of incubation at 37°C. Bacterial viability was determined by using plate-counting procedure after MDMs were lysed in 0.1% saponin (15 minutes on ice).

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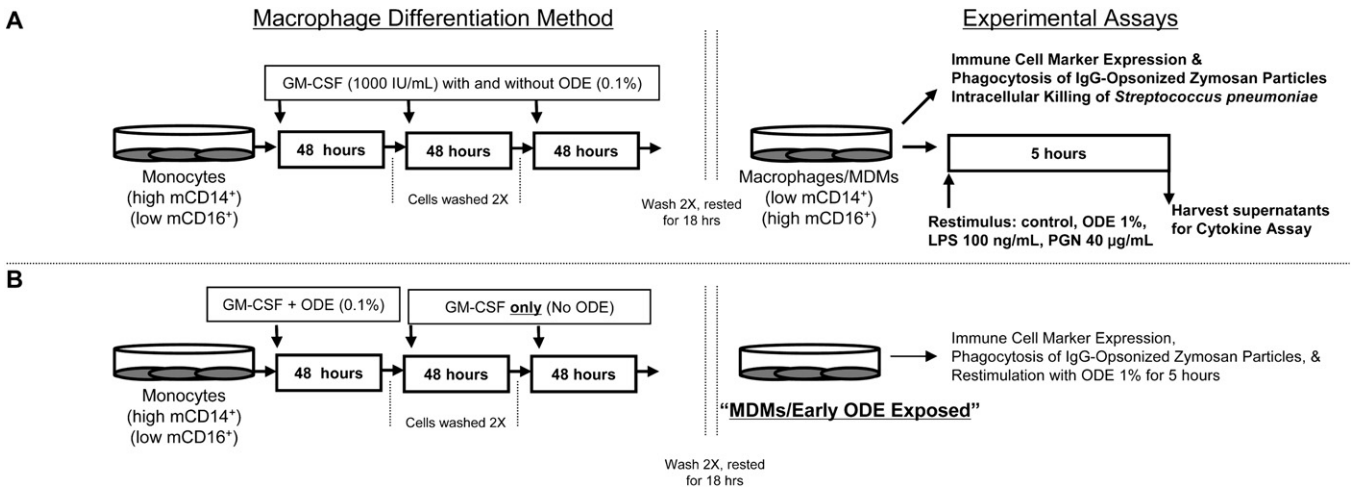

FIG E1. **A**, Schematic representation of experimental protocol showing elutriated monocytes cultured with GM-CSF (1000 IU/mL) with and without low-concentration ODE (0.1%) for 6 days. **B**, Monocytes cultured with GM-CSF and ODE (0.1%) for 48 hours and washed and maintained in culture for the remaining 4 days (MDMs/early ODE exposed). Macrophages rested for 18 hours before experimental assays.

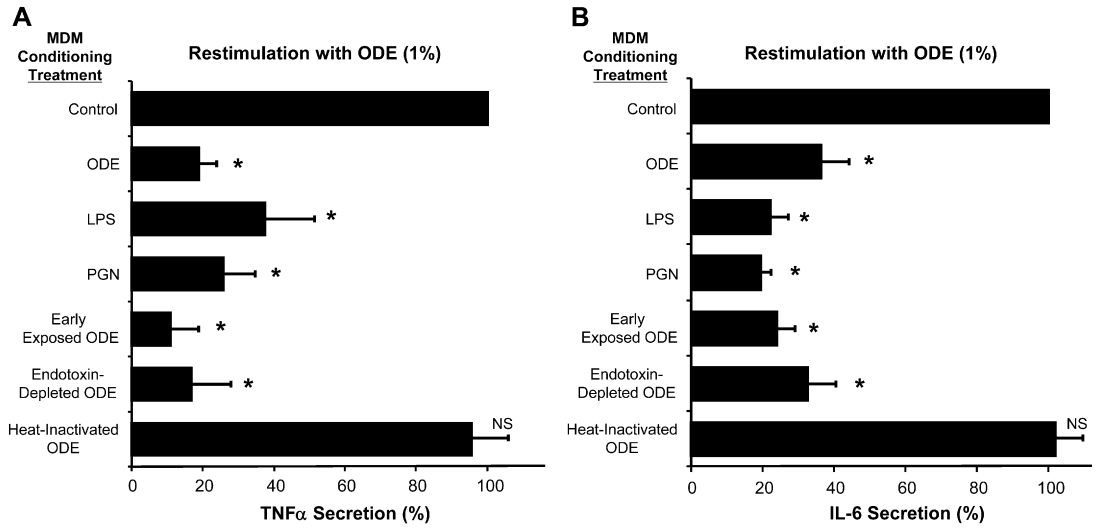

FIG E2. Cytokine secretion in MDMs at 1 week after restimulation with ODE (1%) for 5 hours. TNF **(A)** and IL-6 **(B)** secretion was dampened in all conditioned MDMs compared with that seen in control MDMs, except for heat-inactivated ODE MDMs (n = 3). Mean results are presented as the percentage of ODE-induced cytokine secretion in control MDMs ± SEM. *Statistically significant, $P < .05$). *NS*, Not significant.

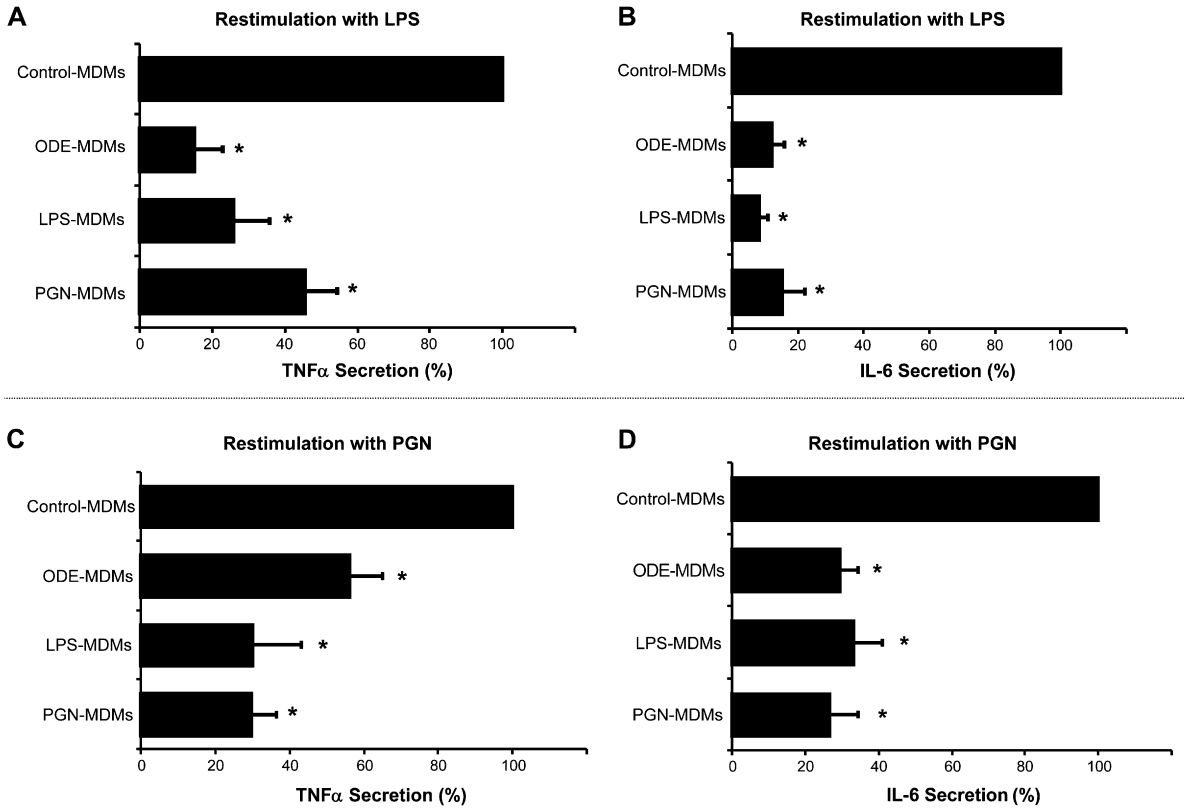

FIG E3. Cytokine secretion in MDMs at 1 week after restimulation with LPS (100 ng/mL; **A** and **B**) and PGN (40 μg/mL; **C** and **D**). TNF (Fig E2, *A* and *C*) and IL-6 (Fig E2, *B* and *D*) secretion was dampened in all conditioned MDMs compared with that seen in control MDMs (n = 3). Mean results are presented as the percentage of LPS- or PGN-induced cytokine secretion in control MDMs ± SEM. *Statistically significant, $P < .05$).