

Biological material on inhaled coarse fraction particulate matter activates airway phagocytes *in vivo* in healthy volunteers

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Background: *In vitro*, endotoxin on coarse fraction particulate matter (PM_{2.5-10}) accounts for the majority of the ability of PM_{2.5-10} to induce cytokine responses from alveolar macrophages.

Objective: We examined *in vivo* whether inhaled PM_{2.5-10} from local ambient air induce inflammatory and immune responses in the airways of healthy human beings and whether biologic material on PM_{2.5-10} accounts for these effects.

Methods: On 3 separate visits, 9 healthy subjects inhaled nebulized saline (0.9%, control), PM_{2.5-10} collected from local ambient air that was heated to inactivate biological material (PM_{2.5-10}⁻), or nonheated PM (PM_{2.5-10}⁺). PM_{2.5-10} deposition (~0.65 mg/subject) targeted the bronchial airways (confirmed by using radiolabeled aerosol), and induced sputum was obtained 2 to 3 hours postinhalation for analysis of cellular and biochemical markers of inflammation and innate immune function.

Results: Inhaled PM_{2.5-10}⁺ induced elevated inflammation (% PMNs, macrophage mRNA TNF- α), increased eotaxin, upregulated immune surface phenotypes on macrophages (mCD14, CD11b, HLA-DR), and increased phagocytosis (monocytes) versus saline ($P < .05$). Biological inactivation of PM_{2.5-10} (PM_{2.5-10}⁻) had no effect on neutrophilia but significantly ($P < .05$) attenuated mRNA TNF- α , eotaxin levels, cell surface marker responses, and phagocytosis.

Conclusion: Biological components of PM_{2.5-10} are not necessary to induce neutrophil responses but are essential in mediating macrophage responses. The ability of PM_{2.5-10} to

activate monocytic cells and potentially skew the airways toward an allergic phenotype by enhancing eotaxin levels may enhance responses to allergens or bacteria in individuals with allergy.

Clinical implications: PM_{2.5-10} might enhance the response of individuals with allergy to airborne bacteria. (J Allergy Clin Immunol ■■■■;■■■:■■■-■■■.)

Key words: PM_{2.5-10}, airway macrophages, TNF- α , mCD14, eotaxin

Exposure to respirable particulate matter (with a mean-median airway diameter [MMAD] <10 μ m [PM₁₀]) is an important cause of exacerbation of asthma and chronic obstructive pulmonary disease and is also associated with increased morbidity because of cardiovascular diseases.¹⁻³ PM₁₀ can be further categorized by size as ultrafine particulate matter (PM; <0.1 μ m MMAD), fine PM (0.1-2.5 μ m MMAD), and coarse PM (2.5-10 μ m MMAD), with most studies suggesting that fine particles exert a stronger effect on cardiopulmonary outcomes than coarse fraction PM.⁴ However, recent investigations suggest that the role of coarse fraction PM_{2.5-10} on asthma and other diseases has been significantly underestimated.

Recent animal and human *in vitro* work by our group suggests that on an equal mass basis, PM_{2.5-10} is more potent in inducing alveolar macrophage responses (TNF, IL-6, mRNA COX-2) than fine and ultrafine PM, and in fact contains 90% to 95% of the stimulatory material present in PM₁₀.⁵⁻⁷ We and others have observed that the effect of PM_{2.5-10} on macrophages and monocytes is a result primarily of endotoxin and other biological components. These studies found that macrophage cytokine responses were mediated in large part by mCD14 and Toll-like receptor (TLR)-4, and to a lesser extent TLR2,^{8,9} indicating that bacterially derived components of PM_{2.5-10} are central to this action. However, IL-8 production by airway epithelial cells induced by PM_{2.5-10} was unaffected by mCD14 inhibitors and TLR4 antagonists. Thus, it is likely that range of inflammatory effects of PM_{2.5-10} on the airway may include the effects of biological material on macrophages, as well as other components such as metals acting on epithelial cells.

There is evidence that innate and acquired immune responses may be involved in PM_{2.5-10} exposure. Animal

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Abbreviations used

C:	Central
Mac:	Macrophage
MFI:	Mean fluorescence intensity
MMAD:	Mean-median airway diameter
P:	Peripheral
PM:	Particulate matter
PM _{2.5-10} :	Particulate matter with a mean median aerodynamic diameter between 2.5 and 10 μ m
PM ₁₀ :	Particulate matter with a mean median aerodynamic diameter of 10 μ m
ROI:	Region of interest
SC:	Sulfur colloid
TLR:	Toll-like receptor

studies have shown that certain particles are strong adjuvants for recall responses to antigen,^{10,11} whereas heterogeneous mixtures of particles can affect host defense function and immunity.⁶ In human beings, diesel exhaust particles have been shown to have adjuvant activity and influence the development of allergic sensitization,^{12,13} whereas we have demonstrated that controlled exposure to LPS and LPS-associated PM₁₀ increased responsiveness to inhaled allergen in atopic patients with asthma.^{14,15} More recently, we reported that direct inhalation of low dose LPS (10,000 EU) by healthy volunteers activated airway phagocytes and produced a T_H2 cytokine profile *in vivo*.¹⁶

In this study, we investigated in healthy volunteers whether inhalation challenge with ambient PM_{2.5-10} induced airway inflammation (neutrophil influx) and activated airway macrophages, as demonstrated by alterations in immune cell surface phenotypes, phagocytic function, and oxidative burst generation. We also determined whether biological components of PM_{2.5-10} were essential in mediating any observed immune-inflammatory responses. Furthermore, because PM_{2.5-10} has been shown to be preferentially deposited in bronchial airways in human beings *in vivo*,^{17,18} we used a controlled breathing inhalation challenge system that preferentially deposited PM_{2.5-10} to the bronchial airways. Preferential deposition was confirmed by using radiolabeled aerosol delivery and γ -scintigraphy techniques. In addition, we used induced sputum as our sampling method to assess airways inflammation, because it selectively retrieves samples from the surfaces of the bronchial airways.¹⁹ We report here that inhaled PM_{2.5-10} from local ambient air induced airway inflammation and modified macrophage biology in the airways of healthy volunteers. Macrophage responses, however, were significantly attenuated when biological components were removed from the PM_{2.5-10}.

METHODS

Subjects

Nine healthy subjects (3 men, 6 women) between the ages of 18 and 35 years underwent exposure to coarse fraction particulate matter

(PM_{2.5-10}). All subjects were nonsmoking volunteers with no history of lung disease (asthma, chronic obstructive pulmonary disease, fibrosis) and had been free of upper and lower respiratory tract infection for at least 4 weeks before beginning the study. All women provided a urine sample for pregnancy testing, and a positive pregnancy test resulted in exclusion from the study. All subjects had a FEV₁ of greater than 80% of predicted values for a population of similar height, weight, sex, age, and race. On a separate visit before the study, all subjects received a medical examination where informed written consent was obtained, and where the ability to produce an adequate number of sputum inflammatory cells for functional analyses ($>1.0 \times 10^6$ cells) was assessed. This study was approved by the Committee on the Protection of the Rights of Human Subjects, the School of Medicine, the University of North Carolina at Chapel Hill.

Study protocol

On 2 separate visits, subjects inhaled ambient PM_{2.5-10} that was collected from Chapel Hill, NC, and sterilized by UV radiation. On 1 visit, subjects inhaled PM_{2.5-10} that had been heat-treated (120°C for 20 hours) to inactivate the biological material (PM_{2.5-10}⁻), and on the other visit, they inhaled PM_{2.5-10} that was not heat-treated (PM_{2.5-10}⁺). On a third visit, subjects inhaled saline (0.9%) to serve as the inhalation control. All visits were randomly assigned, and investigators and subjects were blind to which PM_{2.5-10} (PM_{2.5-10}⁺ or PM_{2.5-10}⁻) was being inhaled. During the final minute of the saline control inhalation, Tc99m-sulfur colloid (SC) particles were added to the saline mixture, and a γ -camera scan of 2 to 3 hours followed to validate delivery of PM_{2.5-10} to the bronchial airways. Induced sputum was performed 3 hours after each PM_{2.5-10} inhalation and at the end of the γ -scan on the control visit. Sputum samples were analyzed for inflammatory cells and cytokines, mRNA for inflammatory cytokines, cell surface phenotypes, and phagocyte function (phagocytosis, oxidative burst). A minimum of 1 week separated each visit by all subjects.

PM_{2.5-10} collection

A particle impactor (ChemVol Model 2400 High Volume Cascade Impactor; Thermo Electron Corp, Waltham, Mass) collecting coarse particles in the size range 2.5 to 10 μ m diameter (PM_{2.5-10}; >80% of particles <5 μ m diameter) was located on the roof of the Environmental Protection Agency Human Studies Facility (Chapel Hill, NC), where the study was conducted. The impactor ran 4 d/wk from August to October (2001) and April to May (2003) to collect sufficient mass of particles (3 mg/subject). PM_{2.5-10} was collected on foam filters, removed by sonication into sterile water, lyophilized, and then pooled into 2 batches (August batch and May batch). Nine subjects received particles from the October 2001 batch, and 2 subjects received particles from the spring 2003 batch. The dried PM_{2.5-10} was sterilized by UV irradiation (UV Strata-Linker 1800; Stratagene, La Jolla, Calif) for 15 minutes to eliminate viable bacteria. After irradiation, bacterial growth was assessed, and if growth was present, irradiation was repeated until the PM_{2.5-10} was void of all viable bacteria. In a separate set of *in vitro* experiments, the biological activity of our PM_{2.5-10} samples was tested by examining their cytokine inducing capability. PM_{2.5-10}⁺ particles were capable of stimulating TNF- α and IL-6 responses in peripheral blood monocytes above control levels, whereas heat-inactivated particles (PM_{2.5-10}⁻) were unable to provoke TNF- α and IL-6 responses beyond control levels (data not shown). Both the TNF- α and IL-6 responses after PM_{2.5-10}⁺ incubation were significantly inhibited by Polymixin B Sulfate (Sigma, St Louis, Mo; 10 μ g/mL), an endotoxin inhibitor. Endotoxin concentrations on PM samples were not directly measured, because previous experience using the Limulus assay for measuring PM-bound endotoxin has proven unreliable. We therefore

used the cytokine induction assay with Polymixin B as a better alternative to reflect the level of endotoxin activity in our samples.

PM_{2.5-10} elemental and carbonaceous composition

Elemental analysis using inductively coupled plasma mass (and atomic emission) spectroscopy on Chapel Hill, NC, PM_{2.5-10} from October 2001 revealed the presence of the following components: Al, Fe, Si, Cu, Ti, As, Cr, Pb, Ni, Se, V, and Zn.²⁰ Relative to fine and ultrafine PM, PM_{2.5-10} contained higher levels of Al, Fe, Si, Cu, and Ti. Data on the carbonaceous/biological material present on PM_{2.5-10} revealed the presence of pollens, spores, bacteria, and other carbonaceous material. In ambient Chapel Hill air, these biological materials make up approximately 25% by number and 13% by mass of the total PM_{2.5-10}. The mass contribution of pollens, spores, or bacteria to coarse PM in Chapel Hill air was approximately 9%, with other carbonaceous material making up the remaining 4%. Bacteria breakdown analysis of 2001 Chapel Hill PM has been performed and showed that 30% of the colonies were Gram-negative (*Pseudomonas* spp identified by morphology), with the remainder being Gram-positive cocci. Twelve to 16 colonies of fungal spores were identified as belonging to *Penicillium*, *Cladosporium*, and *Alternaria* species classes.⁸

PM_{2.5-10} delivery and deposition

According to the blind study schedule for the day, either the nonheated or heat-inactivated PM_{2.5-10} was resuspended in sterile 0.9% saline for nebulization (DeVilbiss 646; Sunrise Medical HHG, Somerset, Pa). Subjects inhaled the aerosol (5 μ m MMAD, 2.0 geometric standard deviation) directly from the nebulizer mouthpiece using a controlled breathing pattern. The reservoir of the nebulizer was filled with 3 mg PM_{2.5-10} in 4 mL saline solution. The nebulizer had a delivery efficiency of approximately 30% when pulsed during inspiration; therefore, the estimated amount of PM_{2.5-10} delivered to the mouth of the subjects was approximately 1 mg. This exposure was selected to be comparable to the amount of dust inhaled over a period of 24 hours in an environment having a PM₁₀ mass particle concentration of approximately 70 μ g/m³. Subjects followed a controlled, shallow breathing pattern of 500 mL tidal volume at 25 breaths/minute frequency on an oscilloscope screen. This breathing pattern was displayed and recorded from a pneumotachograph attached downstream to the nebulizer. Each inhalation exposure proceeded until the nebulizer would no longer pulse aerosol (6-700 breaths, approximately 30 minutes) and matched for the 3 exposure days. The volume remaining in the nebulizer at the end of the exposure was measured to assure comparable dosing on each day. These residual volumes were less than 1 mL and not different between study days or within a subject. Assuming a 65% deposition efficiency for 5- μ m particles inhaled under these conditions,¹⁸ approximately 0.65 mg PM_{2.5-10} was deposited in the respiratory tract of subjects.

Targeting aerosols to the central airways: Confirmation by radiolabeled aerosol inhalation technique

Regional deposition of an inhaled Tc99m-SC aerosol under the same inhalation conditions as the PM_{2.5-10} was performed to confirm preferential deposition of PM_{2.5-10} in the bronchial airways. The labeled SC particles were assumed to act as a surrogate marker of PM in solution, whose deposition in the airways would be dependent on the nebulized particle size and breathing pattern used. The nebulized particle size (5 μ m MMAD) and breathing pattern were chosen to optimize deposition to the bronchial airways. One to 2 mCi Tc99m-SC in 3 mL saline was added to the nebulizer after completion of the saline control inhalation. Inhalation of the Tc99m-SC then proceeded

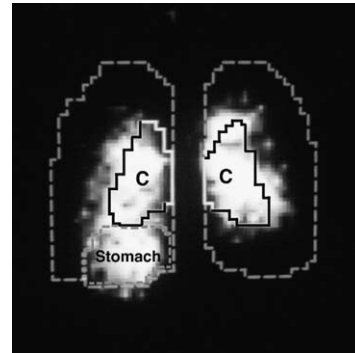


FIG 1. γ -Camera image of the whole lung (left and right, stomach shown bottom quadrant left lung) immediately after a central airway deposition of radiolabeled 99mTc-SC particles for 1 subject. Outlines of the right and left lung are based on the xenon equilibrium scan. The central (C) airways are shown for both lungs. Increased brightness represents greater presence of particles.

under the controlled breathing condition described until approximately 20 μ Ci radioactivity had deposited in the subject's lungs (less than 2 minutes), as measured by a single crystal NaI detector placed at the subject's back. This was followed immediately by sequential γ -camera scanning (2 minute scans) of the subject's lungs for the 2 to 3 hours before induced sputum and immediately after sputum induction to measure particle retention in the lung. Subjects returned the following day to obtain a 24-hour measure of particle retention.

Fig 1 shows a posterior γ -camera image of the whole lung (left and right) 2 minutes after a central/bronchial airway deposition of radiolabeled Tc99m-SC particles for 1 subject. Shown is the outline of the right and left lung based on a Xenon equilibrium scan performed before study on the saline control day. The central airways region of interest (ROI; interior white line) is shown for both lungs (area = 25% of the whole lung ROI). Greater deposited activity is shown in white in the central airways. The ROI for the stomach is shown in the bottom quadrant of the left lung to delineate and subtract stomach activity.

Central (C) to peripheral (P) activity for the initial deposition image (both right and left) normalized to lung volume was determined for each subject.^{19,21} A C/P of near 1.0 indicates deposition has primarily occurred in airways distal to the segmental bronchi (ie, large bronchi). Increases in C/P to values greater than 1.0 indicate increasing deposition in the large bronchial airways relative to more peripheral airspaces. Clearance of radioactive particles from the whole lung over the 2-hour to 3-hour period of scanning before sputum induction, during the sputum induction, and through 24-hour postradioactive particle deposition was determined for both lungs. C/P ratio and % cleared through 2 to 3 hours and 24 hours are indices of the amount of deposition occurring in the bronchial airways.^{21,22}

Induced sputum

The sputum induction and processing methods have been previously described in detail.¹⁹ In brief, three 7-minute inhalation periods of nebulized (UltraNeb 99; Sunrise Medical) hypertonic saline (3%, 4%, 5%) were followed by expectoration of sputum into a sterile specimen cup. Sputum plugs were macroscopically identified and manually selected from their surrounding fluid and treated with DTT (0.1%), a mucolytic dispersing agent. After filtration (48-52 μ m pore), total cell counts and cell viability were determined by using a Neubauer hemacytometer (Canemco, Inc., St Laurent, Quebec, Canada) and Trypan Blue exclusion staining (Sigma), respectively. Cytospin slides were prepared and stained with Diff Quik for differential leukocyte analysis. Approximately 500,000 cells were prepared

for flow cytometry to analyze cell surface phenotypes (mCD14, CD11b, HLA-DR, CD64, CD16) and cell function (phagocytosis, intracellular oxidative burst). The remaining cell sample (500,000 to 1×10^6 cells) was devoted to isolation of sputum macrophages ($\sim 100,000$ macrophages) using Percoll (Pharmacia, Uppsala, Sweden) gel density centrifugation for quantification of cytokine mRNA expression by RT-PCR. Isolated macrophage recovery by the Percoll technique was typically $>95\%$ pure. Fluid phase cytokines (IL-4, IL-5, IL-6, IL-8, IL-10, IL-13, TNF- α , IFN- γ , GM-CSF, RANTES, eotaxin) were analyzed by using Luminex bead array technology (Luminex¹⁰⁰ total system; Luminex Corp, Austin, Tex) and commercially available ELISA kits (R&D Systems, Minneapolis, Minn).

Flow cytometry and immunofluorescent staining

Immunofluorescent staining and flow-cytometry methodology have been described in detail in previous publications.²³ In brief, flow cytometry was performed by using a FACSORT (Becton Dickinson, Franklin Lakes, NJ). Macrophages, monocytes, neutrophils, and lymphocytes in sputum were initially identified and gated on the basis of light scatter properties and positive expression for CD45 (pan leukocyte marker). Cell populations were then confirmed by using CD16 (PMN), mCD14 (monocytes), HLA-DR (macrophages), and CD3 (lymphocytes). Surface markers analyzed included CD11b/CR3, mCD14/LPS receptor, CD16/Fc γ RIII, CD64/Fc γ RI, and HLA-DR/MHC class II. Analysis was performed by using the Cell Quest software (Becton Dickinson).

Phagocytosis assay

Phagocytosis of fluorescein isothiocyanate-labeled IgG-opsonized *Saccharomyces cerevisiae* zymosan A BioParticles (Molecular Probes, Inc, Eugene, Ore) was performed as previously described.²³ All samples were analyzed by flow cytometry within 24 hours of fixation with 2% paraformaldehyde. Particle uptake was displayed on histograms and identified as a rightward shift in fluorescence into the particle (zymosan)-exposed region from the control (nonparticle-exposed) region. Phagocytosis was defined as the mean fluorescence intensity (MFI) of the cells in the zymosan-exposed region less the MFI of the cells in the control region. A uniform shift in all sputum phagocyte populations was routinely observed when cells were exposed to particles, such that at least 80% of the cells shifted (with particle exposure) into the exposed region compared with the unexposed region.

Isolation of RNA

Total RNA from purified sputum macrophages was isolated by using the TRIzol Reagent method as per the manufacturer's instructions (Invitrogen, Carlsbad, Calif).

Real-time quantitative PCR

Relative gene expression in sputum macrophages was quantified by using real-time quantitative PCR. Oligonucleotide primer pairs for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and TNF- α and fluorescent probes were designed by using a primer design program (Primer Express; Applied Biosystems, Foster City, Calif) and obtained from Integrated DNA Technologies (Coralville, Iowa). Quantitative fluorogenic amplification of cDNA was performed by using the ABI Prism 7700 Sequence Detection System (Applied Biosystems), TaqMan Universal PCR Master Mix (Applied Biosystems), and the primers and probes. The relative abundance of mRNA levels was determined from standard curves generated from a serially diluted standard pool of cDNA prepared from LPS-stimulated sputum macrophages. The relative abundance of GAPDH mRNA was

TABLE I. Mean $\pm$ SEM inflammatory cell and cytokine response in sputum

	Saline (0.9%) inhalation	PM _{2.5-10} ⁺ inhalation	PM _{2.5-10} ⁻ inhalation
Total cell counts (10^6)	7.5 $\pm$ 1.7	4.8 $\pm$ 0.9	8.2 $\pm$ 1.6
Viability (% alive)	84 $\pm$ 2	87 $\pm$ 3	82 $\pm$ 3
PMN/mg	221 $\pm$ 45	389 $\pm$ 78*	577 $\pm$ 253*
Mac/mg	903 $\pm$ 190	791 $\pm$ 166	988 $\pm$ 184
IL-8 (ng/mL)	43.8 $\pm$ 12	59.6 $\pm$ 12	83.7 $\pm$ 21
IL-6 (pg/mL)	1933 $\pm$ 1621	3728 $\pm$ 1638	3376 $\pm$ 1133
TNF- α (pg/mL)	355 $\pm$ 78	436 $\pm$ 64	502 $\pm$ 201
Eotaxin (pg/mL)	816 $\pm$ 55	1280 $\pm$ 183*#	784 $\pm$ 101
IL-10 (pg/mL)	58 $\pm$ 0.9	59 $\pm$ 0.7	58 $\pm$ 0.3
GM-CSF (pg/mL)	111 $\pm$ 7	123 $\pm$ 8	109 $\pm$ 5
RANTES (pg/mL)	48 $\pm$ 4	46 $\pm$ 5	48 $\pm$ 6

* $P < .05$ vs saline.

#vs PM_{2.5-10}⁻.

used to normalize levels of the mRNA of interest. Primer sequences have been previously cited.⁵

Intracellular oxidative burst

Oxidative burst was measured in sputum cells using the Fc OxyBurst assay as per the manufacturer's instructions (Molecular Probes, Eugene, Ore) and analyzed on the flow cytometer using FACSORT software and expressed as MFI.

Statistical analysis

Differences in study endpoints across all 3 inhalation conditions were analyzed by 1-way ANOVA. Differences between specific inhalation conditions (ie, PM_{2.5-10}⁺ vs PM_{2.5-10}⁻) were analyzed by using nonparametric pairwise statistics (Wilcoxon sign-rank). An overall α level set at 0.05 was used, and data are presented as means $\pm$ SEMs unless otherwise noted.

RESULTS

Inflammatory cells and cytokines

Table I shows the mean ($\pm$ SEM) absolute number of neutrophils (PMN/mg) and macrophages (Mac/mg) recovered from induced sputum 3 hours after inhalation of heat-inactivated PM_{2.5-10} (PM_{2.5-10}⁻), untreated/naive PM_{2.5-10} (PM_{2.5-10}⁺), and saline (0.9%). The neutrophil response (cells/mg) was significantly elevated after PM_{2.5-10}⁺ and PM_{2.5-10}⁻ inhalations compared with saline, but the mean number of PMNs did not differ after PM_{2.5-10}⁺ and PM_{2.5-10}⁻ inhalation. Absolute macrophages (Mac/mg) as well as monocytes and lymphocytes (data not shown) were not significantly altered after PM_{2.5-10}⁺ and PM_{2.5-10}⁻ inhalations compared with saline. Fig 2 shows that the proportion (%) of neutrophils was also significantly increased after PM_{2.5-10}⁺ and PM_{2.5-10}⁻ inhalations compared with saline, and like PMN/mg, no difference in %PMNs was observed between PM_{2.5-10}⁺ and PM_{2.5-10}⁻ inhalations.

Several cytokines were measured in the fluid phase of induced sputum, and the values (pg/mL) are shown in

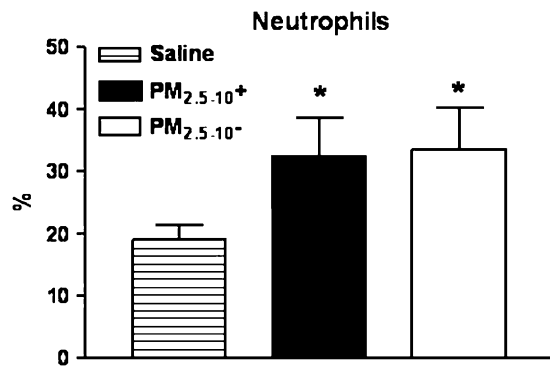


FIG 2. Mean ($\pm$ SEM) neutrophil (%PMN) response in induced sputum 2 to 3 hours after inhalation of saline (hatched bar), coarse fraction particulate matter ($\text{PM}_{2.5-10}^+$; solid bar), and heat-inactivated coarse fraction particulate matter ($\text{PM}_{2.5-10}^-$; open bar). $\text{PM}_{2.5-10}^+$ and $\text{PM}_{2.5-10}^-$ responses were significantly ($P < .05$) increased versus saline (*).

Table II. Eotaxin levels were significantly elevated after $\text{PM}_{2.5-10}^+$ inhalation compared with both saline and $\text{PM}_{2.5-10}^-$ inhalation. All other cytokines showed no difference among saline, $\text{PM}_{2.5-10}^+$, and $\text{PM}_{2.5-10}^-$ conditions. The use of DTT affected the recovery of IL-4, IL-5, IL-13, and IFN- γ in several sputum samples; therefore, these cytokines were excluded from the analyses.

TNF- α mRNA expression

TNF- α mRNA expression on purified sputum macrophages was measured 3 hours after each inhalation condition. **Fig 3** shows that TNF- α mRNA expression was significantly ($P < .05$) increased after $\text{PM}_{2.5-10}^+$ inhalation compared with saline (*). No significant difference in TNF- α mRNA was observed after $\text{PM}_{2.5-10}^-$ inhalation compared with saline.

Cell surface phenotypes

Cell surface phenotypes associated with LPS-mediated responses (mCD14), host defense (CD11b/CR3, CD64/Fc γ RIII, CD16/Fc γ RI) and antigen presentation (HLA-DR/MHC class II) were examined on sputum cells. **Fig 4, A**, shows significantly ($P < .05$) increased macrophage and monocyte mCD14 expression (MFI) after $\text{PM}_{2.5-10}^+$ inhalation compared with saline (*) and $\text{PM}_{2.5-10}^-$ (#). Expression of mCD14 was also significantly increased after $\text{PM}_{2.5-10}^-$ inhalation compared with saline, but this increase was less than the increase observed after $\text{PM}_{2.5-10}^+$ inhalation. In **Fig 4, B**, macrophage CD11b/CR3 expression was significantly ($P < .05$) increased after $\text{PM}_{2.5-10}^+$ inhalation compared with saline (*), and in **Fig 4, C**, HLA-DR expression (MFI) on macrophages was also significantly ($P < .05$) enhanced after $\text{PM}_{2.5-10}^+$ inhalation compared with both saline (*) and $\text{PM}_{2.5-10}^-$ inhalation (#). No significant differences were observed in expression of CD64/Fc γ RI and CD16/Fc γ RIII on macrophages, monocytes, or neutrophils after $\text{PM}_{2.5-10}^+$ or $\text{PM}_{2.5-10}^-$ inhalation versus saline, or between $\text{PM}_{2.5-10}^+$ and $\text{PM}_{2.5-10}^-$ inhalation (data not shown).

TABLE II. Mean $\pm$ SEM intracellular oxidative burst response (MFI) in sputum macrophages

Cell type	Saline (0.9%)	$\text{PM}_{2.5-10}^+$	$\text{PM}_{2.5-10}^-$
Macrophages (MFI)	69 $\pm$ 8	75 $\pm$ 11	80 $\pm$ 7

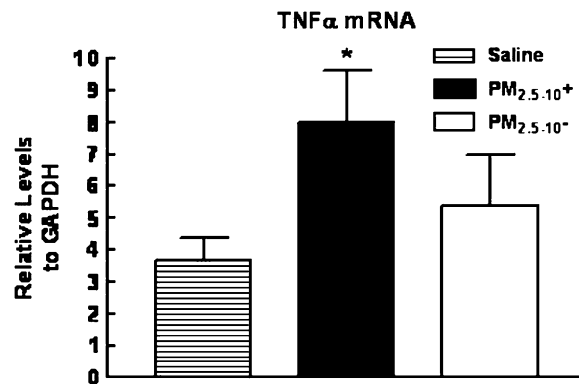


FIG 3. Mean ($\pm$ SEM) mRNA TNF- α expression on Percoll isolated sputum macrophages 2 to 3 hours after inhalation of saline (hatched bar), coarse fraction particulate matter ($\text{PM}_{2.5-10}^+$; solid bar), and heat-inactivated coarse fraction particulate matter ($\text{PM}_{2.5-10}^-$; open bar). Expression of mRNA TNF- α was significantly ($P < .05$) increased after $\text{PM}_{2.5-10}^+$ versus saline inhalation (*).

Phagocytosis and intracellular oxidative burst

The ability of sputum cells to phagocytose IgG-opsonized zymosan particles was assessed by flow cytometry, and values are expressed in MFI. As shown in **Fig 5**, monocyte phagocytosis was significantly ($P < .05$) enhanced after inhalation of $\text{PM}_{2.5-10}^+$ compared with $\text{PM}_{2.5-10}^-$ (#). Macrophage, monocyte, and neutrophil phagocytosis was elevated after $\text{PM}_{2.5-10}^+$ inhalation compared with saline, and with macrophages, an increased trend was observed ($P = .06$), but none of these comparisons reached formal significance ($P < .05$). Intracellular oxidative burst on sputum macrophages was also examined by flow cytometry, and the values are expressed as MFI and shown in **Table II**. Compared with saline inhalation, oxidative burst after $\text{PM}_{2.5-10}^+$ or $\text{PM}_{2.5-10}^-$ did not differ, nor did these values differ when compared with each other.

Targeted deposition of radiolabeled aerosol to the bronchial airways

As validation that our inhalation technique targeted $\text{PM}_{2.5-10}$ delivery to the bronchial airways, ie, those airways sampled by induced sputum, we found a mean C/P ratio of 1.61 ± 0.28 (SD), indicating preferential deposition to the central versus peripheral airways. In addition, we found $54\% \pm 11\%$ (SD) clearance through 24 hours, suggesting that at least half of the $\text{PM}_{2.5-10}$ that was deposited in the lung likely occurred in the ciliated, bronchial airways where induced sputum was sampled. Moreover, these values are similar to those obtained in our previous

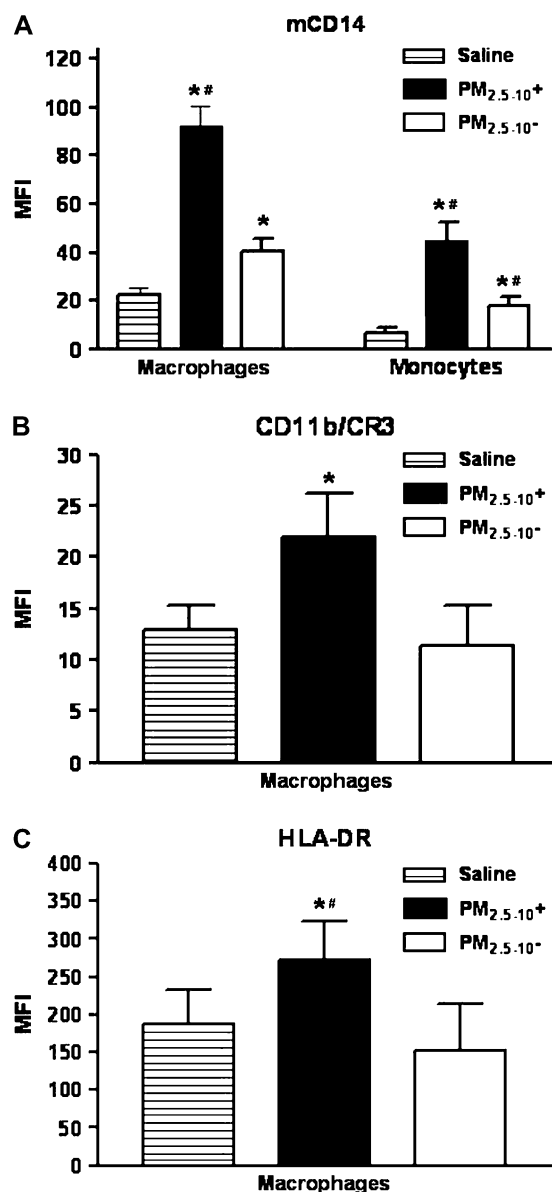


FIG 4. Mean ($\pm$ SEM) mCD14 (A), CD11b (B), and HLA-DR (C) expression (MFI) on sputum macrophages and monocytes 2 to 3 hours after inhalation of saline (hatched bar), coarse fraction particulate matter (PM_{2.5-10}⁺; solid bar), and heat-inactivated coarse fraction particulate matter (PM_{2.5-10}⁻; open bar). A, mCD14 expression (MFI) on macrophages and monocytes was significantly ($P < .05$) elevated after PM_{2.5-10}⁺ versus saline (*) and versus PM_{2.5-10}⁻ (#) inhalation. mCD14 was also significantly ($P < .05$) elevated after PM_{2.5-10}⁻ inhalation versus saline (*). B, CD11b/CR3 expression (MFI) on macrophages was significantly ($P < .05$) elevated after PM_{2.5-10}⁺ versus saline (*). C, HLA-DR expression (MFI) on macrophages was significantly ($P < .05$) elevated after PM_{2.5-10}⁺ versus saline (*) and versus PM_{2.5-10}⁻ (#) inhalation.

study in which we used shallow, inhaled aerosol boluses to target the bronchial airways to show that these airways were the source of induced sputum.¹⁹ We found no correlation between C/P values or percent clearance and sputum inflammatory endpoints with either PM_{2.5-10}⁺ or PM_{2.5-10}⁻

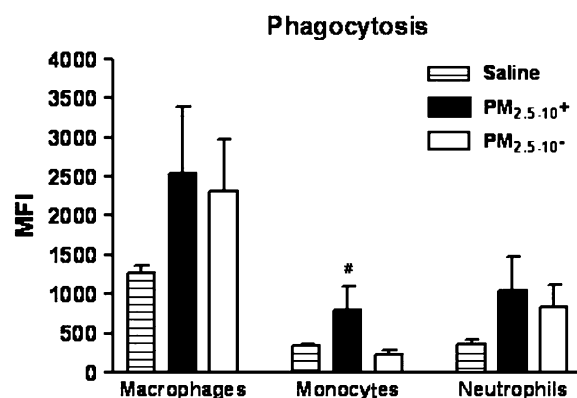


FIG 5. Mean ($\pm$ SEM) phagocytosis (MFI) of IgG-opsonized *Saccharomyces cerevisiae* zymosan bioparticles by sputum macrophages, monocytes, and neutrophils 2 to 3 hours after inhalation of saline (hatched bar), coarse fraction particulate matter (PM_{2.5-10}⁺; solid bar), and heat-inactivated coarse fraction particulate matter (PM_{2.5-10}⁻; open bar). Phagocytosis (MFI) was significantly ($P < .05$) elevated after PM_{2.5-10}⁺ versus PM_{2.5-10}⁻ inhalation (#).

exposure. This is likely a result of our efforts to control aerosol inhalation tightly and thus maintain a relatively constant airway dose for all inhalations.

DISCUSSION

In this study, we examined the effect of inhalation of local ambient coarse fraction PM_{2.5-10} on airway inflammation and phagocytic cell responses *in vivo* in healthy volunteers. In particular, we examined whether PM_{2.5-10} activated or primed airway phagocytes, and if so, whether PM_{2.5-10}-associated biological components were responsible for this effect. This is important because microbial agents such as bacteria, viruses, and fungi are part of outdoor PM and when tested *in vitro* are the most potent activators of alveolar macrophages.⁹ Moreover, bacterial components are found to be mainly associated with PM_{2.5-10}²⁴⁻²⁶ versus fine and ultrafine PM. Consequently, we hypothesized that bacterial products on inhaled PM_{2.5-10} would mediate PM_{2.5-10}-induced effects *in vivo*.

Our data show that inhalation of ambient PM_{2.5-10} by healthy individuals induces neutrophilic airways inflammation. Moreover, our results demonstrate for the first time *in vivo* in humans that inhaled PM_{2.5-10} can induce subtle proinflammatory changes in airway phagocytes at the mRNA level as well as activate cell surface phenotypes and modify cell function associated with innate immunity. We report that PM_{2.5-10} induced increased expression of TNF- α mRNA in sputum macrophages and stimulated increased expression of mCD14, CD11b/CR3, and HLA-DR/MHC class II on sputum macrophages and monocytes. These activating responses were induced by untreated PM_{2.5-10} but were eliminated when these particles were heated for 20 hours at 120°C before inhalation. Heat inactivation of PM_{2.5-10} significantly reduced the ability of coarse PM to induce increased cell-surface marker expression and enhance phagocytosis, and to a lesser extent,

stimulate TNF- α mRNA expression on macrophages. Interestingly, heat inactivation had no effect on the ability of PM_{2.5-10} to induce neutrophil influx to the airways. Neutrophil influx to the airways is driven in large part by IL-8, a potent neutrophil chemoattractant produced by airway epithelial cells.²⁷ Our own *in vitro* studies show that biological components present on coarse PM do not appear to play a role in the production of IL-8 by human airway epithelial cells. Heated PM_{2.5-10} served to inactivate or destroy heat-sensitive biological components such as Gram-negative bacteria (LPS), as well as Gram-positive peptidoglycans and lipoteichoic acid,²⁸ both of which can stimulate alveolar macrophages through CD14-dependent mechanisms.^{8,29} Analysis of ambient Chapel Hill, NC, PM_{2.5-10} from 2001 showed that it contained 30% Gram-negative bacteria (LPS, *Pseudomonas*), with the remainder mostly composed of Gram-positive cocci and fungal spores (*Penicillium*, *Cladosporium*).⁸ Of those components, Gram-negative bacteria were 3 times more potent in inducing cytokine responses from alveolar macrophages than Gram-positive bacteria, highlighting the preferential response of macrophages to Gram-negative bacteria. Among the elemental compounds present in the PM_{2.5-10}, relatively high concentrations of Al, Fe, Si, Cu, and Ti were found. Compared with biological components, however, metals likely played a minor role in the responses observed here because we saw no effect from heat inactivation on neutrophil influx or oxidative burst, responses largely attributed to metals like Fe and Cr.

It is important to also consider what responses PM alone would induce in the absence of biological or residual chemicals. We have previously shown that certain responses by macrophages such as apoptosis and oxidative burst may be a result of the particle itself or are at least independent of such factors like endotoxin and metals.¹⁰ However, ambient PMs pick up biological matter when becoming aerosolized in the atmosphere and contain variable but sometimes high numbers of bacteria.³⁰ We have previously shown that when environmentally relevant levels of bacteria are incubated with macrophages (0.1 bacteria per macrophage), they illicit a cytokine response equivalent to approximately 25 $\mu\text{g/mL}$ freshly collected PM_{2.5-10}.⁸

Our laboratory has demonstrated that LPS accounts for a large part of the cytokine-inducing activity of PM_{2.5-10} on human alveolar macrophages,⁹ but showed that LPS is not involved in mediating IL-8 production by cultured human airway epithelial cells exposed to PM_{2.5-10}.⁸ In the alveolar macrophage studies, mCD14 antagonists and LPS inhibitors attenuated the majority of IL-6 production by macrophages exposed to PM_{2.5-10},⁸ and when macrophage cytokine (IL-6) responses to Gram-positive (*Staphylococcus*, *Streptococcus*) and Gram-negative (LPS) bacteria were compared, LPS induced a 7-fold greater level of IL-6 production than the Gram-positive organisms.⁸ Although these studies provide good *in vitro* evidence that LPS may in large part be responsible for activating lung phagocytes, they were not performed *in vivo* in humans. We recently showed that low-dose LPS inhalation challenge primes airway phagocytes

in vivo in healthy individuals.¹⁶ In that study, we reported that LPS (10,000 EU/ $\sim$ 200ng LPS deposited) enhanced mCD14 and CD11b expression and increased phagocytosis on sputum macrophages and monocytes, results that are strikingly similar to those reported here. Interestingly, in that study, direct LPS inhalation challenge did not modify neutrophil levels in the airways, an observation that fits with the notion that neutrophil influx is related to the production of IL-8 by airway epithelial cells and that LPS has little direct effect on these cells. A recent study by Roos-Engstrand et al³¹ also demonstrated that after inhalation of relatively high levels of LPS (50 μg), neutrophil levels and IL-8 concentration were unchanged in bronchoalveolar lavage and bronchial wash fluids compared with saline. Although it is difficult to draw direct comparisons between studies that use different inhalation methodologies (nebulized aerosol vs controlled breathing pattern) and different exposure compounds (pure LPS vs PM_{2.5-10}), the similarity in results is intriguing despite these differences and range in LPS concentrations.

The ability of certain types of PM (diesel exhaust) to act as an adjuvant in individuals with allergy^{12,13} and the action of LPS in enhancing the response of atopic patients with asthma to inhaled or nasally applied allergen^{14,15} suggest that allergic processes may be stimulated by PM and their biologic components. Although we could not assess the effect of PM_{2.5-10} on specific T_H2-type cytokines (IL-4, IL-5, IL-13) in this study because of their variable recovery in induced sputum, we did find elevated levels of eotaxin, the potent eosinophil chemoattractant compound. Eotaxin has been reported to be an early response gene and present in both nonallergic and allergic airways inflammation, specifically identified in epithelium and alveolar macrophages after an allergic stimulus.²⁷ Consequently, elevated levels of this cytokine even in nonatopic individuals may skew the airway toward an allergic phenotype, similar to what we reported with IL-4 exposure to the upper airways of nonatopic volunteers.³² Furthermore, we speculate that individuals with allergic airway disease may be more susceptible to the inflammatory effects of PM_{2.5-10} because of the constitutive presence of eotaxin in macrophages, and as reported here, the ability of PM_{2.5-10} to activate those macrophages.

In summary, we report that coarse fraction PM induces neutrophil responses that are not dependent on biological components of this pollutant, as well as changes in monocytic cells, which are dependent on the biological components of these particles. Because macrophages likely mediate responses to several stimuli in the airway, including pathogens and allergens, the effect of coarse fraction PM on these cells may account for the observed effect of low levels of PM on human health.

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