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Suppression of the NF-κB Pathway by Diesel Exhaust Particles Impairs Human Antimycobacterial Immunity

Srijata Sarkar,* Youngmia Song,* Somak Sarkar,* Howard M. Kipen,† Robert J. Laumbach,‡ Junfeng Zhang,*‡§ Pamela A. Ohman Strickland,¶ Carol R. Gardner,** and Stephan Schwander*†‡§

Epidemiological studies suggest that chronic exposure to air pollution increases susceptibility to respiratory infections, including tuberculosis in humans. A possible link between particulate air pollutant exposure and antimycobacterial immunity has not been explored in human primary immune cells. We hypothesized that exposure to diesel exhaust particles (DEP), a major component of urban fine particulate matter, suppresses antimycobacterial human immune effector cell functions by modulating TLR-signaling pathways and NF-κB activation. We show that DEP and H37Ra, an avirulent laboratory strain of Mycobacterium tuberculosis, were both taken up by the same peripheral human blood monocytes. To examine the effects of DEP on M. tuberculosis-induced production of cytokines, PBMC were stimulated with DEP and M. tuberculosis or purified protein derivative. The production of M. tuberculosis and purified protein derivative-induced IFN-γ, TNF-α, IL-1β, and IL-6 was reduced in a DEP dose-dependent manner. In contrast, the production of anti-inflammatory IL-10 remained unchanged. Furthermore, DEP stimulation prior to M. tuberculosis infection altered the expression of TLR3, -4, -7, and -10 mRNAs and of a subset of M. tuberculosis-induced host genes including inhibition of expression of many NF-κB (e.g., CSF3, IFNG, IFNA, IFNB, IL1A, IL6, and NFIB) and IFN regulatory factor (e.g., IFNG, IFNA1, IFNB1, and CXCL10) pathway target genes. We propose that DEP downregulate M. tuberculosis-induced host gene expression via MyD88-dependent (IL6, IL1A, and PTGS2) as well as MyD88-independent (IFNA, IFNB) pathways. Prestimulation of PBMC with DEP suppressed the expression of proinflammatory mediators upon M. tuberculosis infection, inducing a hyposresponsive cellular state. Therefore, DEP alters crucial components of antimycobacterial host immune responses, providing a possible mechanism by which air pollutants alter antimicrobial immunity. The Journal of Immunology, 2012, 188: 2778–2793.

Air pollution and tuberculosis (TB) each contribute significantly to global burden of disease. The World Health Organization estimates that air pollution, which is linked to a variety of illnesses including cardiopulmonary diseases and cancer, causes ~2 million premature deaths worldwide per year (1). According to the World Health Organization’s 2009 Global Health Risk report (2), indoor air pollution (from biomass fuel and coal combustion) and urban outdoor air pollution rank 10th and 14th, respectively, among 19 leading risk factors for mortality in low- and middle-income countries. Exposure to ambient-air fine particulate matter <2.5 μm in aerodynamic diameter (PM2.5) is estimated to cause ~1% of mortality from acute respiratory infections in children >5 y worldwide (3).

TB was estimated to afflict ~10 million people and cause 2 million deaths in 2010 alone (4). Epidemiologic associations have been established between the incidence of TB and, respectively, cigarette smoking (5–11), occupational exposure to aerosolized silica (12–14), and indoor air pollution (10, 11, 15). Despite these associations, to the best of our knowledge, no studies have been conducted to assess the mechanisms that underlie associations between air pollution and TB development in primary human cells.

Human exposure to diesel exhaust particles (DEP), a major component of urban PM2.5 in most industrialized urban areas (16), typically occurs by inhalation. Due to their small size, DEP remain airborne for prolonged time periods and deposit in the lungs upon inhalation. Likewise, infection with Mycobacterium tuberculosis typically occurs by inhalation of aerosolized M. tuberculosis-containing droplet nuclei (1–5 μm) that are released from patients with active TB during respiratory maneuvers (17–20). Thus, concurrent respiratory exposure to DEP and M. tuberculosis can be expected to occur under real-life conditions and may subsequently alter host immune responses.

Protective antimycobacterial human host immunity involves innate and adaptive immune mechanisms that are primarily cell-

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The online version of this article contains supplemental material.

Abbreviations used in this article: BCG, bacillus Calmette-Guérin; CB, carbon black; DEP, diesel exhaust particle; IRAK, IL-1R-associated kinase; IIFN, IFN regulatory factor; LAL, Limulus amebocyte lysate; MOI, multiplicity of infection; PAH, polycyclic aromatic hydrocarbon; PI, propidium iodide; PM2.5, particulate matter <2.5 μm in aerodynamic diameter; PPD, purified protein derivative; qRT-PCR, quantitative RT-PCR; TB, tuberculosis; TEM, transmission electron microscopy; UMDNJ, University of Medicine and Dentistry of New Jersey.
mediated, involving monocytes/macrophages, dendritic cells, CD4+ and CD8+ T cells, NK, and γδT cells. *M. tuberculosis* Ag-specific immunity is characterized predominantly by a Th1 response (IL-2, IFN-γ, and TNF-α) (21) that is strongly enhanced and compartmentalized at the infection sites, most commonly the lungs (22–24). IFN-γ, secreted from activated T cells and NK cells, is known to activate macrophages and promote bacterial killing (25) by permitting phagosomal maturation and production of antimicrobial inductive NO synthase and reactive oxygen intermediates (26). IFN-γ has an essential role in the control of mycobacterial infections both in the murine model (27, 28) and in humans (29–33). Likewise, TNF-α plays an important role in killing of intracellular *M. tuberculosis* (34, 35) and granuloma formation. As evidenced by the increased risk of reactivation TB during TNF-α inhibitor treatment (36, 37), TNF-α is required in the maintenance of latent *M. tuberculosis* infection. IL-6 and IL-10 are important regulatory cytokines during the early phase of the mycobacterial infection of macrophages and in the inflammatory responses to *M. tuberculosis* and the regulation of IFN-γ, respectively (22).

Innate host resistance against *M. tuberculosis* depends to a large extent on the engagement of TLRs (38–43) and nucleotide oligomerization domain-like receptors (44). TLR2, TLR4, and TLR9 are activated by *M. tuberculosis*-derived ligands such as lipoolarabinomannan and mycobacterial 19-kDa protein (39, 40, 45), *M. tuberculosis* heat shock proteins 65 and 71 (38), and mycobacterial DNA (42), respectively. Stimulation of these TLRs (38, 41–43) on monocytes, alveolar macrophages, or dendritic cells (46–49) activates signal transduction pathways that culminate in the activation of MAPK, transcription factor NF-kB, and the IFN regulatory factor (IRF) family (47), leading to the release of antimicrobial effector molecules (50), proinflammatory cytokines (51–57), and chemokines (58).

DEP exposure has been shown to shift Th1 to Th2 cytokine production by T, monocyte-derived dendritic, and spleen cells (59, 60), decrease secretion of IFN-γ, and increase secretion of IL-10 in murine bone marrow-derived dendritic cells (61). A study of the direct effects of DEP on *M. tuberculosis* immune responses showed that DEP increases the pulmonary *M. tuberculosis* burden concomitant with decreased production of proinflammatory cytokines (e.g., IL-1β, IL-12p40, and IFN-γ) in experimentally infected mice (62). DEP exposure also decreases the phagocytosis and bactericidal activity of rat alveolar macrophages exposed to *Listeria monocytogenes* infection (63, 64).

The current study was undertaken to examine the effects of DEP on human host immune responses to *M. tuberculosis*. We show that DEP significantly alter live *M. tuberculosis* and purified protein derivative (PPD)-induced cytokine production in primary human PBMC. Furthermore, DEP modulate *M. tuberculosis*-induced host gene expression by inhibiting TLR-mediated signaling including NF-kB and IRF pathways crucial for *M. tuberculosis*-induced host immunity. Suppression of *M. tuberculosis*-induced activation of these two pathways by DEP provides insights into the possible mechanisms by which particulate air pollution may modify pathogen-induced human host immune responses.

**Materials and Methods**

Approval to perform this study, collect personal health information, and perform venipunctures was given by the Institutional Review Boards of the University of Medicine and Dentistry of New Jersey (UMDNJ) in Newark and New Brunswick (Institutional Review Board protocol number 0120060235).

**Human subjects**

A total of 20 healthy individuals (13 male, 7 female, mean age 33.5 y [range 20–52 y]) were recruited from students and staff of the UMDNJ-School of Public Health, Rutgers University, the Environmental and Occupational Health Sciences Institute, and volunteers at the Chandler Clinic in New Brunswick, NJ. Inclusion and exclusion criteria were as follows: inclusion criteria: healthy women and men, 18–65 y of age; exclusion criteria: concurrent infections, use of immunosuppressive medications (steroidal and/or nonsteroidal anti-inflammatory medications, TNF-α inhibitors, antineoplastic chemotherapy), illnesses affecting host immunity (diabetes, HIV-1 infection, chronic liver or kidney diseases, and malignancies), smoking, and illicit drug use. A total of 80 ml heparinized venous whole blood was obtained from each individual from a cubital vein by venipuncture. Not all subjects were examined in all experiments. Subject numbers for each experiment are shown in the figures or figure legends. All study subjects provided written informed consent before personal health information was acquired and whole heparinized peripheral blood obtained by venipuncture.

**Reagents**

Reagents were obtained from the following sources: ELISPOT assays: capture and biotinylated detection Abs for IL-1β and IL-6 (Cell Sciences, Canton, MA), IFN-γ, TNF-α (Endogen Pierce, Rockford, IL), IL-10, IL-4 (BD Pharmingen, San Diego, CA), streptavidin-peroxidase (Sigma-Aldrich, St. Louis, MO), chromogen 1% 3-amin-9-ethylcarbazole (Pierce, Rockford, IL), and Multiscreen®_ON high protein binding 96-well plates (Millipore, Bedford, MA); PBMC isolation, culture, and stimulation: PHA (Sigma-Aldrich), LPS (Escherichia coli 026:B6; Sigma-Aldrich), PPP (Statens Serum Institute, Copenhagen, Denmark), RPMI 1640 (Bio-Whittaker, Walkersville, MD), t-glutaminate (Cellgro, Manassas, VA), pooled human AB serum (Gemini Bioproducts, Woodland, CA), and Ficoll-Paque (GE Healthcare Biosciences, Pittsburgh, PA); RNA extraction and quantification: QIAamp mini kit (Qiagen, USA). Gene expression: RNase-free DNase set (Qiagen), RT2 First Strand kit and RT2 qPCR Master Mix (SuperArray Bioscience, Frederick, MD), Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA), TaqMan reverse transcription reagents (Applied Biosystems); pathway-specific gene expression arrays: human Th1-Th2-Th3 (PAHS-34E) and TLR (PAHS-18E; SuperArray Bioscience, Frederick, MD); cytokines and IL-6 (Cell Sciences, San Jose, CA); flow cytometry: Annexin V/propidium iodide (PI) apoptosis detection assay (BD Biosciences), PE-conjugated CD14 (BioLegend, San Diego, CA), and allophycocyanin-conjugated CD3 (eBioscience, San Diego, CA) mAbs; and DEP and carbon black (CB): DEP was a gift from Dr. Sagai (Tokyo, Japan), generated by a diesel-powered automobile, collected in a condensation trap, and stored at 20˚C until use. Prior to addition to cell cultures, DEP or CB were thawed and vortexed for 5 min (a period determined to provide reliable dispersion of DEP in preliminary studies). DEP were then diluted in complete cell-culture medium to generate desired final concentrations (0.1, 1, 10, 50, and 100 μg/ml).

**Preparation of DEP and CB suspensions**

DEP and CB stock suspensions (10 mg/ml) were prepared by 15-min sonication in PBS containing 0.05% Tween, aliquoted, and kept frozen at –80°C until use. Prior to addition to cell cultures, DEP or CB were thawed and vortexed for 5 min (a period determined to provide reliable dispersion of DEP in preliminary studies). DEP were then diluted in complete cell-culture medium to generate desired final concentrations (0.1, 1, 10, 50, and 100 μg/ml).

**Determination of endotoxin content and sterility of DEP**

Presence of any endotoxin in complete culture fluid with and without DEP (100 μg/ml) was assessed using a commercial endotoxin assay (Lonza Walkersville, Walkersville, MD) that employed the quantitative kinetic chromogenic *Limulus* amebocyte lysate (LAL) method (KQCL). The assay met all validation requirements per U.S. Food and Drug Administration-established guidelines for LAL testing (“Guideline on Validation of the Limulus Amebocyte Lysate Test as an End-Product Endotoxin Test for Human and Animal Parenteral Drugs, Biological Products, and Medical Devices”). The assays were conducted in triplicate, and DEP were analyzed in triplicate. Results were compared to USP <85> Bacterial Endotoxin Test and Lonza’s Quality System requirements. Testing was performed according to standard operating procedure 162.6.

To rule out the possibility of bacterial or fungal contamination of DEP, complete culture media (RPMI 1640 + 10% pooled human serum + L-Gln) and complete culture media containing DEP (100 μg/ml) were examined on blood brain heart infusion and inhibitory mode agar plates as well as on sheep blood and chocolate agar plates (all from Voigt Global Distribution, Lawrence, KS) at the Department of Microbiology at Robert Wood Johnson University Hospital in New Brunswick, NJ.
Preparation of PBMC

PBMC were isolated from whole heparinized venous blood by Ficoll gradient centrifugation (66). Briefly, whole blood was diluted 1:1 with L-glutamine supplemented RPMI 1640 medium and subjected to gradient density centrifugation (1200 rpm, 45 min, 21°C) over Ficoll-Paque. Following removal from the interface, PBMC were washed three times in RPMI 1640, resuspended in complete culture medium, counted, and adjusted at required concentrations. Viability of PBMC was 98–100% by trypan blue exclusion in all experiments.

Preparation of M. tuberculosis for in vitro infection

Suspensions of M. tuberculosis (avirulent M. tuberculosis strain H37Ra) were prepared in Middlebrook 7H9 broth medium supplemented with 10% albumin dextrose catalase and 0.2% glycerol. After a 21-d incubation period at 37°C on an orbital shaker, M. tuberculosis stock suspensions were harvested, aliquoted, and stored at −86°C until use. The concentrations of the M. tuberculosis stock suspensions were confirmed by assessing CFU numbers from serial culture dilutions after 21 d of incubation on 7H10 solid agar plates. For PBMC infection experiments, single-cell suspensions of M. tuberculosis were prepared as follows: frozen M. tuberculosis stock was thawed, centrifuged for 5 min at 6000 × g, and resuspended in complete culture medium. To generate single-bacterial-cell suspensions, M. tuberculosis stock suspensions were depleted by vortexing for 5 min in the presence of five sterile 3-mm glass beads. Remaining M. tuberculosis clumps were removed with additional centrifugation at 3500 rpm for 10 min. Remaining M. tuberculosis was then washed three times with DMEM containing 10% FCS, 2 mM L-glutamine supplemented RPMI 1640, resuspended in complete culture medium, counted, and subjected to gradient density centrifugation (1200 rpm, 45 min, 21°C) over Ficoll-Paque. Remaining M. tuberculosis was then washed three times with PBS and PBS-Tween 0.05% followed by the addition of 0.2% glycerol.

Preparation of enriched CD14+CD3- blood monocytes for microscopy

Enriched CD14+CD3- peripheral blood monocytes were generated by negative selection through immunomagnetic depletion of nonmonocytes using Miltenyi M-120 MicroBeads. CD3+CD14+ monocytes were isolated by negative selection through immunomagnetic depletion of nonmonocytes (bacterial monocytes), and monocytes were assumed to constitute 10% of PBMC. Concentrations of frozen M. tuberculosis stock suspensions were confirmed after thawing of aliquots in each experiment by CFU assays.

Preparation of enriched CD14+CD3- blood monocytes for ELISPOT assays

Enriched CD14+CD3- blood monocytes were generated by negative selection through immunomagnetic depletion of nonmonocytes using Miltenyi M-120 MicroBeads. CD3+CD14+ monocytes were isolated by negative selection through immunomagnetic depletion of nonmonocytes (bacterial monocytes), and monocytes were assumed to constitute 10% of PBMC. Concentrations of frozen M. tuberculosis stock suspensions were confirmed after thawing of aliquots in each experiment by CFU assays.

ELISPOT assays

ELISPOT assays are highly sensitive tools for quantitation of frequencies of cytokine-producing cells and the semiquantitative assessment of their cytokine output (spot size) (67). In the current study, ELISPOT assays were performed to enumerate frequencies of IL-1β-, IL-4-, IL-6-, IL-10-, IFN-γ-, and TNF-α-producing cells. PBMC were stimulated simultaneously with DEP (0, 1, 10, 50, and 100 µg/ml) and M. tuberculosis at MOI 1 or MOI 10, or PPD (10 µg/ml), or LPS (100 ng/ml), or complete culture medium (control) in duplicate wells and incubated at 37°C in 5% CO2. PBMC and stimuli were cultured in a total volume of 200 µl well in high protein-binding 96-well plates coated with appropriate anti-human primary capture Abs. Optimal densities of PBMC per well for the detection of the various cytokines were as follows: 20,000 for IL-6 and TNF-α, 100,000 for IL-1β, and 200,000 for IL-4, IL-10, and IFN-γ. Optimal short-term cell culture incubation periods for the ELISPOT assays were 4 h for TNF-α, 20 h for IL-1β, and overnight at 4°C for all other cytokines. Following washing, cytokine spots were visualized using peroxidase-conjugated goat anti-mouse, HRP, and chromogen 1% 3-amino-9-ethylcarbazole. After washing, plates were dried and scanned, images acquired, and frequencies of cytokine-produc-
FIGURE 2. TEM of DEP and M. tuberculosis uptake in human peripheral blood monocytes. The micrographs ([A] and [B]) overviews of two independent cells; (C) details of the area surrounded by rectangle in (A); (D) intracellular compartment containing both DEP and three M. tuberculosis show uptake of DEP (asterisks) and M. tuberculosis (arrows) within the same transects of monocytes, providing evidence of presence of DEP and M. tuberculosis within the same cell. Enriched peripheral blood CD14+CD3− monocytes were cultured with DEP (10 μg/ml) and M. tuberculosis (MOI 10) for 24 h and processed as described in Materials and Methods. Scale bars and direct magnifications are included in the figure.

Preparation of RNA from PBMC and generation of cDNA

PBMC cultures were incubated at 37°C in 5% CO2 and stimulated with DEP, M. tuberculosis, M. tuberculosis plus DEP simultaneously, M. tuberculosis following a 20-h DEP prestimulation, and M. tuberculosis following a 20-h LPS prestimulation or left untreated (control) in six-well plates (Falcon; BD Biosciences) at concentrations of 1.5 × 106 cells/ml for 24 h. Total RNA was extracted from unstimulated (in complete culture medium only) or stimulated PBMC using the RNeasy mini kit (Qiagen), and DNA was removed from RNA samples by RNase-free DNase treatment (Qiagen). Concentration and purity of RNA were determined using a Nanodrop spectrophotometer (Nanodrop Technologies, Wilmington, DE). Total RNA (400 ng) was reverse transcribed into cDNA with RT2 Master mix (550 ng) according to the manufacturer’s protocol.

Pathway-specific qRT-PCR arrays

Human Th1-Th2-Th3 and TLR pathway-specific qRT-PCR arrays were used to assess mRNA expression in unstimulated and unstimulated PBMC samples. First-strand cDNA diluted in water (102 μl) was mixed with 2× RT-qPCR master mix (550 μl; SuperArray Bioscience), and the total reaction volume was adjusted to 1100 μl with RNase-free water. Sample cocktails (10 μl/well) were loaded onto 384-well plates. A two-step cycling program consisting of 1 cycle of 95°C for 10 min, 40 cycles of 95°C for 15 s, and 60°C for 1 min was performed with an ABI 7900HT (Applied Biosystems) in a two-step cycling program followed by dissociation curve as described in the section above was used.

Statistical analysis

We performed statistical analyses to test the following two hypotheses: the first was whether there was an effect for DEP within each stimulant; the second was whether each DEP dose affected M. tuberculosis MOI 1, MOI 10, and PPD stimulation effects differentially. Histograms of the values were examined for normality and other model assumptions. Linear mixed-effects models were employed with cytokine counts measured as a response and DEP dose as the predictor. Because all DEP doses were applied to each PBMC sample, a random effect for subject was included to account for the repeated measures. Also, based on observed heterogeneity, the variance of the cytokine counts was allowed to vary by DEP dose. The analyses were then stratified by each stimulant (M. tuberculosis MOI 1, M. tuberculosis MOI 10, PPD, and LPS). All the statistical analyses were conducted using the SAS 9.1 for Windows (SAS Institute).

Results

Characterization of DEP

DEP used in this study consist of a carbonaceous core to which organic chemicals such as polycyclic aromatic hydrocarbons (PAH), nitro derivatives of PAH, oxygenated derivatives of PAH (ketones, quinines, and diones), heterocyclic compounds, aldehydes, and aliphatic hydrocarbons are adsorbed (65, 69–71). To determine any potential endotoxin contamination of the DEP used in this study,

<table>
<thead>
<tr>
<th>DEP Concentration (μg/ml)</th>
<th>Annexin V FITC/Propidium Iodide Positive (Mean %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>3.2 6.3 23.6</td>
</tr>
<tr>
<td>0.1</td>
<td>4.3 7.2 24.0</td>
</tr>
<tr>
<td>1</td>
<td>4.9 6.1 22.4</td>
</tr>
<tr>
<td>10</td>
<td>5.4 6.8 24.9</td>
</tr>
<tr>
<td>100</td>
<td>8.5 31.4 40.6</td>
</tr>
</tbody>
</table>

Mean proportions of PBMC from healthy subjects (n = 5) showing positivity for Annexin V and PI by flow cytometry.

Gene expression data obtained by qRT-PCR arrays were validated by real-time PCR with gene-specific forward and reverse primer pairs. Forward and reverse primers were designed using Oligoperfect software (Invitrogen), and cDNA was generated from RNA from PBMC using TaqMan reverse transcription reagents (Applied Biosystems) according to the manufacturer’s protocols. Briefly, 400 ng total RNA was used for the generation of cDNA in a 25-μl reaction (1× reverse transcription buffer, 5.5 mM MgCl2, 500 mM 2′-deoxynucleoside 5′-triphosphate, 2.5 μM random hexamer, 0.4 U/μl RNase inhibitor, 1.25 U/μl Multiscribe Reverse Transcriptase, 0.4 M forward primer, and 0.4 M reverse primer). Thermal cycling parameters of the reverse transcription reaction were 25°C for 10 min, 48°C for 10 min, and 95°C for 5 min. Real-time PCR was performed with Power SYBR Green PCR master mix in an ABI 7900HT (Applied Biosystems). A two-step cycling program by dissocation curve as described in the section above was used.
a quantitative kinetic chromogenic LAL method (KQCL) was employed (see Materials and Methods). Endotoxin content of culture media without and with DEP (100 μg/ml) was <0.05 EU/ml, indicating that no additional endotoxin was introduced into the PBMC cultures as a consequence of stimulation with DEP. To further rule out microbial contaminants in the DEP, complete culture media without and with DEP (100 μg/ml) were cultured for bacterial and fungal growth. No bacterial or fungal growth was detected in either of the samples after 1 wk and 1 mo, respectively.

**DEP and M. tuberculosis uptake by CD14+CD3− peripheral blood monocytes**

*M. tuberculosis* is an intracellular bacterium that is taken up in monocytes, macrophages, and dendritic cells by phagocytosis, a prerequisite for the initiation of innate and subsequent adaptive host immune responses. Because human mononuclear phagocytes have been demonstrated to take up DEP (72), uptake of DEP and *M. tuberculosis* was assessed in enriched peripheral CD14+CD3− monocytes. Cytospin analysis of monocytes stimulated with DEP and *M. tuberculosis* (Fig. 1) provided initial evidence for a concurrent cellular uptake of DEP and *M. tuberculosis* into the boundaries of the cells. Subsequent studies by TEM confirmed presence of DEP and *M. tuberculosis* within the same transect of the monocytes (Fig. 2), implying the possibility of functional interactions of DEP and *M. tuberculosis* within the same cells.

**Induction of apoptosis and necrosis by DEP**

To examine the extent of DEP-induced toxicity in PBMC, we first assessed apoptosis and necrosis using a flow cytometry-based Annexin V/PI apoptosis detection assay. PBMC from healthy

![Simultaneous stimulation with DEP alters pathogen-induced cytokine production. Frequencies of cytokine-producing PBMC (IFN-γ [n = 20 subjects], TNF-α [n = 18 subjects], IL-6 [n = 15 subjects], IL-10 [n = 20 subjects, data for DEP 50 μg/ml from n = 18 only], and IL-1β [n = 5 subjects]) stimulated with DEP alone (0, 1, 10, 50, and 100 μg/ml) or the stimuli *M. tuberculosis* (M.tb) MOI 1, MOI 10, and PPD (10 μg/ml) in presence (1, 10, 50, and 100 μg/ml) or absence (0 μg/ml) of DEP, were measured by ELISPOT assay. Cytokine data are arranged horizontally for each of the stimuli, which are arranged vertically. Frequencies of cytokine-producing cells (y-axes) are plotted as a function of DEP concentration in micrograms per milliliter (x-axes shown below the IL-1β panel only). Data are presented in box plots showing from top to bottom: the maximum value (black dot), the 95th (whisker), 75th (top of box plot), 50th (median, dotted line), mean (solid line in middle of box plot), and 25th (bottom of box plot), and 5th (whisker) percentiles and the minimum value (black dot). The p values are shown on top of box plots in which differences in cytokine expression between no DEP and different doses of DEP were regarded to be statistically significant (p ≤ 0.05). Additional statistical comparisons are shown in Supplemental Table I.
subjects ($n = 5$) were exposed to DEP for 2, 24, and 72 h at final concentrations of 0.1–100 µg/ml and proportions of mononuclear cells undergoing combined apoptosis (Annexin V positive) and necrosis (PI positive) determined. Stimulation of PBMC with DEP for 2 h did not result in significant toxicity (combined apoptosis and necrosis) at any of the concentrations of DEP (0–100 µg/ml) tested. Furthermore, no significant cell toxicity with DEP stimulation in the dose range from 0.1–10 µg/ml relative to unstimulated cells was observed at any of the time points examined. Combined apoptosis and necrosis levels were increased only by 20–25% above the spontaneous level in PBMC exposed to DEP at a concentration of 100 µg/ml (Table I). It is worth noting that even at the highest concentration of DEP examined, ∼90, 70, and 60% of cells were viable at 2, 24, and 72 h, respectively. Based on these observations, we chose the DEP concentrations and PBMC stimulation durations for the subsequent ELISPOT assay and mRNA expression studies.

**Simultaneous exposure to DEP and stimuli alters**

*M. tuberculosis*-induced cytokine production

ELISPOT assays were used to assess frequencies of cytokine-producing PBMC as a measure of cytokine protein production. Representative images of cytokine-producing PBMC in developed wells are shown in Supplemental Fig. 1. To assess whether DEP alter antimycobacterial host immune responses, PBMC were stimulated with DEP alone or *M. tuberculosis* MOI 1, MOI 10, and PPD in presence of DEP at 0 (no DEP control), 1, 10, 50, and 100 µg/ml.

Stimulation of PBMC with DEP alone induced negligible amounts of IFN-$\gamma$ (Fig. 3, leftmost column). Frequencies of IFN-$\gamma$-producing cells in PBMC stimulated with *M. tuberculosis* MOI 1 and MOI 10 were reduced significantly at DEP concentrations of ≥10 µg/ml relative to DEP 0 µg/ml. As expected, frequencies of PBMC producing all cytokines tested (IFN-$\gamma$, TNF-$\alpha$, IL-1$\beta$, IL-6, and IL-10) were greater upon stimulation with *M. tuberculosis* MOI 10 than with MOI 1 (DEP 0) (Fig. 3, second and third columns from the left). Following PPD stimulation, IFN-$\gamma$ production was significantly decreased at DEP concentrations of ≥50 µg/ml (Fig. 3, IFN-$\gamma$). Interestingly, suppressive effects of DEP were also observed on the per-cell IFN-$\gamma$ output, as the size of the IFN-$\gamma$ spots (diameters) decreased with increasing DEP concentrations (data not shown).

TNF-$\alpha$ production was barely detectable upon stimulation of PBMC with DEP alone (Fig. 3, leftmost column). TNF-$\alpha$ production by PBMC stimulated with DEP at a concentration of ≥50 µg/ml was significantly lower ($p \leq 0.05$) compared with that of unstimulated PBMC (culture media). TNF-$\alpha$ production upon stimulation with *M. tuberculosis* (MOI 1 and MOI 10) and PPD was significantly decreased at DEP concentrations of ≥50 and ≥10 µg/ml, respectively (Fig. 3, TNF-$\alpha$ panel).

As shown for IFN-$\gamma$ and TNF-$\alpha$, *M. tuberculosis* and PPD-induced IL-1$\beta$ and IL-6 production was significantly decreased by DEP in a dose-dependent manner (Fig. 3, IL-1$\beta$ and IL-6 panels). Unlike IFN-$\gamma$ and TNF-$\alpha$, however, stimulation of PBMC with DEP alone induced significant levels of cytokine production at concentrations of 1 and 10 µg/ml for IL-6 and 10 µg/ml for IL-1$\beta$ compared with PBMC in culture media (Fig. 3, leftmost column). It is worth noting that the DEP-mediated suppression of cytokine production was most striking for IFN-$\gamma$ and IL-1$\beta$, both of which are crucial for *M. tuberculosis*-induced host immune responses (21, 28–30, 73).

We also investigated the effect of DEP on the production of the immunoregulatory cytokines IL-4 and IL-10, which have suppressive effects on innate and acquired immune responses to intracellular pathogens including *M. tuberculosis* (74–77). In contrast to the findings with IFN-$\gamma$, TNF-$\alpha$, IL-1$\beta$, and IL-6, IL-10 production was significantly decreased with increasing DEP concentrations (data not shown).
production in response to *M. tuberculosis* or PPD stimulation remained unchanged in presence of DEP concentrations up to 50 µg/ml relative to unstimulated PBMC (DEP 0) (Fig. 3, IL-10). No significant IL-10 production was detected when PBMC were stimulated with DEP alone (Fig. 3, leftmost column). No IL-4 production by PBMC was detected following stimulation with DEP, *M. tuberculosis*, or PPD (data not shown).

To examine whether DEP effects on *M. tuberculosis*-induced cytokine expression were similar to those induced by LPS, a TLR4 agonist, we determined frequencies of IFN-γ–, TNF-α–, IL-1β–, IFN-γ, and TNF-α–producing PBMC by ELISPOT assay, and frequencies of cytokine-producing cells (y-axes) are plotted as a function of DEP concentration in micrograms per milliliter (x-axes shown below the TNF-α panel only). Mean frequencies ± SEM are shown.
IL-6, and IL-10–producing cells in PBMC stimulated with LPS in presence and absence of DEP. Effects of DEP on frequencies of IFN-γ–, TNF-α–, IL-1β–, IL-6–, and IL-10–producing PBMC following stimulation with LPS were comparable to effects of DEP following stimulation with *M. tuberculosis* or PPD (Fig. 4). Production of all cytokines was completely abrogated at the DEP concentration 100 μg/ml (Figs. 3, 4). It is worth noting that DEP-mediated apoptosis and necrosis of PBMC cannot account for the observed inhibition of cytokine production because 60–90% of PBMC were viable even after 72 h of incubation with DEP (Table I).

Taken together, these results indicate that stimulation of PBMC with DEP inhibited *M. tuberculosis*, PPD, and LPS-induced production of IFN-γ, TNF-α, IL-1β, and IL-6 in a concentration-dependent manner. On the contrary, IL-10 production remained unchanged at all but the highest concentration of DEP examined. The observed suppression of *M. tuberculosis*–induced production of proinflammatory cytokines together with the concomitantly unaltered IL-10 production strongly suggest that DEP interfere with protective host antimycobacterial immunity.

Particle versus chemical effects of DEP

To determine whether the observed effects of DEP were due to its particle nature or the adsorbed surface chemical species (65, 69–71), we assessed the effects of CB as a chemically inert particle control on *M. tuberculosis*–induced production of IFN-γ, TNF-α, and IL-10, in parallel with DEP. CB represents the chemically inert inner carbon core of DEP (78) and is devoid of surface chemical species. In contrast to the findings following DEP exposure, *M. tuberculosis*– or PPD-induced production of IFN-γ, TNF-α, and IL-10 did not change with increasing CB concentrations (0, 1, 10, and 50 μg/ml) (Fig. 5). Taken together, these results suggest that the alterations in cytokine production observed in PBMC stimulated with DEP (Figs. 3, 4) are in large part due to adsorbed surface chemical species (see Characterization of DEP) rather than the inert carbon core of DEP.

**DEP alters *M. tuberculosis*-induced mRNA expression profiles**

A broad spectrum of gene targets was studied using Th1-Th2-Th3 and TLR pathway-specific gene expression qRT-PCR arrays to determine the effect of DEP on *M. tuberculosis*-induced host gene expression.

### M. tuberculosis–induced mRNA expression

In a first step, mRNA expression profiles of PBMC stimulated with *M. tuberculosis* MOI 10 were compared with that of unstimulated PBMC (cultured in complete medium). Levels of *M. tuberculosis*–induced mRNAs (increased or decreased by ≥2-fold; *p* ≤ 0.01) relative to unstimulated cells were evaluated with Th1-Th2-Th3 (Fig. 6A, 6B) and TLR pathway-specific (Fig. 6C, 6D) qRT-PCR arrays. Using human Th1-Th2-Th3 qRT-PCR arrays, a significant increase (100–1000-fold) of mRNAs encoding Th1 cytokines IFNG, IL12B, and CSF2 relative to untreated controls was observed (Fig. 6A). In addition, the level of mRNA encoding Th1 cytokines and related genes IL12RB2, IL2RA, IFN1, IL18R1, SOCS1, STAT4, TBX21, and TNFA was increased by 2–50-fold upon *M. tuberculosis* stimulation (Fig. 6A). The expression of a smaller number of Th2 cytokines and related genes (e.g., CCL7, IL10, IL13, IL5, ICOS, and NFAT2) was increased by 2–50-fold in *M. tuberculosis*-stimulated cells relative to unstimulated PBMC. Increased expression (2- to ≥2000-fold) of genes encoding CD4 T cell markers CD40LG, CTLA4, FASG, IL6, IL7, LAG3, TNFRSF8, TNFRSF9, and TNFRSF4 was observed in *M. tuberculosis*-stimulated PBMC (Fig. 6A), whereas IL18, TLR4, IL13RA1, IL1R2, CD4, and CD86 genes were downregulated by 2–30-fold following *M. tuberculosis* stimulation (Fig. 6B).

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**FIGURE 7.** DEP effects on *M. tuberculosis* (M.tb)-induced Th1-Th2-Th3 pathway-specific mRNA expression. Depicted on y-axes are statistically significant (*p* ≤ 0.01) mean fold changes (≥2-fold) ± SEM in mRNA expression levels of in vitro-stimulated PBMC from healthy subjects (*n* = 11). (A) The DEP-mediated (10 μg/ml) mRNA expression in PBMC relative to mRNA expression levels of unstimulated PBMC in culture medium (set as 0-line). (B) mRNA expression levels from simultaneous DEP (10 μg/ml) and *M. tuberculosis* (MOI 10) stimulation of PBMC compared with mRNA expression levels from PBMC stimulated with *M. tuberculosis* alone (set as 0-line). (C) The effect of DEP prestimulation on *M. tuberculosis*-induced mRNA expression. PBMC were prestimulated with DEP (10 μg/ml) for 20 h and subsequently stimulated with *M. tuberculosis* at MOI 10 for an additional 24 h. The mRNA expression levels of DEP prestimulated and subsequently *M. tuberculosis*-stimulated PBMC were compared with that of PBMC stimulated with *M. tuberculosis* alone (set as 0-line).
Using human TLR signaling pathway-specific qRT-PCR arrays, upregulation of NF-κB target genes CCL2, CSF2, CSF3, IFNA1, IFNB1, IFNG, IL1A, IL1B, IL6, IL8, IL10, TNF, NFKB1, and NFKB1A and of genes downstream of IRF pathway, CXCL10, IFNA1, IFNB1, IFNG, and IRF1, were observed in M. tuberculosis-stimulated PBMC relative to unstimulated PBMC (Fig. 6C). In addition to M. tuberculosis-mediated upregulation (Fig. 6C), downregulation of several genes related to TLR-mediated signal transduction pathway by M. tuberculosis was observed (Fig. 6D).

**DEP-mediated modification of M. tuberculosis-induced mRNA expression.** Engagement of TLRs by M. tuberculosis ligands initiates signaling pathways that ultimately activate transcription factor NF-κB, leading to subsequent release of cytokines and chemokines (38, 41, 42, 73). The effects of DEP were studied upon simultaneous stimulation of PBMC with DEP and M. tuberculosis and upon a 20-h prestimulation of PBMC with DEP followed by stimulation with M. tuberculosis. The prestimulation with DEP simulated the sequential exposures to DEP and M. tuberculosis that may occur in real life. Thus, PBMC were stimulated with DEP, M. tuberculosis plus DEP (simultaneous), M. tuberculosis following DEP (prestimulation), and M. tuberculosis alone or left unstimulated. The mRNA expression profile of PBMC that had been stimulated with M. tuberculosis following DEP pre-exposure was considerably different from that of PBMC stimulated with M. tuberculosis plus DEP simultaneously (compare Fig. 7B, 7C and Fig. 8B, 8C, Table II). The mRNA fold changes plotted on y-axes in Figs. 7B, 7C, 8B, and 8C represent a ratio of the mRNA levels of M. tuberculosis plus DEP and M. tuberculosis alone. Thus, all DEP-mediated mRNA expression changes are represented relative to the mRNA expression levels in PBMC stimulated with M. tuberculosis alone (set as baseline). IFNG, TLR4, and CXCL10 mRNAs decreased to comparable levels during both the simultaneous stimulation (Figs. 7B, 8B, Table II) and the 20-h prestimulation with DEP (Figs. 7C, 8C, Table II). DEP prestimulation followed by M. tuberculosis infection, however, led to the downregulation of a larger number of M. tuberculosis-induced genes with known involvement in antimycobacterial immunity such as costimulatory molecules, type I and type II IFNs, proinflammatory cytokines, chemokines, chemokine receptors, and TLRs 3, 4, 7, and 10. In addition to the downregulation of the genes mentioned above, stimulation of PBMC with DEP upregulated the expression of several M. tuberculosis-induced genes including CSF2, IL1R1 during simultaneous, and CD86, CEBPB, IGSF6, IL13RA1, and IL18 during prestimulation by 2–4-fold (p ≤ 0.01) compared with PBMC stimulated with M. tuberculosis alone (Figs. 7B, 7C, 8B, 8C, Table II). Thus, prestimulation with DEP followed by M. tuberculosis stimulation alters M. tuberculosis-induced host responses more profoundly than simultaneous stimulation with DEP and M. tuberculosis as evidenced by the alteration of 4 and 5 mRNAs in Figs. 7B and 8B, respectively, compared with 23 and 17 mRNAs in Figs. 7C and 8C, respectively. Stimulation of PBMC with DEP alone altered the expression of only a small number of mRNAs (Figs. 7A, 8A). The expression of proinflammatory mediators...
The expression of the receptors
macrophages to subsequent secondary stimulation, which is char-
stimulation induces a state of cellular tolerance of monocytes/
M. tuberculosis
DEP prestimulation modified
responses in a manner comparable to that shown in other studies
following repeated LPS stimulation of cells (40, 79–81). Such LPS
responses against microbial infection.
mediated antimycobacterial immunity, DEP-induced suppression
downregulate the expression of a large number of genes including
arrays.
expression of eight genes altered by simultaneous and/or presti-
samples in the qRT-PCR arrays using primers listed in Fig. 9. The
expression analyses with qRT-PCR arrays were vali-
date by ±2-fold (*p < 0.01) relative to the mRNA expression levels of PBMC stimulated with M. tuberculosis alone are shown.

<table>
<thead>
<tr>
<th>Functional Group</th>
<th>Genes</th>
<th>DEP Stimulation Condition</th>
<th>Fold Change Relative to M. tuberculosis</th>
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<td>Cytokines and cytokine receptors</td>
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<td>Pre</td>
<td>↓ 9</td>
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<td>IL6</td>
<td>Pre</td>
<td>↓ 8.9–9.5</td>
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<td>Simultaneous and Pre</td>
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<tr>
<td></td>
<td>IFNA1</td>
<td>Pre</td>
<td>↑ 6.4</td>
</tr>
<tr>
<td></td>
<td>IFNB1</td>
<td>Pre</td>
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</tr>
<tr>
<td></td>
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</tr>
<tr>
<td></td>
<td>IL1B</td>
<td>Pre</td>
<td>↑ 4.7</td>
</tr>
<tr>
<td></td>
<td>IFNG</td>
<td>Simultaneous and Pre</td>
<td>↓ 2.4–2.7</td>
</tr>
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<td>IL7</td>
<td>Pre</td>
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</tr>
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<td></td>
<td>IL2RA</td>
<td>Pre</td>
<td>↓ 2.3</td>
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<tr>
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<td>Pre</td>
<td>↓ 2.2</td>
</tr>
<tr>
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<td>IL1R1</td>
<td>Simultaneous</td>
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</tr>
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<td></td>
<td>IL13RA</td>
<td>Pre</td>
<td>↑ 2.1</td>
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<tr>
<td>Chemokines and receptors</td>
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<td>Simultaneous and Pre</td>
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<td>CCR4</td>
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<tr>
<td>Transcription factors and inhibitors</td>
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<td></td>
<td>FOS</td>
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<td></td>
<td>GATA3</td>
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<td>↓ 2</td>
</tr>
<tr>
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<td>CEBPB</td>
<td>Pre</td>
<td>↓ 2</td>
</tr>
<tr>
<td>TLR</td>
<td>TLR7</td>
<td>Pre</td>
<td>↓ 4.7</td>
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<tr>
<td></td>
<td>TLR4</td>
<td>Pre</td>
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<tr>
<td></td>
<td>TLR3</td>
<td>Pre</td>
<td>↓ 3.1</td>
</tr>
<tr>
<td></td>
<td>TLR10</td>
<td>Pre</td>
<td>↓ 2.4</td>
</tr>
<tr>
<td></td>
<td>TLR5</td>
<td>Simultaneous</td>
<td>↑ 2.1</td>
</tr>
<tr>
<td>Costimulatory molecules, T cell activation</td>
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<td>Pre</td>
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<td></td>
<td>CD80</td>
<td>Pre</td>
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<td>Pre</td>
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<td></td>
<td>CTLA4</td>
<td>Pre</td>
<td>↓ 2.6</td>
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<td>ICOS</td>
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<td></td>
<td>TNFSF4</td>
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<tr>
<td></td>
<td>EIF2AK2</td>
<td>Pre</td>
<td>↑ 5.6</td>
</tr>
<tr>
<td>IFN-responsive effector</td>
<td>SPP1</td>
<td>Pre</td>
<td>↑ 2.1</td>
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<td>FASLG</td>
<td>Pre</td>
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<td></td>
<td>PTGS2</td>
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<tr>
<td></td>
<td>IGSF6</td>
<td>Pre</td>
<td>↑ 3.9</td>
</tr>
</tbody>
</table>

The mRNA levels of PBMC stimulated simultaneously with M. tuberculosis and DEP (simultaneous) and with M. tuberculosis following DEP
prestimulation (Pre) were compared with that of PBMC stimulated with M. tuberculosis alone. mRNA expression levels that were increased or decreased
including IL1R1, IL8, PTGS2, and TNF was increased, whereas
expression of the receptors CD4 and CD80 (B7-1) was decreased (Figs. 7A, 8A).

The gene expression analyses with qRT-PCR arrays were validated by real-time PCR of cDNAs generated from a subset of RNA samples in the qRT-PCR arrays using primers listed in Fig. 9. The
expression of eight genes altered by simultaneous and/or presti-
mulation with DEP is shown in Fig. 9. In general, real-time PCR
data were consistent with the data obtained from the qRT-PCR
arrays.

Simultaneous and prestimulation with DEP were found to
downregulate the expression of a large number of genes including
that of TLRs (Fig. 8C). Considering the importance of TLR-
mediated antimiycobacterial immunity, DEP-induced suppression of TLR pathways could potentially inhibit robust host immune responses against microbial infection.

Comparison of DEP and LPS prestimulation

DEP prestimulation modified M. tuberculosis-induced cytokine responses in a manner comparable to that shown in other studies
following repeated LPS stimulation of cells (40, 79–81). Such LPS
stimulation induces a state of cellular tolerance of monocytes/ macrophages to subsequent secondary stimulation, which is char-
acterized by impairment of IFN-γ, TNF-α, IL-1β, and IL-6 and
preservation of anti-inflammatory IL-10 production (80). Stimula-
tion with LPS, a potent ligand of TLR4 and CD14, leads to activ-
ation of the NF-κB pathway via MyD88-dependent and MyD88-
-independent signaling (47, 82). To examine whether LPS pre-
stimulation suppresses M. tuberculosis-induced gene expression, the
gene expression profile of PBMC infected with M. tuberculosis,
M. tuberculosis-
induced host immune genes

\[
\begin{align*}
\text{IL-6} & \quad \text{Il-6} \\
\text{TNF-α} & \quad \text{Il-1γ} \\
\text{IFN-γ} & \quad \text{IL-12β} \\
\text{IL-18} & \quad \text{IL-18R} \\
\text{IL-23} & \quad \text{IL-23R} \\
\text{IL-22} & \quad \text{IL-22R} \\
\text{IFN-β} & \quad \text{IFN-βR} \\
\text{IL-10} & \quad \text{IL-10R} \\
\end{align*}
\]

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\begin{align*}
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\text{TNF-α} & \quad \text{Il-1γ} \\
\text{IFN-γ} & \quad \text{IL-12β} \\
\text{IL-18} & \quad \text{IL-18R} \\
\text{IL-23} & \quad \text{IL-23R} \\
\text{IL-22} & \quad \text{IL-22R} \\
\text{IFN-β} & \quad \text{IFN-βR} \\
\text{IL-10} & \quad \text{IL-10R} \\
\end{align*}
\]
FIGURE 9. Validation of qRT-PCR array results. Total RNA (400 ng) from PBMC of a subset of donors (n = 4–6) was reverse transcribed to generate cDNA. Real-time PCR was performed and mRNA fold change in DEP-prestimulated and M. tuberculosis (M.tb) MOI 10-exposed PBMC relative to M. tuberculosis was evaluated. Means ± SEM are shown. IFNG: forward primer, 5'-GCATCCAAAAGATGGAGAC-3', reverse primer, 5'-TACTGGGATGCTTCCGACCT-3'; CXCL10: forward primer, 5'-TGAGCTCCAGGTACCTCTCTC-3', reverse primer, 5'-CTTGGATGCGCTTCATTG-3'; CD86: forward primer, 5'-GGTGCTGCTCCTCTGAGATT-3', reverse primer, 5'-TGA AGTCCAGGTCGCAACTG-3'; IL1RA: forward primer, 5'-GTATCCAGGTAGCCACCAATGA-3', reverse primer, 5'-CCAGGCTCTTGTGGCCAATAG-3'; IL6: forward primer, 5'-AGACACGCCACCTACCCCTTCA-3', reverse primer, 5'-CACCAGGCAAAGTCTCCTCAT-3'; CSF3: forward primer, 5'-ATAGGGGCTTTCCTCTA-3', reverse primer, 5'-GCCATTCCAGCTTCTCCAT-3'; PTGS2: forward primer, 5'-GGTGATGAGCTTGTTGCTCAG-3', reverse primer, 5'-GAAGGGATGTCAGTATGAG-3'; and LAG3: forward primer, 5'-TCCTGGTGATCTGGAACATG-3', reverse primer, 5'-TGGAGTCCACCTCACAAAGCAG-3'.

FIGURE 10. Effect of LPS prestimulation on M. tuberculosis (M.tb)-induced mRNA expression. Depicted in y-axes are statistically significant (p < 0.01) mean fold changes (±2-fold) ± SEM in mRNA expression levels of in vitro-stimulated PBMC from healthy subjects (n = 11 for Th1-Th2-Th3, n = 8 for TLR pathway-specific qRT-PCR arrays). PBMC were prestimulated with LPS (100 ng/ml) for 20 h and subsequently stimulated with M. tuberculosis at MOI 10 for an additional 24 h. Following RNA extraction and generation of cDNA, gene expression was assessed by qRT-PCR arrays. mRNA expression levels were compared with that from PBMC stimulated with M. tuberculosis alone (set as 0-line). (A) Effects of LPS prestimulation on M. tuberculosis-induced Th1-Th2-Th3 pathway-specific mRNA expression. (B) Effects of LPS prestimulation on M. tuberculosis-induced TLR pathway-specific mRNA expression.

Discussion
TB remains a major global public health concern that is aggravated by the HIV pandemic and emergence of drug-resistant M. tuberculosis strains. Deteriorating air quality from rapid industrial growth and urban traffic coincide with high prevalence and incidence rates of endemic TB in densely populated regions of low- and middle-income countries. Although epidemiological evidence suggests that exposure to air pollutants (e.g., silica, indoor pollution from fossil fuel combustion, cigarette smoke) increase the incidence of TB, only a few studies have assessed mechanisms that may underlie the effects of urban air PM2.5 on innate and adaptive human immune responses to mycobacteria (62, 72, 83). Because concomitant aerosol exposure to DEP and M. tuberculosis occur frequently in real-life situations, we hypothesized that DEP may alter human antimycobacterial host immunity. This study is the first, to our knowledge, to assess the effects of DEP, a major component of urban air pollution, on human primary immune cell responses to M. tuberculosis. We focused on the effects of DEP on M. tuberculosis-specific cytokine production and gene expression in PBMC, which, unlike studies in isolated stimulation compared with that in the presence of M. tuberculosis alone was consistent with the reduced IFN-γ, IL-1β, and IL-6 and increased or unchanged IL-10 protein expression in the ELISPOT assays (compare Figs. 7B, 7C, 8B, 8C with Fig. 3). DEP had no effect on M. tuberculosis-induced TNF-α mRNA expression (measured 24 h after stimulation), whereas DEP decreased M. tuberculosis-induced TNF-α protein expression (Fig. 3). This discrepancy in gene and protein expression is likely due to the transient and early expression of mRNA encoding TNF-α. Taken together, these cytokine production and mRNA expression data suggest that DEP exposure modulates and interferes (see hypothetical model in Fig. 11) with antimycobacterial immune responses.
Table III. Alteration of *M. tuberculosis*-induced genes by DEP and LPS

<table>
<thead>
<tr>
<th>M. tuberculosis-Induced Genes (Relative to <em>M. tuberculosis</em>)</th>
<th>M. tuberculosis-Induced Genes Not Altered by DEP Prestimulation (Relative to <em>M. tuberculosis</em>)</th>
<th>M. tuberculosis-Induced Genes Not Altered by LPS Prestimulation (Relative to <em>M. tuberculosis</em>)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCL5 ↓</td>
<td>CCL7 ↓</td>
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<tr>
<td>CCR4 ↓</td>
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<td>CD86 ↑</td>
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<td>FASLG ↓</td>
<td>MAP4K4 ↓</td>
<td>IL13</td>
</tr>
<tr>
<td>IFN↓</td>
<td>NFκB1A ↓</td>
<td>IL2A</td>
</tr>
<tr>
<td>IFNG ↓</td>
<td>NFκB1A ↓</td>
<td>IL10</td>
</tr>
<tr>
<td>IL18R1 ↓</td>
<td>NFκB1A ↓</td>
<td>IL7</td>
</tr>
<tr>
<td>IL23A ↓</td>
<td>NFκB1A ↓</td>
<td>IL8</td>
</tr>
<tr>
<td>LAG3 ↓</td>
<td>NFκB1A ↓</td>
<td>IL2A</td>
</tr>
<tr>
<td>TLR3 ↓</td>
<td>NFκB1A ↓</td>
<td>IL10</td>
</tr>
</tbody>
</table>

The mRNA levels of PBMC stimulated with *M. tuberculosis* following DEP or LPS prestimulation were compared with that of PBMC stimulated with *M. tuberculosis* alone. Increased or decreased (≥2-fold; *p* ≤ 0.01) mRNA expression levels relative to mRNA expression levels of *M. tuberculosis*-stimulated PBMC (set as 0-value) are shown. mRNAs altered by both DEP and LPS (columns 1 and 2) are in boldface.

Our studies show that DEP suppress *M. tuberculosis*, PPD, and LPS-induced IFN-γ, IL-6, IL-1β, and TNF-α protein production as determined by a dose-dependent reduction of the frequencies of cytokine-producing PBMC, whereas the frequencies of IL-10-producing PBMC remain unaltered except at the highest DEP dose (Figs. 3, 4). To gain further insights into the mechanisms of these immunosuppressive effects of DEP, we studied a broad spectrum of genes corresponding to TLRs and their associated pathways as well as Th1-Th2-Th3 cytokines, chemokines, and their receptors. The central finding of these studies is that DEP prestimulation impairs *M. tuberculosis*-induced effector mechanisms by suppressive NF-κB (IL6, IFNG, NFKB1, IFNβ1, IFNα1, IL1α, and CSF3) and IRF (IFNG, NFKB1, IFNβ1, and CXCL10) pathway target genes (Fig. 5C, Table II), thus modulating the production of IFN-γ, IL-6, IL-1β, and TNF-α. It has been shown that *M. tuberculosis*-mediated TLR activation leads to the recruitment of various adaptor proteins and the initiation of TLR signaling, such as the activation of adaptor protein MyD88-dependent and -independent pathways and the activation of various transcription fac-
tors including NF-κB and IRFs (38, 41–43, 46, 47). Our data suggest that DEP prestimulation suppresses these MyD88-dependent signaling pathways, as indicated by the downregulation of several genes such as IL1A, IL6, and PTGS2 (Fig. 8B). In addition, DEP may also affect MyD88-independent pathways (91), as indicated by the inhibition of the expression of M. tuberculosis-induced type I IFN mRNAs (IFNA1 and IFNB1) following DEP prestimulation. Curiously, the observed increase in expression of proinflammatory mediators IL8, PTGS2, and TNF and decrease in expression of costimulatory receptors CTLA4 and CD80 (B7-1) in PBMC stimulated with DEP alone (Fig. 8A) coincide with that in monocyte-derived immature dendritic cells following in vitro exposure to ambient PM (93). Further, our finding of suppressed expression of M. tuberculosis-induced TLR3, TLR4, TLR7, and TLR10 mRNA following DEP prestimulation (Fig. 8C) is consistent with the reported suppression of expression and function of TLRs (TLR2 and TLR4) in monocyte-derived dendritic cells exposed to ambient PM (93, 94). In addition, suppression of TLR signaling may also result from the reduced expression and function of signaling intermediates downstream of the TLRs, as indicated by recent studies (80, 95, 96).

Based on the presented findings and published evidence, we propose a hypothetical model of DEP-mediated suppression of M. tuberculosis-induced TLR signaling (Fig. 11) in which DEP prestimulation of PBMC suppresses both MyD88-dependent and -independent M. tuberculosis-induced signaling pathways, leading to a hyporesponsive state upon subsequent exposure to M. tuberculosis. Such a DEP-induced hyporesponsive state is reminiscent of the effects of tobacco smoke on alveolar macrophages, which have been shown to become refractory to challenge with TLR2 and TLR4 agonists compared with alveolar macrophages of nonsmokers (97). The DEP-induced hyporesponsive state toward M. tuberculosis-stimulation described in this study also shows similarities to LPS-induced tolerance (40, 79–81), a state characterized by a transitory hyporesponsiveness to secondary stimulation following repeated preceding LPS stimulation. It is interesting in this context that in contrast to our DEP prestimulation experiments, simultaneous stimulation of PBMC with DEP and M. tuberculosis affected the expression of fewer M. tuberculosis-induced genes (Figs. 7B, 8B). DEP prestimulation thus appears to be a prerequisite for the observed induction of the cellular hyporesponsiveness to subsequent M. tuberculosis-induced cellular responses. These studies are of relevance, as periods of respiratory exposure to air pollutants may precede subsequent M. tuberculosis infection events frequently in real-life situations.

DEP stimulation has also been linked to a potent Th2 adjuvant activity and induction of allergic conditions (98–102) and increased Th2 cytokine responses (103). These observations are

![FIGURE 11.](http://www.jimmunol.org) A hypothetical representation of DEP-mediated suppression of M. tuberculosis-induced TLR signaling and effector functions. M. tuberculosis-derived ligands (lipopolysaccharide [LPS], 19-kDa protein, heat shock proteins 65 and 71, and M. tuberculosis DNA) bind to TLR2, -4, and -9 and recruit adapter protein MyD88 to the TLR receptor complex, which associates with IL-1R–associated kinase (IRAK) 1 and 4. TNFR-associated factor 6 (TRAF6) is also recruited to the receptor complex following phosphorylation of IRAK1 by IRAK4. Association of TRAF6–IRAK complex with TANK-binding kinase 1 (TBK1), leading to phosphorylation of IRF3. Phosphorylated IRF3 translocates to the nucleus and induces production of type I IFNs (47–49). DEP suppress M. tuberculosis-induced expression of target genes IL6, IL1A, and PTGS2 via MyD88-dependent and type I IFNs via MyD88-independent pathways. DEP-mediated downregulation of expression of TLR4, TLR7, and NF-κB target genes and type I IFNs is shown by downward arrows.
consistent with our findings of a DEP pretreatment-mediated downregulation of the expression of IFN-γ and type I IFNs (Figs. 7C, 8C), which promote Th1 differentiation, inhibit Th2 responses, and are required for robust Th1 responses.

The DEP-induced alterations described in this study suggest that exposure to DEP may promote *M. tuberculosis* survival and progressive *M. tuberculosis* pathogenesis. Indeed, intrapulmonary instillation of DEP in C57BL/6J mice followed by infection with attenuated bovine *M. tuberculosis* strain BCG results in a 5-fold increased pulmonary BCG load compared with mice exposed to BCG without prior DEP exposure and decreases the responsiveness of the murine alveolar macrophages to IFN-γ, IFN-γ–induced NO production, and the recovery of IFN-γ–producing lung lymphocytes (83). Similarly, long-term respiratory exposure of mice to DEP has been shown to result in increased pulmonary burden of *M. tuberculosis* (Kurono strain) following experimental aerogenic infection (62) and decrease in IL-1β, IL-12p40, IFN-γ, and inducible NO synthase production (62). Thus, the net effects of DEP-induced alterations of host immunity in rodent studies translate into decreased clearance of bacteria (63), further promoting progressive *M. tuberculosis* pathogenesis. Functional studies assessing the impact of the observed DEP effects on *M. tuberculosis* growth control in vitro (24), and in vivo human DEP exposure effects on antimycobacterial immunity are under way in our laboratory.

Future studies will have to determine whether the observed decrease in IFN-γ, IL-1β, and IL-6 protein and CXCL10 and IFNβ mRNA, the increased IL13RA1 mRNA, and the unchanged IL-10 protein expression is indicative of a phenotype switch from classically to alternatively activated macrophages (104, 105). Indeed, recent studies suggest that tolerance and alternative macrophage activation (M2 polarization) in human monocytes and monocyte-derived macrophages are related processes (106).

Previous studies have shown that suppressive effects of DEP on macrophages reside with polar aromatic hydrocarbons and resins containing fractions (107). Consistent with these observations, our studies point into the same direction as CB, an inert control for particle effects, did not suppress cytokine responses at the same concentrations of DEP tested (Fig. 5). This observation is also consistent with findings in rats in which DEP, but not CB exposure, decreased the pulmonary clearance of *L. monocytogenes* following intratracheal infection, suggesting that the exposure to the condensed organic and inorganic species adsorbed to the carbon core in DEP were responsible for the failing antimicrobial activity (65).

In conclusion, this study suggests that the DEP-mediated alterations of *M. tuberculosis*-induced NF-κB and IRF pathway target gene expression and a concomitant decrease in Th1 immunity may favor the progression of *M. tuberculosis* infection. This DEP-induced dysregulation of host immunity may thus result in increased susceptibility to *M. tuberculosis* infection and/or facilitate reactivation of latent *M. tuberculosis* infection and increased incidence of TB. Given the wide geographical scales of both air pollution and *M. tuberculosis* infections, the mechanistic insights from this study may have significant public health implications, particularly in cities and regions with high levels of air pollution and high TB prevalence rates.

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**Disclosures**

The authors have no financial conflicts of interest.

**References**


