Urban Airborne Particle Exposure Impairs Human Lung and Blood *Mycobacterium tuberculosis* Immunity

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Online Data Supplement

METHODS

Human Participants

Participants were residents of Iztapalapa (the most populous municipality in Mexico City) or the neighboring Iztacalco for a minimum of one year prior to their study inclusion. Of the 35 participants, six were TST positive (>10 mm, Mantoux method, 5U Turbesol, Canada), 29 TST-negative, two interferon gamma release assay (IGRA)-positive and 33 IGRA-negative. Participant numbers for each experiment are shown in the corresponding figures and their legends

Primary Human Immune Cells

PBMC were obtained by gradient centrifugation from whole heparinized peripheral venous blood using Lymphocyte Separation Medium (Bio Whittaker, LONZA) as

described previously (Supplementary Fig. 1). BAC from participants were obtained by bronchoalveolar lavage at the bronchoscopy suite of the INER using 150mL of sterile 0.9% sodium chloride solution in a segment of each right and left lung as described previously (Supplementary Fig.2).

PBMC and BAC (viability ≥93%, trypan blue exclusion) were suspended in complete culture medium [RPMI (BioWhittaker, Walkersville, MD, USA) + 10% pooled human AB serum (Valley Biomedical, Inc.) + 2 mM L-glutamine. By cytospin analysis (n= 32) BAC consisted of 91.5% AM (mean; max. 99.3%, min. 76.3%), 8.5% alveolar lymphocytes (AL, mean; max. 23.7%, min. 0.7%) and scarce neutrophils. In addition, the macrophage population in BAC (n=8) was also determined based on forward angle and side scatter in flow cytometry (Supplementary Fig.1) and consisted of 85.64% AM (mean; max. 89%, min. 81.9%) and 10.8% AL (mean; max. 16.4%, min. 5.7%).

Collection of Outdoor $PM_{2.5}$ in Mexico City and Preparation for *in vitro* Studies

$PM_{2.5}$ was sampled for 24 h on Mondays, Wednesdays, and Fridays of every week between May 2012 and April 2013 comprising of the three weather seasons cold-dry (CD, November - February), warm-dry (WD, March – May) and rainy (R, June – October) using a high volume sampler (TE6070V-2.5, Tisch Environmental, Inc.; Village of Cleves, OH, USA, airflow rate 1.13 m^3min^{-1}) to obtain adequate amounts of $PM_{2.5}$ on modified nitrocellulose membranes (Supplementary Figs. 3, 4). $PM_{2.5}$ was mechanically recovered from the membranes, pooled by month and stored in endotoxin-free glass vials at 4°C, in the dark, in a desiccator after assessing the total $PM_{2.5}$ monthly mass. A yearly pool and pools from each weather season were created using

monthly $PM_{2.5}$ aliquots according to the proportional contribution to the total $PM_{2.5}$ mass by the monthly PM samples. Immediately prior to *in vitro* exposure experiments, $PM_{2.5}$ was weighed on a precision balance Sartorius CPA225D (Göttingen, Germany), autoclaved (121°C, 15 min), and suspended in complete culture medium. Following sonication for 5 minutes (351OR-DTH; Branson, Danbury, CT, USA), $PM_{2.5}$ suspensions were diluted to final concentrations of 0.1, 1, 5 and 10 μg/ml.

Preparation of *M.tb* for *In vitro* Infections

M.tb H37Ra (ATCC 25177, Manassas, VA) was grown in Middlebrook 7H9 broth (BD Difco, Sparks, Maryland, USA) supplemented with BBL Middlebrook ADC Enrichment (10% albumin dextrose catalase, BD Difco) and 0.2% glycerol (Fisher Scientific, Fair Lawn, NJ, USA). After 21 days of incubation at 37°C in a shaking incubator, *M.tb* cultures were harvested, centrifuged at 2095 x *g* (3000 rpm) for 30 min, room temperature (RT) and suspended in Middlebrook 7H9 broth medium with 6% glycerol (Fisher Scientific, Fair Lawn, NJ). Mycobacteria were then aliquoted in cryotubes and stored at -70°C until use. Colony-forming unit (CFU) counts from serial dilutions on 7H10 agar plates (Sigma-Aldrich, Fluka. MO, USA) were performed to determine concentrations of the resulting *M.tb* stock. For *in vitro* infection experiments, frozen aliquots of *M.tb* H37Ra (ATCC 25177, Manassas, VA) were thawed, centrifuged for 8 minutes at 5220xg (8000 rpm), resuspended in complete medium, disaggregated by vortexing five minutes in the presence of five sterile 3-mm glass beads and centrifuged at 350 x g (1000 rpm) for 2 minutes to generate single-cell suspensions of *M.tb*.

Phagocytosis of *M.tb* by human AM

To assess phagocytosis of *M.tb*, BAC (2 x 10^4/well) were placed in 8-well chamber slides and exposed to $PM_{2.5}$ (1µg and 5µg/ml) for 18 h, subsequently infected with *M.tb* for 2h (37°C, 5% CO_2) at multiplicities of infection (MOI) of 0.1, 0.5, 1 and 5, washed (RPMI 3x) to remove extracellular *M.tb* and stained by Kinyoun (AlphaTec. WA. USA). Proportions of cells containing one or more intracellular *M.tb* were determined by bright field microscopy (1000x, oil immersion) on cytospin preparations in a total of 300 randomly selected AM from each study participant at each experimental condition.

Cell viability, necrosis and apoptosis

Briefly, 10^4 to 10^6 cells were resuspended in 1 ml of protein-free buffer, mixed with 1 µl of diluted staining reagent and incubated 30 minutes on ice. After washing once with 1 ml of PBS, cells were resuspended in 200 µl of PBS and viability analyzed by flow cytometry with fluorescence detection at 660 nm (FACSfusion; BD Biosciences, San Jose, CA). Differences in intensity between live and dead cell populations were typically greater than 50-fold. Cell viability was expressed in percentages of untreated cells (controls).

For MTS assay, BAC and PBMC were seeded in 96-well plates at densities of 200,000 and 50,000 cells/well, respectively, and incubated in a total volume of 200 µL in triplicate. Cells were incubated with $PM_{2.5}$ suspensions (0.1, 1, 10 and 25 µg/ml) and culture medium (viability control) for 24, 48 and 72 h (humidified, 37°C, 5% CO_2). Absorbance was quantified at 490 nm using a microplate reader (Multiskan Ascent, Thermo Electron Co., Shanghai, China). Cell viability was expressed as a percentage of

unexposed cells (control). For the detection of Apoptosis/Necrosis, 10^6 BAC or PBMC were placed in ultra-low attachment 24-well plates, exposed to $PM_{2.5}$ at final concentrations of 0.1, 1, 10 and 25 μg/ml and incubated for 24, 48 and 72h. Cells incubated in culture medium alone or in culture medium with 2 μM of staurosporine (apoptosis inducer) were included as negative and positive controls, respectively.

Assessment of *in vivo* PM burden in AM

The ranges used for the size and shape of the particles were: size 0 - ∞ μm and circularity 0.00 - 1.00, where 1 equals the perfect circle. Image adjustment was used with color threshold images analyzed with a range of (HSB): Hue 50-229, saturation 60 - 255, brightness 25 - 140. Thresholding method: default. Threshold color: network. Color Space: HSB. The Overlay Mask function was used to show particle contours in the superposition of images.

Cytokine ELISPOT Assays

BAC and PBMC were added into Multi Screen HTS 96-flat bottom well plates coated with appropriate antibodies in duplicate (200 μl/well) at optimal densities determined in earlier studies and based on frequency of spot-forming cells (IFN-γ: $2x10^4$ BAC, $2x10^5$ PBMC; TNF-α: $2x10^2$ BAC, $2x10^3$ PBMC; IL-1β: $2x10^4$ BAC, $2x10^5$ PBMC; IL-6: 10^3 BAC, $2x10^3$ PBMC), incubated in the presence of 0, 0.1, 1, 10, 25 μg/ml $PM_{2.5}$ and infected simultaneously with *M.tb* (MOI 1, 5, 10) or stimulated with PPD (10μg/ml), or 6 kDa early secretory antigenic target (ESAT-6, 10μg/ml) at 37°C in 5% CO_2 for 24 h (simultaneous exposure). Unexposed/uninfected control cells were incubated in

complete culture medium. Cytokine spot-forming cells (SFC) were enumerated with an ImmunoSpot Analyzer and Image Acquisition 4.5 Software (Cellular Technology Ltd, Cleveland, OH, USA) and frequencies of IL-1β, TNF-α, IL-6 and IFN-γ-producing cells calculated by averaging spot numbers from duplicate wells after background subtraction. Results are expressed as SFC/10^6 BAC or PBMC.

Western Blotting

To examine the activation of signaling pathways involved in the induction of cytokines, levels of P-IκB-α, P-STAT1, P-ERK1/2 and GAPDH (Cell Signaling Technologies, Danver, MA) were assessed by western blotting. BAC (7.5 x 10^5) and PBMC (2 x 10^6) were placed in 24-well plates and lysed by incubating for 30 min on ice in RIPA (radio immunoprecipitation assay) lysis buffer system (Santa Cruz Biotechnology, Dallas, TX) containing 2 mM PMSF, protease inhibitor cocktail, and 1 mM sodium orthovanadate and supernatants were collected following centrifugation at 10,000 g for 10 min at 4ºC. Protein lysates were analyzed on 10% SDS/PAGE in Tris-Glycine buffer and transferred onto PVDF membrane. After blocking in 5% milk in TBST (150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 0.05% Tween 20) for 1 hour at room temperature, membranes were incubated with appropriate antibody overnight at 4ºC. Following incubation, membranes were washed 3-4 times in TBST and incubated with HRP-conjugated secondary antibody at room temperature for 1 hour. After washing four times in TBST, protein specific bands were visualized with enhanced chemiluminescence substrate Western Lightning Plus-ECL (PerkinElmer, Waltham, MA).

Statistical analysis

Statistical analyses were performed using GraphPad Prism, version 5.0 (GraphPad Software Inc., San Diego, CA, USA). Types of statistical analysis used for experiments and conditions are indicated in the corresponding figure legends in the Results section.

SUPPLEMENTARY FIGURES

Supplementary Figure 1. Cytotoxic effect of $PM_{2.5}$. PBMC or BAC were exposed to PM and incubated for 24-72h to evaluate $PM_{2.5}$ exposure effects on membrane permeability and induction of apoptosis and necrosis. Permeability of PBMC and BAC membranes was evaluated by flow cytometry. Individual cells from an exemplary BAC sample were selected in SS-A and SS-H (A), lymphocyte and macrophage subpopulations identified (B) and single histogram plots generated to identify live lymphocytes and macrophages using LIVE/DEAD Fixable Far Red Dead Cell Stain (C, D).

Supplementary Figure 2. $PM_{2.5}$ does not affect *M.tb* phagocytosis by AM. AM were pre-exposed to $PM_{2.5}$ cold-dry, warm-dry, rainy weather seasons at 0 and 1 µg/ml (A) and 5 µg/ml (B) for 24 h, infected with *M.tb* MOI 0.1, 0.5, 1 and 5 for 2 h and proportions of AM with intracellular *M.tb* determined by bright field microscopy (1000x, oil immersion) on Kinyoun-stained cytospins assessing a total of 300 AM in each experimental condition.

Supplementary Figure 3. Effect of $PM_{2.5}$ on *M.tb*-induced Cytokine Production in BAC and PBMC. Optimal densities of BAC and PBMC (n=9) were exposed to $PM_{2.5}$ (0, 0.1, 1 and 10 µg/ml) and simultaneously infected with *M.tb* or left uninfected for 24h. Frequencies of IL-1β, TNF-α, IL-6 and IFN-γ spot-forming cells were evaluated by ELISpot assay. Images of representative IL-1β, TNF-α, IL-6 and IFN-γ-producing BAC showing cytokine spot formation and identification in *in vitro* PM-exposed and *M.tb*-infected cells for image analysis and automated counting (A). Frequencies of constitutively (unstimulated) IFN-γ, IL-1β, IL-6, and TNF-α-producing BAC (open circles) and PBMC (black circles) (B). Frequencies of IL-1β (C), TNF-α (D) IL-6 (E) and IFN-γ production (F) in response to *in vitro* PM exposure (left column panels) and *M.tb* infection (right column panels) by BAC (open circles) and PBMC (black circles). Each data point represents an individual participant and horizontal lines indicate medians. Asterisks indicate significant differences ($p \leq 0.05$, ANOVA Friedman test followed Dunn's multiple comparisons test using the GraphPad prism software for Mac OS X version 6.0) relative to $PM_{2.5}$-unexposed control cells.

Supplementary Figure 4. Activation of signaling pathways. BAC and PBMC from three participants (1-3) were infected with *M.tb* at MOI1 and 5 or stimulated with PPD or left unstimulated (constitutive) for 24h. Protein lysates were analyzed with SDS/PAGE followed by western blotting for Phospho-IκBα (Ser32/36), Phospho-STAT1 (Tyr701) and Phospho-Erk1/2 (Thr202/Tyr204), and GAPDH.

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