Pulmonary T cell activation in response to chronic particulate air pollution

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In this study, we extended some of these observations to further elucidate the nature of the innate and adaptive immune response to PM2.5 exposure by systematically investigating local immune response to PM2.5 in the lung, mediastinal lymph nodes (MLN), spleen, and in circulation. In addition, we aimed to determine how exposure of macrophages to PM2.5 or oxidized phospholipids affected T cell proliferation and how this compared with a benchmark such as lipopolysaccharide (LPS) activation.

The local immune response to diverse triggers is thought to be dependent, in part, on the local balance between stimulatory and inhibitory pathways mediated by effector CD4+ T lymphocytes (Teff), cytotoxic CD8+ T lymphocytes, FoxP3+ regulatory T cells (Tregs), and members of the innate immune system (macrophages and dendritic cells). Inmate immune cells may either modify the function of the T cells or themselves be modified by direct cell-cell or cytokine interactions with T cells. T cell subsets express specific patterns of chemokine receptors, resulting in migration capacities that depend on T cell function. For example, lung macrophages are an important source of the chemokine CXCL10, the cognate ligand to the T cell receptor chemokine (C-X-C motif) receptor 3 (CXCR3) (36). CD4+ Th1 cells are characterized by expression of CCR5 and CXCR3. Expression of the chemokine receptor, CXCR3, is important in regulating T cell trafficking as well as an indicator of Th1 CD4+ and CD8+ effector cell maturation and activation in response to various triggers (17). Using a mouse model of CXCR3 deficiency and a mouse expressing green fluorescent protein (GFP) under control of a Foxp3 promoter to track Tregs, we delineate the nature of the innate and adaptive immune response to chronic PM2.5 exposure. We hypothesized that PM2.5 exposure results in a proinflammatory Th1/Treg cell imbalance.

METHODS

Animals and exposure to PM2.5 ambient whole body inhalation. Twelve-week-old male C57BL/6, CXCR3 knockout (C57BL/6 background), Foxp3-GFP knockin mice (Foxp3gfp.KI; C57BL/6 background) were used in this study. C57BL/6 mice were obtained from Jackson Laboratories (Bar Harbor, ME). Chemokine receptor 3 knockout (CXCR3−/−) mice were provided by Satoskar’s group and bred in house. Foxp3gfp.KI mice were bred in house and were a generous gift of Mohamed Oukka and Vijay Kuchroo at Brigham and Women’s Hospital Harvard Medical School. These mice express GFP under control of a Foxp3 promoter (5). Mice were randomized to exposure (see below). The Committee on Use and Care of Animals from the Ohio State University (OSU) approved all experimental procedures.

Animal exposure and the monitoring of exposure atmosphere and ambient aerosol were performed as previously described using a versatile aerosol concentration enrichment system that was modified for long-term exposures (2, 22, 34). Briefly, mice were exposed to...
concentrated PM$_{2.5}$ or filtered air (FA) in a mobile trailer at The Ohio State University in Columbus, OH (OASIS-1 chamber, “Ohio air pollution exposure system for the interrogation of systemic effects”). The system is designed to expose mice to concentrated ambient particles of a size <2.5 μm (22, 34). FA-exposed mice received identical treatment with the exception of a high-efficiency particulate air filter (Pall Life Sciences, East Hills, NY) positioned in the inlet valve to remove PM$_{2.5}$ in the FA stream, as described previously (3). The exposure protocol included exposures for 6 h/day, 5 days/wk. The CXCR3$^{−/−}$ and wild-type (WT) mice were exposed for a total duration of 28 wk from May to December 2010. The Foxp3gfp.KI mice were exposed between August and February 2011 (24 wk).

Mice were removed from the exposure facility the evening (~16 h) before death by CO$_2$ asphyxiation.

**Analysis of PM$_{2.5}$ concentration in the exposure chamber.**
To calculate exposure mass concentrations of PM$_{2.5}$ in the exposure chambers, samples were collected on Teflon filters (Teflo, 37 mm, 2 μm pore; PALL Life Sciences, Ann Arbor, MI) and weighed before and after sampling in a temperature- and humidity-controlledweighing room using a Mettler Toledo Excellence Plus XP microbalance. Weight gains were used to calculate exposure concentrations. Concentrations of PM$_{2.5}$ were determined in a total of 13 chambers before and after sampling in a temperature- and humidity-controlled weigh-chambers, samples were collected on Teflon filters (Teflo, 37 mm, 2 μm pore; PALL Life Sciences, Ann Arbor, MI) and weighed before and after sampling in a temperature- and humidity-controlled weighing room using a Mettler Toledo Excellence Plus XP microbalance. Weight gains were used to calculate exposure concentrations. Concentrations of PM$_{2.5}$ were determined in a total of 13 chambers before and after sampling.

**Energy-dispersive X-ray fluorescence.** All PM$_{2.5}$ samples were analyzed for gravimetric and elemental analyses were collected on filters (see above). Analyses for major elements were followed by nondestructive X-ray fluorescence (model EX-6600-AF; Jordan Valley) using five secondary fluorescers (Si, Ti, Fe, Ge, and Mo) and spectral software. Elemental composition can be found in Table 1. Elemental composition of PM$_{2.5}$ from OASIS in Table 1.

**Liquid chromatography mass spectrometry of oxidized phospholipids.** Bronchoalveolar lavage (BAL) was performed using a blunt 22-gauge needle ligated into the trachea as previously described (25). A 1.0-ml Bronchoalveolar lavage (BAL) was performed using a blunt 22-gauge needle ligated into the trachea as previously described (25). A 1.0-ml lavage was collected and then slowly aspirated 10 times. The BAL fluid was centrifuged at 500 g for 5 min, and the supernatant was collected. Determination of oxidized phospholipids was performed as previously described (19). Briefly, lipids were extracted with chloroform-methanol (2:1 by volume) and separated on a Zorbax RX-SIL 4.6 × 250-mm HPLC column followed by infusion in a Applied Biosystems 3200 QTRAP mass spectrometer with TurbolonSpray source. The mass spectrometer was operated in multiple reactions monitoring (MRM) positive ionization mode. Specific monitor Q1/Q3 ion pairs were mass-to-charge (m/z) 782/184 for 1-palmitoyl-2-arachidonyl-sn-glycero-3-phosphorylcholine (PAPC), m/z 594/184 for 1-palmitoyl-2-(5-oxovaleryl)-sn-glycero-3-phosphocholine (POPC), and m/z 610/184 for 1-palmitoyl-2-glutaryl phosphatidylcholine (PGPC). All data were acquired and processed by Analyst software (version 1.4.2; Applied Biosystems, Foster City, CA).

**Lung digestion and processing for flow staining and CD4 cell isolation.** Lungs were washed in PBS and weighed before digestion. Lung tissue was cut into small pieces using a scalpel and then incubated in DMEM 10% FBS with collagenase II (2 mg/ml, C1764; Sigma Aldrich), collagenase I (2 mg/ml, C0130; Sigma Aldrich), and dispase I (0.04 mg/ml, D818; Sigma Aldrich) in a shaking water bath at 37°C and 140 rpm. After 45 min, tissue was completely dissociated; the suspension was filtered through a 100-μm cell strainer, pelleted, and resuspended in hypotonic red blood cell lysis buffer (420301; Biolegend, San Diego, CA) for 5 min. Suspension was diluted with PBS, pelleted, resuspended in fluorescence-activated cell sorter (FACS) buffer (PBS 5% FBS), and counted on a hemocytometer before staining for flow cytometry or CD4 cell isolation.

**Flow staining and cytometry.** Cells were stained according to the manufacturer’s instructions. Briefly, ~1 μg of antibody was used per million cells followed by incubation at 4°C for 15 min. Cells were subsequently washed with flow buffer. GFP-expressing cells were run immediately while other samples were resuspended in 1% neutral buffered formalin and analyzed by flow cytometry (BD FACS LSR II flow cytometer; Becton-Dickinson, San Jose, CA). Data were analyzed using BD FACS Diva software (Becton-Dickinson). Antibody concentration and fluorophore conjugate selection were optimized for each tissue based on cellular autofluorescence and appropriate staining specificity based on cellular size and granularity. Antibodies used were purchased from Biolegend as follows: CD11b-PE (clone M1/70), CD11c-APC (clone N418), Ly6-G/Ly6-C-PE/Cy7 (clone RB6–8C5), CD3-PE/Cy5 (clone 145–2C11), CD4-APC/Cy7 (clone RM4–5; lung staining only), CD4-PE (clone GK1.5; blood, spleen, MLN staining), CD8a-PE/Cy7 (clone 53–6.7), CD44-PE/Cy7 (clone IM7), CD62L-APC (clone MEL-14), and CCR7-PE/Cy5 (clone 4B12).

**Quantitative real-time PCR assessment of gene expression.** RNA was isolated using Absolutely RNA (Stratagene) according to the manufacturer’s instructions, including DNase digestion. RNA quality and quantity were assessed by agarose gel electrophoresis and a Nanodrop spectrophotometer. RNA was reverse transcribed according to the manufacturer’s instructions (Transcriptor; Roche) using random primers. PCR was performed using SYBR Green 1 master mix (Roche) on a Roche Lightcycler 480. All real-time reactions had the following profile conditions: 10 min hot start at 95°C followed by 45 cycles (94°C for 10 s, 60°C for 20 s, and 72°C for 20 s). Reference and target gene dilution standards were run in triplicate for each primer set to calculate PCR efficiency using the above profile. The concentration ratios were determined after PCR efficiency correction by relative quantification analysis using Lightcycler 480 software. All target genes were expressed as fold increase compared with control. Melting/dissociation curves were run on each plate to assure the production of one amplicon of the same melting temperature for each primer set. Real-time primers were designed to span genomic introns, thus avoiding amplification of genomic DNA possibly present in the RNA samples.

**Immunohistochemical staining of lung.** The postcaval lobe of the right lung was excised and fixed in 1.5% paraformaldehyde at 4°C for 16 h before death by CO$_2$ asphyxiation.

Table 1. Elemental constituents of PM$_{2.5}$ from OASIS in Columbus September 2010 to January 2011 by energy-dispersive X-ray fluorescence

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Units are ng/mg; n = 17 filters. PM$_{2.5}$, particulate matter less than 2.5 μm.
1 wk before paraffin embedding and sectioning. Immunostaining was provided by the immunohistochemistry core at OSU. The avidin-biotin complex method was used, including antigen retrieval (Dako-Cytomation Target Retrieval Solution), protein blocking (DakoCytomation Serum-Free Protein Block), and hydrogen peroxide treatment before incubation with primary antibody. Anti-mouse CD3 (dilution 1:800, catalog no. A0452; Dako) and anti-mouse F4/80 primary (dilution 1:100, catalog no. MCA497G; AbD Serotec) antibodies were used followed by a biotinylated secondary antibody. Chromagen (DAB) was applied followed by hematoxylin staining.

**Particulate matter for in vitro culture.** Two sources of PM$_{2.5}$ were used for in vitro experiments. Columbus, OH, PM$_{2.5}$ was isolated from randomly sampled filters during the time frame of the in vivo exposure. The filters were submerged in purified LPS-free water and sonicated in an ice bath, followed by lyophilization, weighing, and resuspension in PBS to 5 mg/ml. In addition, to address the generalizability of these findings, we also tested Chapel Hill, NC, PM$_{2.5}$ (Chapel Hill, July 2002, fine, 5 mg/ml), which was kindly provided by Dr. Robert Devlin from the Environmental Protection Agency. PM$_{2.5}$ was concentrated ambient air using a ChemV ol model 2400 high-volume cascade impactor as previously described (2, 3, 18). Particle composition was previously described (18).

**Bone marrow-derived macrophage culture.** Primary bone marrow-derived monocytes (BMM) were cultured by flushing the femur and tibia with flow buffer, PBS ± 5% FBS. The cells were resuspended in BMM growth media (DMEM, 10% FBS plus L cell conditioned media) in a CO$_2$ incubator at 37°C for 5 days in a 150-mm suspension culture dish (Corning, Corning, NY). Differentiated BMMs were treated overnight with smooth LPS (50 ng/ml, L7261; Sigma Aldrich), PM$_{2.5}$ (20 µg/ml), or polyomixin B (25 µg/ml) and washed with PBS before addition of T cells. Cells treated with polyomixin B followed by LPS or PM received polyomixin B 45 min before stimulation.

**T cell proliferation assay.** Splenic CD4$^+$ and CD8$^+$ mouse T cells were isolated using an R&D Systems (MTCC-525) column according to the manufacturer’s instructions (Minneapolis, MN). T cells were stained using the Molecular Probes CellTrace CFSE Cell Proliferation Kit (C34554) in 1 ml of PBS with 0.1% BSA and 5 µM carboxyfluorescein succinimidyl ester. Cells were incubated at 37°C for 10 min followed by two washes of 1 ml of RPMI with 10% FBS and then spun in a microcentrifuge at 250 g for 5 min. T cells were resuspended in T cell growth media before seeding at 2x $10^5$ cells/well of a 96-well plate with confluent, pretreated BMM (see above). T cell growth media containing 2 µl/ml anti-CD3 (16–0031-85 clone 145–2c11; eBiosciences) antibody was added to RPMI with 20% FBS. Recombinant mouse macrophage colony-stimulating factor (R&D systems), 10 ng/ml, was added to the T cell growth media whenever T cells were cocultured with BMMs. After 72 h of culture, T cells were run on a flow cytometer, and proliferation index was calculated according to the equation outlined by Wallace and Muirhead (38).

**RESULTS**

PM$_{2.5}$ exposure increases lung macrophage activation, macrophage numbers unaltered. Ambient mean daily PM$_{2.5}$ concentration at the exposure facility was 17.3 ± 2.96 µg/m$^3$ (Fig. 1A) as measured by filter weighing as described in METHODS. Mean concentration of PM$_{2.5}$ in the PM$_{2.5}$ exposure chamber was determined to be 115.5 ± 9.25 µg/m$^3$, demonstrating an approximately 7-fold concentration of ambient particles by the system. Mice were exposed 6 h/day, 5 days/wk to filtered or PM$_{2.5}$-concentrated air. All mice were returned to maintenance housing after exposure.

The total estimated exposure to PM$_{2.5}$ during the experimental time range (including time spent in maintenance housing, which includes ambient exposure) was calculated assuming a ventilation rate of 105 breaths/min and 0.2 ml/breath in mice: WT and CXCR3$^{-/-}$-FA = 99.7 ± 18.7 µg, WT and CXCR3$^{-/-}$-PM$_{2.5}$ = 206.5 ± 24.2 µg, GFP.Foxp3.KI-FA = 85.4 ± 16.1 µg, and GFP.Foxp3.KI-PM$_{2.5}$ = 177.0 ± 20.7 µg (8). Thus, once corrected for time spent in the exposure chambers and at ambient exposure (outside the chamber) with an average respiratory rate and volume, the total PM$_{2.5}$ dose over the experimental period was approximately twofold higher in the PM$_{2.5}$ group compared with the FA group.

Histological analysis of the lung showed no overt pathological changes in the lung as determined by a board-certified veterinary pathologist at the Mouse Phenotyping Shared Resource at the OSU (data not shown). Histological findings were consistent and characterized by occasional, incomplete cuffs of immunoreactive cells in various groups. These cells were primarily scattered in alveolar lumens, around bronchioles, and within vascular lumens; staining was most intense when distributed around bronchioles. Immunohistochemical analysis of F4/80 staining, a marker of mature macrophages in mice, showed no difference in lung macrophage content between WT-FA and WT-PM (data not shown). Flow cytometric analysis confirmed this finding; chronic PM$_{2.5}$ did not significantly increase CD11b$^+$ macrophages in WT or CXCR3$^{-/-}$-FA mice (4.3 ± 10^6 ± 0.44 cells/g in WT-FA vs. 4.78 ± 10^6 ± 0.99 cells/g in WT-PM; Fig. 1B). CXCR3 deficiency markedly attenuated CD11b$^+$ macrophages in the lungs of CXCR3$^{-/-}$-FA compared with WT-FA mice (0.75 ± 10^6 ± 0.47 and 4.13 ± 10^6 ± 0.44 cells/g, respectively; Fig. 1B), suggesting that CXCR3 is critical in determining basal levels of macrophages in the lung. Interestingly, CD11b$^+$CD11c$^+$ macrophages increased from 0.8 ± 10^6 ± 0.07 cells/g of lung in WT-FA mice to 1.8 ± 10^6 ± 0.18 in WT-PM$_{2.5}$ (P < 0.0002; Fig. 1, C and G, representative flow plots). The difference in CD11b$^+$CD11c$^+$ macrophages in CXCR3$^{-/-}$-FA compared with WT-FA mice (0.23 ± 10^6 ± 0.23) increased compared with CXCR3$^{-/-}$-PM$_{2.5}$ (0.75 ± 10^6 ± 0.23; P = 0.09) was not significant, but this may be due to the sample size. Nonetheless, CXCR3 depletion greatly reduced the number of CD11b$^+$CD11c$^+$ macrophages in the lung of FA-exposed mice (WT-FA vs. CXCR3$^{-/-}$-FA, P = 0.008) and PM-exposed mice (WT-PM vs. CXCR3$^{-/-}$-PM, P = 0.006) compared with background controls. No difference was observed in Ly6C expression, a putative marker of macrophage classical activation, on CD11b$^+$ cells.

**Particulate air pollution results in increased oxidized PAPC derivatives in BAL fluid.** Lipid extracts of the BAL fluid of WT C57BL/6 mice exposed for 28 wk to FA or PM$_{2.5}$ were analyzed by HPLC with positive electrospray ionization mass spectrometry. Parent PAPC and oxidized derivatives (POVPC and PGPC) of lipid oxidative stress (Fig. 1F). In light of the integral role for activated antigen-presenting cells (APCs) (macrophage/dendritic cells) and oxidized lipid moiety (35) in T cell responses, we next investigated the effects of PM$_{2.5}$ exposure on T cell activation.
Activation and CXCR3 induction in lung and MLN T cells.

Chronic PM$_{2.5}$ exposure led to an approximate doubling of CD$_3^+$ lymphocytes in the lung (5.3 × 10$^6$ ± 0.31 CD$_3^+$ in WT-FA vs. 10.1 × 10$^6$ ± 0.20 cells/g in WT-PM$_{2.5}$, $P = 0.005$; Fig. 2A). CXCR3$^{-/-}$ mice demonstrated a markedly attenuated T cell response to PM$_{2.5}$ with abrogation of differences in CD$_3^+$ cells between FA and PM$_{2.5}$ groups (CXCR3$^{-/-}$-FA vs. CXCR3$^{-/-}$-PM$_{2.5}$, $P > 0.05$). FA control CXCR3$^{-/-}$ mice exhibited lower basal levels of CD$_3^+$ cells, suggesting a role for CXCR3 in modulating total CD$_3^+$ T cell content in the lung (1.4 × 10$^6$ ± 0.21 cells/g in WT-FA, $P = 0.0002$); the same effect was observed in the CD4 and CD8 subsets. In response to PM$_{2.5}$ exposure, CD4$^+$ cells significantly increased in WT mice (2.1 × 10$^6$ ± 0.15 in WT-FA vs. 3.2 × 10$^6$ ± 0.27 cells/g in WT-PM$_{2.5}$, $P = 0.007$; Fig. 2A and B, representative flow plots). CXCR3 deficiency attenuated the response to PM$_{2.5}$ markedly with no statistically significant differences noted between PM$_{2.5}$ and FA groups in the CXCR3$^{-/-}$ mice following 16 wk of exposure ($P > 0.05$). The number of CD3$^+$ CD4$^+$ cells in CXCR3$^{-/-}$-PM$_{2.5}$ mice never reached that of the WT-FA control and were significantly lower than WT-PM$_{2.5}$ mice (Fig. 2C).

To understand the activation status of lung-infiltrating Teff cells in response to PM$_{2.5}$, we analyzed surface expression of CD44 and CD62L (L-selectin) in WT and CXCR3$^{-/-}$ mice (Fig. 2D and E, representative flow plot). CD44 is upregulated on naïve T cells after T cell receptor activation and has been shown to be required for activated T cell extravasation into sites of inflammation (12, 30). PM$_{2.5}$ exposure significantly increased the number of activated CD44$^+$ cells in the lung of mice compared with WT-FA controls (2.5 × 10$^6$ ± 0.33 in WT-FA vs. 5.3 × 10$^6$ ± 0.15 in WT-PM$_{2.5}$, $P = 0.01$; Fig. 2D), whereas CXCR3 deficiency prevented this increase. Central
memory cell populations as determined by CCR7 expression on CD44+/H11001 CD62L+/H11001 cells was significantly increased in response to PM2.5 in both WT-PM2.5 and CXCR3+/H11002/CXCR3+/H11002 mice compared with FA groups, with no difference in the magnitude of this response, suggesting that CXCR3 is not involved in the trafficking of this subset of cells.

We then assessed the expression of CXCR3 on CD4+/H11001 and CD8+/H11001 T cells. We found that CD4+/H11001 T cells coexpressing CXCR3 were significantly higher in PM2.5-exposed mice (P = 0.0005; Fig. 2F), whereas CXCR3+/H11001 CD8+/H11001 T cells exhibited no change. We next assessed the percentage of these cells in the MLN, a major lymph structure that drains the lung, fostering T cell and macrophage interactions. In contrast to the lung, CXCR3 expression on CD3+/H11001, CD4+/H11001, and CD8+/H11001 cells was increased in all groups to a statistically significant level (P < 0.002, 0.004, and 0.04, respectively; Fig. 2, G and H, representative flow plots).

Central T cell activation status is modulated by chronic PM2.5. We next determined the changes in splenic T cell populations to address the question if the changes in the lung microenvironment were paralleled by changes in the spleen.

Figure 3A depicts CD4+ and CD8+ T cells in the spleen following PM2.5 exposure as a percentage of total CD3+ cells. Activated splenic CD4+ Teff cells decreased in the spleen of both WT and CXCR3−/− mice in response to PM2.5 exposure (43 ± 2.6% in WT-FA vs. 33 ± 1.9% in WT-PM2.5, P = 0.009; 55 ± 2.1% in CXCR3−/−-FA vs. 44 ± 2.0% in CXCR3−/−-PM2.5, P = 0.1; Fig. 3B). CXCR3−/−-PM2.5 mice had a higher percentage of activated T cells in the spleen (44 ± 20%) than WT-PM2.5 mice (33 ± 1.9%, P = 0.004), and CXCR3−/−-FA mice had a higher percentage of activated cells compared with WT-FA, consistent with an attenuated homing ability of activated T cells from the spleen to sites of inflammation. Central memory cell numbers increased dramatically in the WT-PM2.5 mice with the CXCR3−/− group, demonstrating abrogation of this increase in response to PM2.5 (Fig. 3B). Naïve T cells in the spleen were unaltered in response to PM2.5 exposure in WT mice; however, CXCR3-deficient mice demonstrated an increase in this cell population in response to PM2.5. Taken together with the fact that naïve T cells in the lung did not increase in CXCR3−/− mice, this suggests sequestration of these cells in the spleen and a role for CXCR3 in trafficking of naïve and
activated T cells to sites of inflammation. CXCR3 expression as measured by flow cytometry was significantly higher on splenic CD3+ lymphocytes in WT-PM2.5 mice (Fig. 3C), similar to the lung findings.

Circulating lymphocyte populations are altered by PM2.5 exposure. Plasma CD4+ cell numbers remained unchanged, whereas CD8+ cell numbers increased in WT-PM2.5 mice (Fig. 4A). Circulating activated T cells did not increase significantly within a mouse model with PM2.5 exposure (Fig. 4B); however, CXCR3+/− FA and -PM2.5 mice had dramatically higher levels of activated T cells in circulation than WT-FA or WT-PM2.5, respectively (23 ± 3.1% WT-FA vs. 35 ± 2.9% CXCR3-FA, P = 0.05; 28 ± 3.3% WT-PM2.5 vs. 40 ± 1.4%, P = 0.02). Memory cells decreased by nearly 80% with PM2.5 exposure in the CXCR3+/− group (16 ± 2.6% FA vs. 3.4 ± 1.3%), whereas the WT mice showed no change (Fig. 4, B and C, representative flow data). Naïve CD4+ cells were not affected in WT mice but were decreased in CXCR3+/−-PM2.5 mice by ~25%. CXCR3 expression increased dramatically on circulating CD3+, CD4+, and CD8+ cells (P < 0.0001, 0.0001, 0.002, respectively, for FA vs. PM; n = 6) in WT-PM2.5 mice (Fig. 4D).

Treg numbers and activation are systemically increased with PM2.5, but not in the lung. Finally, we examined the response of Tregs as measured by expression of GFP, a marker of Foxp3 expression, in CD3+ T cells. Interestingly, Tregs were not found in the lung in significant numbers (<100 cells) in either FA or PM2.5 mice by flow cytometry (data not shown). Total CD3+, CD3+CD4+, and CD3+CD8+ T cells, however, were increased in the lung of GFP.Foxp3.KI mice (Fig. 5A). Splenic Foxp3.GFP+ Tregs increased from 8.4 ± 1.3% in the FA to 12.2 ± 0.97% in the PM2.5 group (FA vs. PM2.5, P = 0.02; Fig. 5B). Activated splenic Tregs (GFP+CD4+CD44+CD62L−) were also dramatically increased in the PM2.5 group (13 ± 1.8% FA vs. 21 ± 1.4% PM2.5, P = 0.002; Fig. 5B). Splenic Treg CXCR3 expression also proved to be increased with PM2.5 (P = 0.004; Fig. 5C, representative flow plots). In the MLN, we found abundant Tregs with no change in total Treg numbers with exposure (16.5 ± 0.7% FA vs. 17.6 ± 1.2% PM2.5, P > 0.05; Fig. 5D). However, there was a significant change in CXCR3 expression on MLN-derived Tregs (P = 0.03; Fig. 5D) with PM2.5 exposure. Circulating Treg numbers significantly increased (P = 0.04) with PM2.5 in the blood (Fig. 5E). Increased systemic Tregs may reflect a homeostatic response to an inflammatory stimulus with a similar response noted by our group in response to high-fat feeding (13).

Gene expression changes in lung CD4+ T cells isolated after PM2.5 exposure. We isolated CD4+ T cells from lung digesta to assay altered cytokine and transcription factor expression in cells exposed to PM2.5 pollution. The purpose was to further explore phenotypic alteration in T effector cells to a pro- or anti-inflammatory state, in addition to our extensive flow cytometric analysis. We assayed IL-17 gene expression, a cytokine characteristic of highly inflammatory Th17 CD4+ effector cells. Interestingly, we found IL-17α gene expression was significantly increased (Fig. 6). These data are not conclusive of an increase in Th17 cells but do indicate that such a switch may be occurring. GATA3, a key transcription factor required for Th2 differentiation, was unchanged as was Foxp3 gene expression. Increased CXCR3 gene expression in lung CD4+ T cells confirms increased CXCR3 surface positivity by flow cytometric analysis. Importantly,
CXCR3 expression can be considered a marker of Th1 differentiation, since it is mostly expressed on this cell type. Additionally, to further assess a Th2 response to PM2.5, we measured CCR3, CCR4, and IL-4 expression in CD4^+ T cells isolated from the lung and found no difference compared with FA controls. This suggests that PM2.5 exposure does not have a significant physiological effect on Th2 populations, while a Th1 response may dominate.

Fig. 4. CXCR3 knockout mice have elevated activated T cells in circulation. A: circulating CD8^+ T cells were higher in WT-PM2.5 vs. WT-FA (P = 0.004); CD4^+ T cells were not altered. B: CXCR3^−/− mice had a higher percentage of circulating activated T cells vs. the WT group (WT-FA vs. CXCR3^−/−-FA, P = 0.05; WT-PM2.5 vs. CXCR3^−/−-PM2.5, P = 0.02). Circulating memory and naïve T cells were much lower in CXCR3^−/−-PM2.5 mice vs. CXCR3^−/−-FA. C: representative flow figure used for quantifying CD44 and CD62L expression on blood T cells. D: PM2.5-treated mice had increased CXCR3 expression as measured by flow cytometry on circulating T cells.

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Fig. 5. T regulatory cells (Tregs) increase in no. and activation state centrally but not in the lung. GFP, green fluorescent protein. A: total CD3^+, CD3^+CD4^+, and CD3^+CD8^+ cells in the lung of GFP Foxp3.KI mice responded to PM2.5 exposure similar to the WT model, with increased nos. B: total splenic GFP-expressing Treg cells as well as activated Tregs were higher with PM2.5 exposure vs. FA control (P = 0.02 and 0.002, respectively). C: flow cytometry with indicated gating was used to quantify GFP-positive Tregs and CXCR3-positive cells; splenic Tregs expressing CXCR3 are nearly double that of the FA group (P = 0.004). D: CXCR3 expression was also higher in MLN-derived Tregs of PM2.5 mice vs. control (P = 0.03), although total Treg nos. were unchanged. E: circulating GFP-positive Tregs were higher in the PM2.5 group compared with FA controls (P = 0.04). Experiments included 10 mice/group.
To enhance T cell proliferation? If so, does PM2.5 activate macrophages directly or is activation via an intermediate, such as oxidized phospholipids? To investigate the impact of oxidized phospholipids on macrophage-mediated T cell proliferation, we pretreated macrophages in vitro with PM2.5 derived from filters during the time frame of exposure, and as an additional comparator, particles collected from Chapel Hill, NC (see METHODS), and assessed their relative ability to stimulate T cells. To investigate the impact of oxidized and native pulmonary phospholipids on BMMs, we pretreated macrophages with PAPC and PGPC and measured their ability to stimulate T cell proliferation within the context of PM2.5 and LPS-mediated effects. We used LPS- and IL-4-stimulated BMMs as controls for the effect of proinflammatory/classical and anti-inflammatory/alternative activation on T cell proliferation in vitro. Polymyxin B, an antibiotic that blocks the effects of LPS (7, 9, 31, 37), was used to block/measure the effects of LPS contaminator of PM2.5, allowing the measurement of LPS-independent effects on T cell proliferation. Columbus PM2.5 treatment resulted in a significant increase in T cell proliferation index (3.16 ± 0.11 vs. 1.96 ± 0.9 in untreated BMMs; Fig. 7B). Chapel Hill PM2.5-stimulated BMMs had a small but significant increase in T cell proliferation index vs. untreated BMM (1.098 ± 0.01 vs. 1.06 ± 0.003, respectively; Fig. 7C). Polymyxin B pretreatment did not inhibit the effect of PM on BMM-mediated T cell proliferation, demonstrating an LPS-independent effect of PM2.5 on macrophage activation. Overnight treatment of BMMs with smooth LPS resulted in a dramatic increase in T cell proliferation with an index of 7.27 vs. 1.96 in untreated BMM (1.098 ± 0.01 vs. 1.06 ± 0.003, respectively; Fig. 7C). Polymyxin B, however, completely attenuated the increase in T cell proliferation by LPS with LPS + polymyxin B not significantly higher than polymyxin B alone or untreated BMMs (Fig. 7, B and C). IL-4 treatment resulted in no change in T cell proliferation compared with untreated BMM stimulation. We hypothesized that PM2.5 may directly mediate oxidation of lung phospholipids or via oxidant stress derived from inflammatory cells, with the oxidized phospholipid moieties then leading to macrophage activation; however, we found that the oxidized derivative POVPC did not increase macrophage-mediated T cell proliferation compared with PAPC or untreated controls. These data support a direct effect of PM2.5 on macrophage activation state and possible enhancement of T cell proliferation.

**DISCUSSION**

We demonstrate that chronic exposure to environmentally relevant concentrations of particulate air pollution has an important effect on innate and adaptive immune cell populations in lung, lymphatic, and systemic immune populations. Although prior reports have suggested that inhaled particulate matter may potentiate innate immune function, the role of chronic exposure to PM2.5 as a stimulus for pulmonary and systemic T cell activation is not well described. Exposure to PM2.5 resulted in increased T cell infiltration and increased activation of Teff cells (evidenced by an increase in CD4+/CD44+/CD62L– and CXCR3+ T cells in the lungs) and suggests a phenotypic switch to a Th1/Th17 phenotype in lung Teff cells. These results have important implications for how PM2.5 may detrimentally modulate pulmonary and systemic immune responses. Air pollution exposure typically occurs over long durations and is a pervasive environmental risk factor well known to increase susceptibility to cardiopulmonary mortality. The extent of lung inflammation in several studies of ambient exposure has been reported to be minimal, despite robust systemic inflammatory responses. Many of these studies have used relatively insensitive measures of inflammation (15, 21). Our study points to a rather prominent pulmonary and systemic inflammatory response characterized by T cell infiltration and activation with chronic exposure.

The attenuation of proinflammatory effects in the lungs of CXCR3–/– mice demonstrates a critical role for CXCR3 in T cell recruitment/homing to the lung and in the regulation of inflammation in response to PM2.5 exposure. We believe continued activation (CD44 stimulation) of Teff cells in the lungs leads to the reexpression of CD62L, resulting in increased memory cell populations (Fig. 2) (1). We also provide evidence for heightened levels of resident and central memory T cell populations in the lung and spleen that could potentially prime the lung for rapid inflammatory response to future particulate insults. PM2.5 exposure concomitantly induced significant increases in immune-suppressive and anti-inflammatory Treg cells in the spleen and blood. There were minimal changes in Treg populations in the lung or MLN, suggesting a blunted role in preventing macrophage/dendritic cell-mediated T cell activation and proliferation. Additionally, inflammatory macrophages in the lung may create a local cytokine milieu that is inhospitable to Treg differentiation or maintenance.

The importance of CXCR3 in lung injury and infection has been reported previously. CXCR3 functionality was vital to virus-specific effector CD4+ T cell migration to the lung in pneumonia infection in vivo (20). Deficiency in CXCR3 impaired the trafficking of adaptively transferred antigen-specific Th1 cells to the lung in C57BL/6 mice (24). In a CC10-OVA lung rejection model, CXCR3-deficient CD8+ T effector cells exhibited impaired migration to lung compared with WT Teff, resulting in a dramatic reduction in pulmonary inflammation (33). However, there are conflicting reports on the importance of the CXCR3-ligand pathway on T cell homing to the lung.
Manicone et al. (23) reported in a Ly5a/Ly5b graft-vs.-host model of lung injury that antibody neutralization of IP-10/CXCL10 and MIG/CXCL9 reduced Th1 cell-mediated lung inflammation but did not alter Th1 cell influx to the lung of WT C57BL/6 mice. Interestingly, a lack of CXCR3 in CXCR3/H11002 mice had no effect on the influx of adoptively transferred Th1 cells or acute inflammation (23). These findings may be unique to alloimmune injury, with the effects of particulate matter evoking separate pathways leading to adaptive immune activation. Interestingly, they did show that IP-10/CXCL10 and MIG/CXCL9 may be important in Th1 cell proliferation (23).

In addition, CXCR3-deficient mice showed attenuated acute lung inflammation in response to cigarette smoke (27). The majority of the current literature is complementary to our finding that CXCR3 plays a significant role in the T cell-mediated response to PM2.5.

Lung macrophage and dendritic cell response to PM2.5 exposure most likely plays an important first step mediating some of the T cell responses reported in this study. Increased T cell infiltration in the lung may be the result of PM2.5-mediated macrophage release of cytokines. Recruited exudative macrophages show enhanced production of CXCL10 post noninfectious lung injury (36). However, the three major CXCR3-binding chemokines (CXCL9, CXCL10, and CXCL11) are induced in a variety of cells, including lung macrophages and dendritic cells, in response to other cytokine mediators such as interferon (IFN)-γ. This may provide an amplification loop for Th1 immune responses by attracting more CXCR3-expressing Th1 cells (10). This is evidenced by results demonstrating a marked increase in CXCR3⁺CD4⁺ cells in response to CXCR3-expressing Th1 cells (10). This is evidenced by results demonstrating a marked increase in CXCR3⁺CD4⁺ cells in response to PM2.5 exposure. CXCR3⁻/⁻ mice in our experiments had a significantly lower amount of resident CD11b⁺ macrophages in the lungs under FA conditions, suggesting that this receptor determines basal macrophage content. Prior experiments with antagonism of CXCL10 have demonstrated diminished immune response to infection due to decreased macrophage and granulocyte recruitment (10).

PM2.5 markedly increased activated T cells in the lung microenvironment defined by the expression of CD4⁺CD62L⁻CD4⁺ cells and an increase in CXCR3⁺CD4⁺ T cells in two

Fig. 7. PM2.5-treated macrophages are more effective at promoting T cell proliferation. A: representative histograms of carboxyfluorescein succinimidyl ester (CFSE)-positive T cells grown in the presence of untreated (UT) and stimulated primary bone marrow-derived monocytes (BMMs): PM, PM2.5, 20 μg/ml; PM + Poly, polymyxin B pretreated followed by PM2.5; LPS, lipopolysaccharide, 50 ng/ml; LPS + Poly, polymyxin B pretreatment followed by LPS stimulation; y-axes are cell counts, and x-axes are log10 FITC channel intensity. Quantification of CFSE-expressing cells by generation allowed for calculation of a T cell proliferation index (see METHODS). The parent population is labeled “0,” and filial populations are serially numbered. B: T cell proliferation index values using Columbus PM2.5. C: T cell proliferation index values using Chapel Hill PM2.5 (* and *** vs. UT; # and ### vs. Poly).
models (WT C57BL/6 and Foxp3gfp.KI). This increase in activated CD4+ cells in the lungs and mediastinal nodes was paralleled by a decrease in the spleen in both models, with cells accumulating in the blood of CXCR3-/-PM2.5 but not WT-PM2.5-exposed mice. Taken together with a shift in gene expression, decreased GATA3, and increased Th17 and CXCR3 expression, these findings are consistent with a polarization by PM2.5 exposure toward an inflammatory, Th1/Th17 phenotype. Gene expression for IL-17 alone is not conclusive evidence for increased Th17 cells with PM2.5 exposure; further investigation is needed upon additional exposures. PM2.5 also significantly increased central memory cell populations in the lungs of WT and CXCR3-/- mice and increased central memory cells in the spleen of WT-PM2.5 mice. The increase in memory T cells in the lungs of CXCR3-/-PM2.5 mice was contrasted by the lack of a PM2.5 response in the spleen of CXCR3-/- mice. These data support a role for CXCR3 in homing of activated/memory CD4+ T cells back to the spleen.

Chronic air pollution had an interesting effect on Treg populations in tissues, with increased Tregs in the spleen, MLN, and blood while lung levels remained low to undetectable. To our knowledge, this is the first demonstration that PM2.5 may induce an increase in this cell population using a knockin model that links Foxp3 expression with a fluorescent marker previously shown to be highly specific for Tregs in response to PM2.5 exposure (5). The increase in Tregs in the spleen and systemic circulation may indicate a homeostatic counterregulatory response to PM2.5. However, in contrast to this increase in the spleen and blood, there was no increase in Tregs in the lungs or MLN in response to PM2.5. Interestingly, Treg numbers in the lung were very low compared with other tissue niches where inducible Tregs have been thought to play a role, such as in the adipose and vasculature (11, 13). While this could represent a variation related to the animal models used, we believe this finding points toward alternate mechanisms modulating T cell activation in the lung. Additionally, CXCR3 expression on splenic and MLN Tregs was dramatically increased, suggesting enhanced homing ability, albeit not to the lung. This response is somewhat similar to responses in other tissue niches that are characterized by chronic macrophage inflammation and is similar to results we previously reported in the same mouse model with diet-induced obesity (13). One may speculate that classical activation of macrophages may mitigate Treg proliferation, differentiation, or maintenance via direct cell-cell interactions and/or release of proinflammatory cytokines.

T cell activation involves T cell receptor recognition of antigen as presented by MHC molecules on APCs; additional stimulation is accomplished by binding of the B7 protein on APCs to the CD28 receptor on T cells. APC cytokine release is important in modulating T cell proliferation and differentiation. We demonstrate that in vitro PM2.5-exposed macrophages increased T cell proliferation compared with untreated macrophages. Although these responses were much lower compared with LPS, the fact that PM2.5 induced T cell proliferation in the presence of polymyxin B suggests a role for effects of PM2.5 independent of LPS content. It is important to note that PM10, and to a lesser extent PM2.5, may contain low levels of LPS, and this may drive inflammatory responses locally and systemically. While the precise nature of the signals (cell to cell vs. cytokine mediated) through which PM2.5-exposed macrophages may stimulate T cell proliferation remains to be characterized, our results that show an increase in CD11b+CD11c+ macrophages may point to this population playing a role in antigen presentation or cytokine responses that may engender a robust T cell response in the lung.

Our data have several important limitations, namely that we failed to directly link our findings to a particular disease model or physiological outcome measure. PM2.5 exposure in young healthy mice, to our surprise, was not sufficiently deleterious to result in gross pathophysiological pulmonary change. In addition, because our focus was the lung, we did not collect cardiac endpoints that might be more sensitive to exposure. Our aim was to provide immunological insights into how PM2.5 may be exacerbating local lung and systemic inflammation, possibly leading to increased thrombosis, atherosclerosis, pulmonary disease, and insulin resistance pathologies that are already in place. We did not identify the components of PM2.5 that resulted in T cell infiltration. Moreover, we have not provided evidence regarding the cellular source of CXCR3 ligand(s). It is possible that proinflammatory cytokines released directly in response to PM2.5 or through intermediate signals such as oxidized phospholipids may induce the expression of chemoattractants such as CXCL9, -10, and -11 by a variety of cells, including lung epithelial cells. These could then participate in the migration of activated CXCR3+ cells into the lung. Interestingly, we demonstrate an increase in oxidized forms of PAPC that have been previously shown to activate TLR4 signaling in a variety of different cell types and may elicit the induction of Th1 cytokines such as IFNγ (19). It is also possible that exposure to modified phospholipids may induce a chronic antigenic response as has been demonstrated in atherosclerotic plaques (35). Our study was not designed to study these responses although acutely these oxidized derivatives did not directly induce T cell proliferation responses. In conclusion, chronic PM2.5 exposure mediates a proinflammatory Th1 phenotype in the lung mediated via a CXCR3-dependent mechanism. These findings have important implications for PM2.5-mediated inflammatory diseases.

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DISCLOSURES

The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institute of Diabetes and Digestive and Kidney Diseases or the National Institutes of Health. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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