AIR POLLUTION PARTICULATE MATTER EFFECTS ON ADAPTIVE HUMAN ANTIMYCOBACTERIAL IMMUNITY

By

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ABSTRACT OF THE DISSERTATION

Air Pollution Particulate Matter Effects on Adaptive Human Antimycobacterial Immunity

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Tuberculosis (TB) and air pollution both contribute significantly to the global burden of disease. Epidemiological studies provide evidence that indoor (household) air pollution increases the risk of new infections with *Mycobacterium tuberculosis* (*M.tb*) and development of TB. The mechanisms by which exposure to 'real-world'-derived urban ambient (outdoor) particulate matter (PM) adversely affects *M.tb*-specific human host T cell functions *in vitro* have not been studied. In this thesis research, we explored the effects of air pollution $PM_{2.5}$ (≤2.5 μm, median aerodynamic diameter) collected in the Iztapalapa municipality of Mexico City on *M.tb*-specific T cell functions in human peripheral blood mononuclear cells (PBMC). Upon *in vitro* exposure, $PM_{2.5}$ was observed in clusters of free, non-membrane-bound particle agglomerates in the cytoplasm of the exposed cells. $PM_{2.5}$ exposure did not alter the expression of activation marker CD54 on antigen presenting cells (APC), however, increased the expression of CD80 while decreasing the constitutively expressed CD86 on monocytes during *M.tb* infection. Exposure to $PM_{2.5}$ of *M.tb*-infected PBMC led to an increase of intracellular growth of *M.tb*, indicating loss of *M.tb* growth controlling capacity of the cells that occurred independent of PM-induced changes to PBMC viability. Exposure of PBMC to $PM_{2.5}$ also altered *M.tb*-specific T-cell immune responses by (1) decreasing the surface expression of early T cell activation markers CD69

and CD25 on T cells, (2) inhibiting the intracellular expression of both interferon-gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α), and (3) decreasing the expression of T-box transcription factor TBX21 (T-bet) known to directly regulate the expression of IFN-γ. In contrast, $PM_{2.5}$ exposure increased the intracellular expression of the anti-inflammatory cytokine interleukin 10 (IL-10) and the phosphorylation of transcription factor STAT-3. The observed $PM_{2.5}$-induced decrease in the expression of human pro-inflammatory *M.tb*-specific T cell cytokines, and the loss of intracellular *M.tb* growth control are associated with the increased expression of anti-inflammatory cytokine IL-10 and decreased expression of transcription factor T-bet. Together, the findings of this study suggest that the $PM_{2.5}$-induced decrease of critical human host immune cell functions against *M.tb* represents the mechanistic correlate of epidemiological observations that outdoor air pollution exposure is associated with increases in the incidence of TB and with adversely modified TB treatment outcomes.

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LIST OF ABBREVIATIONS

AM	Alveolar macrophages
APC	Antigen presenting cells
BCG	Bacillus Calmette-Guerin
CD	Cluster of differentiation
CFU	Colony forming units
DEP	Diesel exhaust particles
ELISA	Enzyme-linked immunosorbent assay
IFN-γ	Interferon gamma
IL	Interleukin
LPS	Lipopolysaccharides
M.tb	*Mycobacterium tuberculosis*
MHC	Major Histocompatibility complex
MDM	Monocyte-derived macrophages
MOI	Multiplicities of infection
PBMC	Peripheral blood mononuclear cells
PHA	Phytohemagglutinin
PHS	Pooled human serum
PM	Particulate matter
PPD	Purified protein derivative
STAT	Signal transducer and activator of transcription
TB	Tuberculosis
TNF-α	Tumor necrosis factor alpha

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1.1 INTRODUCTION

The immunological control of *Mycobacterium tuberculosis (M.tb)*, the causative agent of tuberculosis (TB), the world's leading infectious disease cause of death (WHO, 2016), requires the functioning of innate and adaptive antimycobacterial immune responses [1]. Most people who are infected with *M.tb* do not develop active disease because their immune system can control the infection and maintain it in an asymptomatic stage called latent *M.tb* infection. Untreated TB most often leads to death. Factors that weaken the immune system increase the risk of developing TB. Known mechanisms and agents that alter adaptive immune mechanisms *e.g.* by causing improper activation of T cell responses that facilitate progression of TB disease, include human immunodeficiency (HIV) infection [2], malnutrition [3], long-term corticosteroid therapies [4], antineoplastic chemotherapies [5] or tumor necrosis factor (TNF) inhibitors [6]. Recent epidemiological studies have linked exposure to cigarette smoke with increased incidence rates of TB [7-9]. Mechanistic studies have also demonstrated that exposure to cigarette smoke weakens immunity to *M.tb* by inhibiting pulmonary T cell responses [10-13]. There is strong evidence that indoor air pollution is another risk factor for the development of active TB [14, 15] and that exposure to outdoor air pollution modifies the course of anti-tuberculous therapy [16-18]. Epidemiologic evidence suggests a strong association between exposure to particulate matter (PM), a key pollutant in ambient air, and disease progression of *M.tb* [19, 20]. However, while it has been shown that innate immune functions of alveolar macrophages [21], monocytes [22] or monocyte-derived macrophages (MDM) [23] can be impaired by PM exposures, studies assessing the effects of PM on adaptive, T-cell, immunity are

lacking. In this study, we assessed the impact of $PM_{2.5}$ exposure on human peripheral blood T cell responses to *M.tb*.

Human host immunity against *M.tb* requires cell-mediated immune mechanisms, and Th1 immunity [24, 25] with production of gamma interferon (IFN-γ) [26, 27] and tumor necrosis factor alpha (TNF-α) [28-30]. Additional integral effector functions of T cells during *M.tb* infection include the ability of T-cells to produce IFN-γ, lyse *M.tb*-infected phagocytes and to kill intracellular bacteria [31]. TNF-α produced upon *M.tb* infection from human and murine macrophages *in vivo* [24, 32-35], has been shown to have a vital role in protective host immunity against mycobacterial infection [30, 36-38], and is required in synergy with IFN-γ for the complete inhibition of mycobacterial growth in murine bone marrow-derived macrophages [33, 39]. Thus, both TNF-α and IFN-γ are essential for optimal macrophage activation and mycobacterial growth inhibition [40].

Conversely, Interleukin-10 (IL-10), a cytokine with anti-inflammatory properties, has been shown to dampen Th1 cell responses to *M.tb* infection [41], T cell proliferation [42] and IFN-γ production [43] in PBMC from patients with pulmonary TB. IL-10 also promotes *M.tb* survival and correlates with more severe clinical phenotypes of TB. IL-10–transgenic mice are highly susceptible to progressive TB infection and IL-10–deficient mice have increased antimycobacterial immunity [44, 45].

Earlier studies in our laboratory have shown in PBMC that diesel exhaust particles (DEP, a significant component of urban outdoor $PM_{2.5}$) alter *M.tb*-induced inflammatory cytokine and IRF-1 and NF-κB target gene expression in a dose-dependent manner [46]. Recently, our lab studies also showed that outdoor air pollution PM impairs innate antimycobacterial immune responses in the human alveolar type II epithelial cell line A549. Exposure to

$PM_{2.5}$ and PM_{10} (aerodynamic diameters ≤2.5μm and 10μm, respectively) decreased the expression of the antimicrobial peptides human β-defensin 2 (HBD-2) and HBD-3 upon infection with *M.tb*, induced cellular senescence and led to increased intracellular *M.tb* growth in the A549 cells [47].

In this thesis study, we examined the effects of $PM_{2.5}$ on the *M.tb* growth-controlling capacity of PBMC from healthy persons in New Jersey. We also investigated the effects of $PM_{2.5}$ on the expression of early T cell activation markers CD69 and CD25 in *M.tb*-infected PBMC. Finally, we assessed the effects of $PM_{2.5}$ on *M.tb*-induced IFN-γ, and TNF-α production and on the associated transcription factor T-bet in peripheral blood T cells of the healthy donors.

1.2 BACKGROUND

1.2.1 Particulate matter (PM)

PM is a complex mixture of solid and liquid particles that is released into the air during the combustion of coal, wood, gasoline, diesel or fossil fuels, as well as from natural sources (road dust, fires, volcanic emissions, etc.) [48]. PM has a carbon core coated with a range of chemical species including reactive transition metals and organic hydrocarbons **(Figure 1).** The size of PM during inhalation determines which organs they occupy. Based on its aerodynamic diameter, PM is classified as $PM_{2.5}$ and PM_{10}. **(Figure 2: PM sizes in comparison to human hair)**. Particles smaller than aerodynamic diameter of 10 μm (PM_{10}) are commonly assessed in monitoring particulate air pollution. Recently, particles with aerodynamic diameters smaller than $2.5_{\mu}m$ ($PM_{2.5}$) have been studied because of their ability to reach the alveolar spaces with the air stream during breathing [49]. PM_{10} is largely derived from exhaust emissions, oil combustion, and natural sources, such as silicate particles. A significant portion of $PM_{2.5}$ originates from diesel cars, heavy oil combustion, steel industries and secondary aerosols formed from nitric and sulfuric gasses after discharge into the ambient air [19]. Exposure to ambient air $PM_{2.5}$ is estimated to cause ~1% of worldwide mortality from acute respiratory infections in children under 5 years [50].

Particulate Matter (PM)

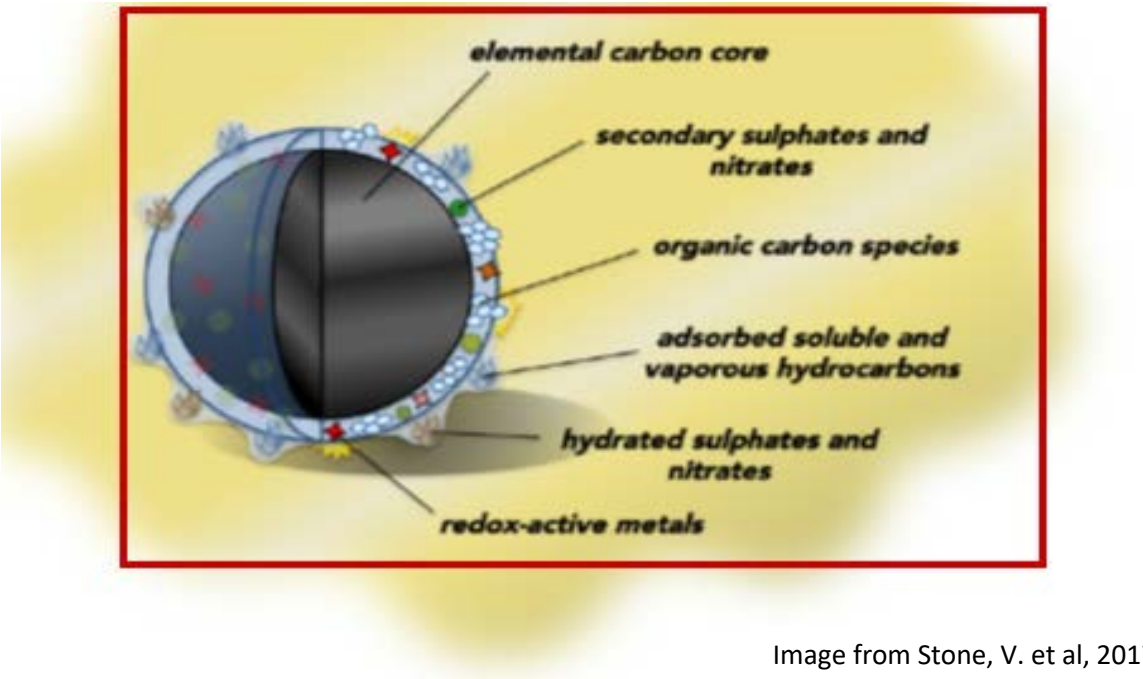
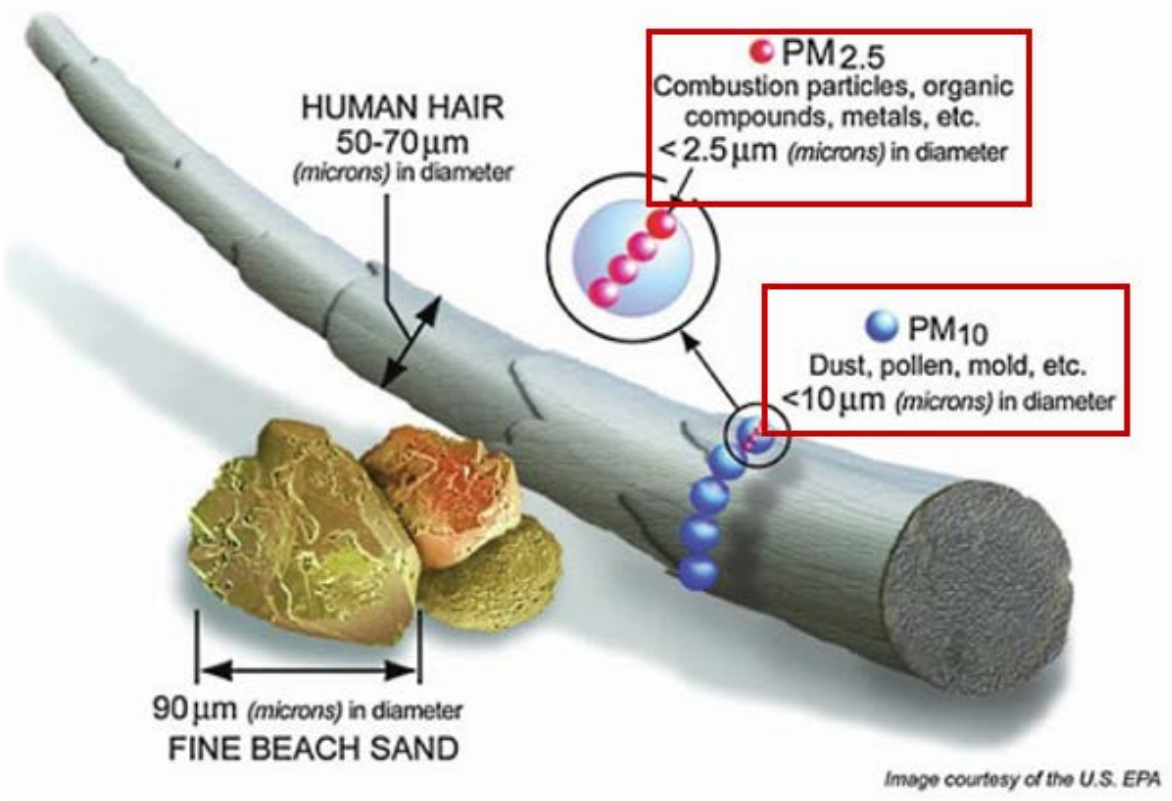


Image from Stone, V. et al, 2017

Figure 1. Urban particulate matter (PM): a carbon core coated with a range of chemical species including reactive transition metals and organic hydrocarbons [51].

PM Sizes (as compared to human hair)



1 µm is 1/1000 millimeter

Image from Zhisheng Li et al, 2017

Figure 2. Comparison of particle diameter [52].

PM are classified as $PM_{2.5}$ and PM_{10} based on aerodynamic diameter. The size of PM during inhalation determines the organs they affect. $PM_{2.5}$ penetrates deep into the alveoli while PM_{10} deposits in the upper airways.

1.2.2 *Mycobacterium tuberculosis* infection

M.tb, the causative agent of TB is a slow-growing, facultative, intracellular, gram-positive, nonspore-forming, and aerobic [53] bacterium that multiplies within phagocytic cells, particularly macrophages and monocytes. Primary infection with *M.tb* occurs most frequently by inhalation of the organism in droplet nuclei that are aerosolized by respiratory maneuvers of infectious TB patients [54]. *M.tb* replicates initially in cells of the terminal airways, after which it is taken up by and replicates in alveolar macrophages (AM) and dendritic cells. AM and dendritic cells carry *M.tb* the organism to other areas of the lungs and the regional lymph nodes. Most of the people who become infected with *M.tb* do not develop TB because they are capable of either resolving the infection completely or of maintaining the infection in an immunologically controlled latent stage [55]. About one-third of the world's population is believed to be latently *M.tb* infected. Over the lifetime, there is a 10% chance for latently *M.tb*-infected persons to develop active TB [56]. This lifetime risk is much higher in people with compromised immune systems such as during HIV infection [2], malnutrition [3], extended corticosteroid therapies [4], chemotherapies [5]and treatments with TNF inhibitors [6]. Host defense against *M.tb* is mediated by a combination of innate and adaptive immunity (**Figure 3**) [54].

Infection with *M.tb*

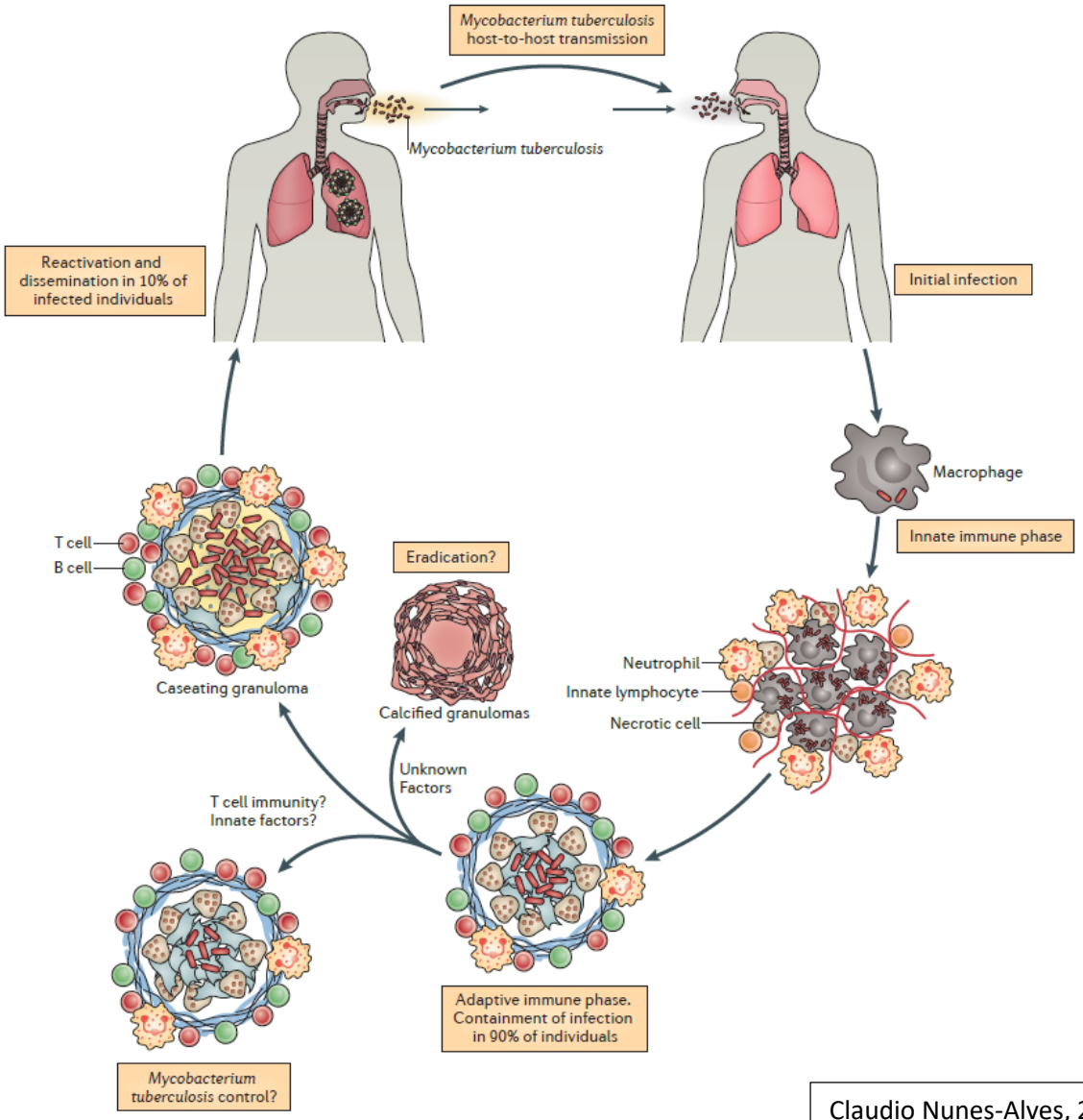


Figure 3. M.tb infection is initiated by the inhalation of aerosol droplets that contain M.tb. The initial stages of the infection are characterized by innate immune responses that involve the recruitment of inflammatory cells to the lung. Following bacterial dissemination to the draining lymph node, dendritic cell presentation of bacterial antigens leads to T cell priming and triggers an expansion of antigen-specific t cells, which are recruited to the lung. The recruitment of T cells, B cells, activated macrophages and other leukocytes leads to the establishment of granulomas, which can contain M.tb. Most infected individuals will remain in a 'latent' state of infection, in which no clinical symptoms are present. A small percentage of these people may eventually undergo a progression of their infection and develop active disease, which can lead to the release of M.tb from granulomas that have eroded into the airways. When individuals with active TB cough, they can generate infectious droplets that transmit the infection to other people allowing continuous infection

1.2.3 Innate immunity to *M.tb.*

The initial stages of *M.tb* infection are characterized by innate immune responses, which represent an important, nonspecific, defense that involves the recruitment of inflammatory cells to the lungs [54]. Innate immune responses begin with the process of bacterial phagocytosis (the uptake of *M.tb)* by professional phagocytes such as AM, which are the primary cell types involved in the initial uptake of *M.tb*. After this first encounter, dendritic cells, neutrophils, as well as epithelial cells take part in the phagocytic process [57]. The phagocytosis process is accompanied by recognition of *M.tb* or its products by pattern recognition receptors (PRRs) leading to initiation of an immune response.

Toll-like receptors (TLRs) are PRRs expressed on/in macrophages and dendritic cells that recognize molecules expressed on invading bacteria [58]. Although at least 10 TLRs have been identified to date, only TLR2, TLR4 and TLR9 have been shown to be involved in cellular responses to *M.tb* resulting in production of IL-12, a strong pro-inflammatory cytokine [59]. TLR2 is believed to be important in the initiation of innate host defense through its stimulatory effects on TNF-α production in macrophages. A mutation in TLR2 was found to inhibit *M.tb*-induced TNF-α production [60]. TLR2 has also been shown to be important for IL-12 release from macrophages [61]. $TLR2^{-/-}$ mice show defective granuloma formation, and when infected with high doses of *M.tb*, display greatly enhanced susceptibility to *M.tb* infection compared to the wild type (WT) mice. In addition, $TLR2^{-/-}$ mice display defects in controlling chronic infection with *M.tb* [62]. Studies with TLR4-transfected Chinese hamster ovary (CHO) cells and murine macrophages showed the importance of TLR4 in recognition of *M.tb* [63].

Macrophages of TLR4-deficient mice produce less TNFα upon *M.tb* infection than macrophages of WT mice [64]. In addition, expression of TLR4 confers responsiveness to both virulent and attenuated *M.tb* [63]. Finally, TLR9 is known to recognize unmethylated CpG (cytosine and guanine separated by only one phosphate) motifs in bacterial DNA. *In vitro* studies have shown that *M.tb*-induced IL-12 release in dendritic cells is TLR9-dependent. *In vivo* experiments also showed that when mice are infected with a high infectious dose of *M.tb*, animals lacking TLR9 succumb earlier to infection than WT animals [65]. Infection of phagocytic cells by *M.tb* and recognition by TLRs lead to cellular responses that activate transcription factors such as NF-ƙB which in turn induce production of cytokines [66] and chemokines [67]. Cytokines and chemokines are secreted primarily by white blood cells, T cells, and epithelial cells and are involved in cellular immune responses. In most cases, the innate immune response is not sufficient to protect against *M.tb* and an adaptive immune response is mounted to control or eliminate the infection [68].

1.2.4 Adaptive immunity to *M.tb*

Adaptive immunity becomes important following the failure of innate immune mechanisms to control *M.tb* growth and sterilize the infection. At this point, the increasing immune pressure mounted by the adaptive immunity may restore the immunological control [69]. There are two types of adaptive immune responses: humoral immunity, mediated by antibodies produced by B cells; and cell-mediated immunity, mediated by T cells. T cell-mediated immunity is considered the central element of anti-*M.tb* immunity and includes the development of antigen (Ag)-specific T cells (lymphocytes) to eliminate *M.tb* [70].

Unlike the innate immune system that consists of cells and proteins that can readily be mobilized to control an infection, the T cell-mediated immune response is normally silent and has to be initiated by antigen presenting cells (APC) which are capable of capturing and presenting antigens to naïve T cells. When stimulated by an antigen, the receptors of APC cause the stimulating antigen to be endocytosed and display the antigen on their surface via major histocompatibility complex (MHC) class II molecules. Activated APC then synthesize chemokines which are chemotactic cytokines that facilitate their migration to lymph nodes where they can display those antigens to groups of naïve T cells to cause their activation. Activated T cells divide and mature, producing effector cells that cause (i) secretion of macrophage-activating cytokines; (ii) lysis of infected host cells by cytotoxic T cells (CTL) and (iii) direct death or elimination of the microbe. **(Figure 4).** (**Figure 29 in appendices**).

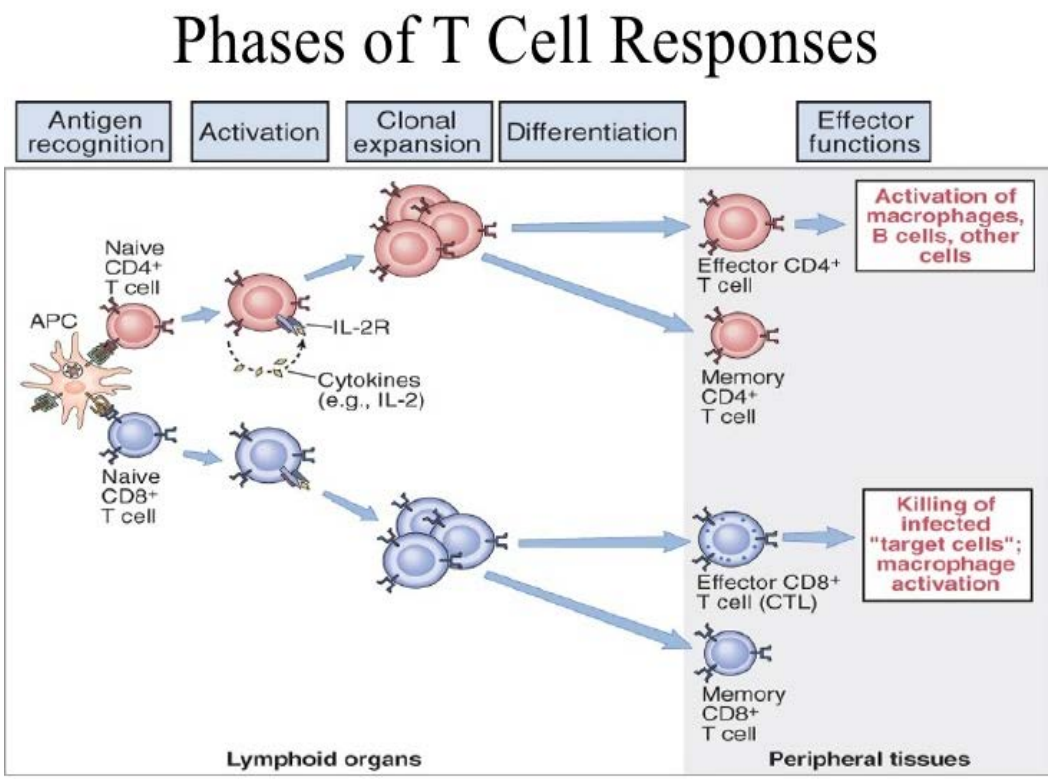


Figure 4. *T cell-mediated immune responses are initiated by APCs that present antigens to naïve lymphocytes resulting in their activation. Activated T cells divide and mature, producing effector cells that cause activation and killing of infected*

Modified from Abbas and Lichtman, 2008

Human immunity against *M.tb* is primarily a cell-mediated immune response promoted by Th1 cells. The induction of antigen-specific Th1 immunity is characterized by the production of IFN-γ and is a crucial prerequisite for efficient protective immunity against *M.tb* [28]. In contrast, Th2 cells secrete IL-4 and IL-10 which block *in vitro* production of IFN-γ and microbial killing leading to suppression of Th1 responses [71-73]. Primary deficiencies such as mutations in interferon gamma receptors 1 and 2 (IFNGR1 and IRNGR2), STAT1, IL-12B, IL-12B1 etc. have dramatically shown the importance of cellular immunity in TB [74]. T-cell mediated elimination or sterilization of *M.tb*-infected macrophages depends to a large extent on the ability of T cells to differentiate into CD4 and CD8 effector T cells. Evidence from both animal and human models suggests an important role for both CD4 and CD8 T cells in successful immune control of *M.tb* infection.

CD4 T cells have been shown to be the predominant source of IFN-γ and interleukin 2 (IL-2), and are (i) capable of cytotoxicity against *M.tb*-infected target cells [75] and (ii) critical for the induction of protective-memory immunity, delayed-type hypersensitivity responses, and the development and maintenance of CD8 T-cell responses during *M.tb* infection [76]. The critical role of CD4 T cells in *M.tb* infection has been demonstrated clearly in HIV-positive patients with CD4 T cell depletion who show an increased susceptibility to primary *M.tb* infection as well as reactivation of latent *M.tb* infection and development of TB [77]. Further studies provide evidence that mice lacking CD4 T cells show increased susceptibility to development of TB, compared with WT mice [78, 79].

CD8 T cells contribute to the cellular immune defense against TB [80] early during *M.tb* infection [81]. The role of *M.tb*-specific CD8 T cells was confirmed by their

appearance in the airway lumen at the beginning of experimental *M.tb* infections in mice [82]. The effector functions of CD8 T cells during *M.tb* infection are represented by the ability of CD8 T cells to lyse infected phagocytes and to directly kill intracellular bacteria through the production of granzymes and perforins [83]. Recent studies suggest progressive dysfunction of CD8 T cells in chronic *M.tb* infection. For example, Lazarevic et al. reported that CD8 T cells in *M.tb*-infected mice gradually lose their lytic potential during the progression to the chronic phase of infection [84]. Studies have shown that CD8 T cells from individuals with pulmonary TB display decreased cytolytic activity and expression of cytotoxic molecules, compared with cells from uninfected healthy controls [85, 86].

1.2.5 Environmental factors affecting T cell immunity to *M.tb*

T cell immunity to *M.tb* can be affected by exposure to environmental air pollutants. For example, inhalation exposure to cigarette smoke has been identified as a major factor that increases susceptibility to *M.tb* infection and the risk of development of TB. In a mechanistic study cigarette smoke was shown to impair antigen-specific T cell responses to *M.tb* by blunting the recruitment of $CD4^{+}IFN\text{-}\gamma^{+}$ T cells to the lung [11]. Another study reported a decreased influx of TNF-α-producing and IFN-γ–producing CD4 and CD8 effector and memory T cells into the lungs and spleens of cigarette smoke-exposed mice infected with *M.tb* [12].

Studying the effects of air pollution PM on *M.tb* infection is crucial as air pollution from growing industrial production and vehicular traffic in rapidly growing cities is increasing, particularly in low and middle income countries with high levels of endemic TB. Also, both aerosolized PM and inhalable pathogens share the primary portal of entry i.e. the respiratory tract. Some epidemiological studies have reported a correlation between exposure to biomass fuels (wood or dung) and susceptibility to *M.tb* infection [14, 87, 88]. In a study, atmospheric pollution (with dust, nitric oxide, nitric dioxide, carbon monoxide and sulfur dioxide) was found to increase the incidence of TB [89].

While there is evidence from epidemiological studies that PM plays a major role in increasing susceptibility to *M.tb* infection, no studies have mechanistically examined the role of urban PM exposure in the modulation of *M.tb*-specific T cell-mediated immunity. Studies investigating effects of air pollution on anti-mycobacterial host immune responses have for the most part focused on innate immunity [90, 91].

1.3 RATIONALE

Preliminary results from Dr. Schwander's laboratory showed defective production of pro-inflammatory cytokines IFN-γ and TNF-α during *M.tb* infection upon PM exposure. It is known that cell-mediated immune responses mediated by functional T cells depend to a great extent on the production of IFN-γ, the cytokine that activates macrophages for *M.tb* infection control. Based on this preliminary result, we investigated the potential adverse mechanisms of PM exposure on *M.tb*-specific T cell functions and the control of *M.tb* infection.

1.4 HYPOTHESIS

We hypothesize that exposure to urban air pollution-derived PM adversely affects adaptive anti-mycobacterial immunity by modulating *M.tb*-specific T cell responses.

1.5 OBJECTIVES

This study aimed to study the effects of PM exposure on T cell functions that contribute to the control of *M.tb* infection. Our approach involves

(i) Examining the uptake of PM and *M.tb* within the same human monocytes in order to investigate the process of immune responses initiation and the possibility of functional interactions of PM and *M.tb*

(ii) Assessing the cellular cytotoxicity from PM exposures in order to ascertain whether any observable functional alterations by PM are a result of cell death

(iii) Studying the effects of PM exposure on antigen presentation, costimulatory signals, and T cell activation

(iv) Determining the effects of PM on *M.tb*–induced cytokine production

(v) Determining the effects of PM on *M.tb* growth control

(vi) Seeking to elucidate the mechanisms by which PM adversely affects *M.tb*-specific human host T cell functions.

1.6 SPECIFIC AIMS

1. To assess $PM_{2.5}$ and *M.tb* uptake by monocytes, assess $PM_{2.5}$-induced cellular toxicity and investigate the role of $PM_{2.5}$ in altering growth control of *M.tb* by human PBMC.

2. To investigate $PM_{2.5}$ effects on the markers of antigen-presenting cell activation, co-stimulation, and T-cell activation during *M.tb* infection.

3. To examine the effects of $PM_{2.5}$ on *M.tb*-induced cytokine production.

4. To study $PM_{2.5}$ effects on cellular activation processes involved in IFN-γ production.

2.0 MATERIAL AND METHODS

2.1 Study Approval

This study in healthy persons in New Jersey, the collection of personal health information, venipunctures to obtain blood, exposure of blood cells to PM collected in the Mexico City area, and infection of the blood cells with *M.tb,* were approved by the Institutional Review Board of Rutgers, The State University of New Jersey in Newark and New Brunswick (Protocol number 2012001383).

2.2 Human Subjects

A total of twenty-one healthy volunteers (7 male, 14 female, median age 28 yrs., range 20 - 62 yrs.) were recruited from students and staff of Rutgers University and the local community in Piscataway at the Environmental and Occupational Health Sciences Institute (EOHSI). A total of 100 mL whole venous blood was obtained by venipuncture from each of the study subjects. Healthy women or men in the age range from 18-65 yrs., with a minimum weight of 110 pounds who were willing to provide informed consent and blood were eligible. Persons with immunosuppressive or chronic medical conditions, undergoing long-term medications, or who were smokers or users of illicit drugs were excluded. A summary of basic demographic information of donors included in different experiments is given in **table 1**.

	LDH	Apoptosis	Killing assay	CD69	IFN-γ	TNF-α/IL-10	Western blot
Number (male)	10(6)	3(0)	11(6)	7(1)	9(1)	6(1)	5(1)
Mean age (range)	30.6(20-62)	42(36-52)	29.9(20-62)	30.5(20-49)	35.3(20-52)	37.5(25-52)	40(28-52)

Table 1. A summary of basic demographic information of donors included in different experiments.

100ml of heparinized venous blood was obtained from 21 healthy volunteers in NJ included in different experiments.

2.3 Preparation of PM

Urban outdoor $PM_{2.5}$ was collected on the roof top of the National Institute of Ecology and Climate Change (CENICA) on the premises of the Autonomous Metropolitan University (UAM) in the Iztapalapa municipality of Mexico City. PM collections were done in the context of the NIEHS-funded project *Air Pollution Particle Effects on Human Antimycobacterial Immunity* (5R01ES020382, PI S. Schwander). PM were collected with high-volume samplers (GMW Model 1200, VFC HVPM10, airflow rate 1.13m^3/min, as previously described [19] in 2012/2013 (**Figure 29 in appendices**). Following removal of the PM from nitrocellulose filters [47], $PM_{2.5}$ was weighed using a semi microbalance (CPA225D; Sartorius, Bohemia, NY, USA) and stored at 4°C in glass flasks until use. Stock suspensions of $PM_{2.5}$ (1 mg/ml) were prepared by 5 min of sonication (model 351OR-DTH; Branson, Danbury, CT, USA) in RPMI 1640 (Bio Whittaker, Lonza Walkersville, MD) supplemented with L-glutamine (Thermo Fisher, Waltham, MA) and

further diluted in complete culture medium (RPMI 1640 supplemented with L-glutamine and 10% pooled human AB serum (HS) (Valley Biomedical, Inc., Winchester, VA) to final concentrations of 1 and 5 µg/ml.

2.4 Preparation of PBMC

PBMC were isolated from whole heparinized venous blood collected from study subjects by Ficoll gradient centrifugation [92]. Briefly, whole peripheral venous blood was diluted with L-glutamine supplemented RPMI 1640 medium (ratio 1:1), overlaid on Ficoll-Paque and subjected to gradient density centrifugation (1200rpm at 21°C for 45 min). The cell interface was then removed and washed three times in RPMI 1640. Resulting PBMC were re-suspended in complete culture medium (RPMI 1640 supplemented with L-glutamine and 10% pooled human AB serum), counted and adjusted at required concentrations. Viability of PBMC was 98-100% by trypan blue exclusion in all experiments.

2.5 PBMC Exposures to PM *in vitro*

In each experimental condition, PBMC were seeded in duplicate wells into 96-well plates (200,000 cells in 100µL complete culture medium per well) or 5 ml round bottom polypropylene tubes (10^6 cells /ml RPMI-10% PHS) and exposed to either $PM_{2.5}$ alone or to $PM_{2.5}$ prior to infection with *M.tb*. For exposures to $PM_{2.5}$ alone, PBMC were exposed to $PM_{2.5}$ at final concentrations of 0 (No PM as negative control), 1, and 5 µg/ml in complete culture medium and incubated (37°C, 4% CO_2, humidified environment) for 20 h. For cell exposures to PM prior to infection with *M.tb*, PBMC were exposed to $PM_{2.5}$ for 20 h (0, 1, and 5 µg/ml, 37°C), washed twice with 1X PBS, and then infected with *M.tb* in complete culture medium for an additional 18 h.

2.6 Preparation of *M.tb* for *in vitro* Infections

Suspensions of *M.tb* (H37Ra, ATCC 25177, Manassas, VA) were prepared in Middlebrook 7H9 broth medium supplemented with 10% albumin dextrose catalase (BD Bioscience) and 0.2% glycerol. After a 21-day incubation period at 37°C on an orbital shaker, *M.tb* stock suspensions were harvested, aliquoted, and stored at −86 °C until use.

For PBMC infection experiments, single cell *M.tb* suspensions were prepared as follows: frozen *M.tb* stock was thawed, centrifuged for 5 min at 8000 × g and re-suspended in complete culture medium. To generate single bacterial cell suspensions, *M.tb* stock suspensions were then de-clumped by vortexing for 5 minutes in the presence of 5 sterile 3-mm glass beads. Any remaining *M.tb* clumps were removed in an additional centrifugation step at 350 × g for 5 minutes. For each infection experiment, percentages of monocytes in PBMC were confirmed by flow cytometry. This allowed the determination of appropriate volumes of *M.tb* suspensions to obtain the desired multiplicities of infection (MOI, i.e. the ratios of *M.tb* to monocytes) of 1 (MOI1) and 5 (MOI5) for the *in vitro* infections. Concentrations of frozen *M.tb* stock suspensions were confirmed after thawing of the aliquots and the declumping procedures in each infection experiment by assessing colony-forming unit (cfu) numbers from serial culture dilutions after 21 day-incubations on 7H10 solid agar plates.

2.7 Infection with *M.tb* and CFU Assays

PBMC were infected with *M.tb* at MOIs of 1 and 5 (37°C), washed twice with warm PBS after 2 h to remove extracellular *M.tb* and further incubated (37°C and 5% CO2) for 1 h (day 0), 1, 4, or 7 days. *M.tb* growth in PBMC, *i.e*. the mycobactericidal capacity of PM-exposed PBMC, was assessed as described previously [76]. Briefly, PM-exposed, *M.tb*-infected, or PM-exposed, and *M.tb*-infected PBMC were washed twice with 1X PBS and then lysed with 0.1% Sodium Dodecyl Sulfate (SDS) (10 min at room temperature) to release any remaining viable intracellular *M.tb* cells. The SDS action was neutralized by the addition of Middlebrook 7H9 broth enriched with 20% bovine serum albumin (BSA) to each well. Four serial cell lysate dilutions (1:10) were then prepared and plated in triplicate (10µl each) onto 7H10 agar plates. The plates were incubated at 37°C for cfu assessments at 21 days using a stereomicroscope at a magnification of X40 (Fisher Scientific, Massachusetts USA).

2.8 Lactate dehydrogenase (LDH) assay

To assess the viability of cells during PM exposure and *M.tb* infection experiments, cell culture supernatants were collected to measure the concentrations of LDH following PBMC exposures to PM and infections with *M.tb.* Release of LDH indicates cell damage. Supernatants (50 µL) from three replicate wells per sample were transferred into 96-well assay plates and 50 µL of substrate (CytoTox 96 Non-radioactive cytotoxicity Assay, Promega, Madison, WI) immediately added to each well. Following incubation at room temperature for 30 minutes in the dark, stop solution (50 µL) was added to each well and absorbance recorded at 493 nm with an ELISA reader (ThermoScientific Multiskan FC, Finland). Cellular toxicity was defined as percent (%) LDH leakage from cells calculated

from the ratios of ODs of PM-exposed / *M.tb*-infected PBMC to the ODs of unexposed PBMC x 100.

2.9 Cell Surface Immunostaining and Intracellular Cytokine Staining

Using flow cytometry, we assessed PM effects on cellular viability (using fixable viability dye eFluor 780) as well as the expression of T cell surface markers (CD3, CD4, CD8, CD16, CD69) as well as intracellular IFN-γ, TNF-α, and IL-10. Uninfected, PM-pre-exposed, *M.tb*-infected or PM-pre-exposed and *M.tb*-infected PBMC (1 X 10^6/ml in 5 ml round bottom polypropylene tubes) were washed twice with phosphate-buffered saline (PBS) and re-suspended in flow cytometry staining buffer (Affymetrix eBioscience, San Diego, CA). This was followed by staining with fluorescence-conjugated monoclonal antibodies (Affymetrix eBioscience, San Diego, CA except where noted otherwise): anti-Human CD3 (clone OKT3)-Alexa Fluor 700; anti-Human CD4 (clone OKT4)-Alexa Fluor 488; anti-Human CD8a (clone RPA-T8)-PE-Cyanine7; and anti-Human CD16 (clone 3G8)-PE/Dazzle 594 (BioLegend, San Diego, CA); and CD69 (clone FN50)-PE-CF594 (BD Biosciences, San Jose, CA). For intracellular cytokine staining and detection of IFN-γ, TNF-α and IL-10, protein transport inhibition cocktail (Brefeldin A and Monensin, Affymetrix eBioscience, San Diego, CA) at 2µl/ml was added to the respective cell culture media during the last 6 - 10 hours of culture. At the end of the culture period, cells were washed twice with PBS, and fixable viability dye eFluor 780 (Affymetrix eBioscience, San Diego, CA) added. Following surface staining, fixation and permeabilization buffer (Affymetrix eBioscience, San Diego, CA) was added. Cells were then stained with anti-Human IFN gamma (clone 4S.B3)-phycoerythrin, anti-Human TNF alpha (clone Mab11)-allophycocyanin and anti-Human IL-10 (clone BMS131-2FI)-Fluorescein isothiocyanate.

Samples stained with antibodies were acquired by Gallios flow cytometer (Beckman Coulter 405nm, 488nm, 633nm laser) and data analyzed with Kaluza Analysis software (Beckman Coulter).

2.10 Apoptosis Assay by Annexin V Staining

To determine PM effects on apoptosis and necrosis of monocytes and lymphocytes in PBMC, uninfected; PM-pre-exposed; *M.tb*-infected; or PM-pre-exposed and *M.tb*-infected PBMC (1 X 10^6/ml in 5ml round bottom polypropylene tubes) were washed twice with PBS. FITC Annexin-V Apoptosis Detection Kit (BD Pharmingen cat: 556547) was used to detect phosphatidylserine (PS) exposure. PS which is usually present on the inner leaflet of plasma membrane of healthy cells, is translocated to the outer leaflet in apoptotic cells, thereby exposing PS to the external cellular environment. Detection of PS exposure thus indicates cell death. Annexin-V FITC/propidium iodide double-staining to assess the proportion of mononuclear cells that were simultaneously undergoing apoptosis (Annexin V positive) and necrosis (Propidium Iodide positive) was performed according to manufacturer's protocol. Induction of apoptosis and necrosis was evaluated by flow cytometry using a Gallios flow cytometer (Beckman Coulter) and Kaluza Analysis software (Beckman Coulter).

2.11 Flow Cytometry Analysis

To analyze IFN-γ, TNF-α, IL-10, and CD69 expression by T cells and monocytes, we used a sequential gating strategy. An initial gate was set on FSC vs. SSC dot plot to contain cells and exclude debris and cell aggregates. The live cells were gated to exclude dead cells on the channel of Fixable Viability dye eFluor 780. Compensation was performed using single

color controls prepared from negative control and anti-mouse Ig compensation beads (BD Biosciences, Franklin lakes, NJ). Logical scaling was used when necessary for compensation using Kaluza Analysis Software. Appropriate and matched isotype controls were used for negative controls and FMO (Fluorescence Minus One) controls were used for gating analysis to distinguish positive from negative cell populations. 70,000 live cell gates were created for acquisition and subsequent analysis of cells for each experimental sample.

2.12 Transmission Electron Microscopy

Transmission electron microscopy (TEM) was employed to examine cellular uptake of PM and *M.tb* by monocytes in PBMC and explore their intracellular localizations. Preparations of PBMC for TEM were done as follows: uninfected, PM-exposed (20 h), *M.tb*-infected (18 h) or PM-pre-exposed and *M.tb*-infected (18 h infection following initial 20 h PM-exposure) PBMC were fixed in 2.5% glutaraldehyde-4% paraformaldehyde in 0.1 M cacodylate for 1 hr at room temperature. Cells were then washed with PBS, post-fixed in buffered 1% osmium tetroxide, dehydrated in a graded series of acetone, and embedded in Epon resin. Fixed cells were cut into 90-nm thin sections using a Leica EM UC6 ultramicrotome. Sectioned grids were then stained with a saturated solution of uranyl acetate and lead citrate. Images were captured with an AMT XR111 digital camera (Advance Microscopy Techniques, Woburn, MA) on a Philips CM12 transmission electron microscope.

2.13 Evaluation of Transcription Factors T-bet, STAT-1 and STAT-3 by Western Blot

T-bet and STAT-1 are transcription factors involved in the expression of IFN-γ, while STAT-3 is a transcription factor that represses pro-inflammatory cytokines, including IFN-γ. To examine PM effects on the expression levels of T-bet, and phosphorylation of STAT 1 and STAT 3, PBMC were exposed to 0 and 5 µg/ml of $PM_{2.5}$ in culture media for 20 h followed by *M.tb* infection at MOI1 or MOI5 or exposed to purified protein derivative (PPD), 10 µg/ml, used in tuberculin skin testing (TST). Following an infection period of 24 h, PBMC were lysed with RIPA (radio immunoprecipitation assay) lysis buffer system (Santa Cruz Biotechnology, Dallas, TX) and quantified by Bradford Protein Assay (Bio-Rad laboratories, Hercules, CA). Protein lysates were analyzed by SDS/PAGE followed by transfer onto polyvinylidene difluoride (PVDF) membranes. T-bet (cat no 13232S), STAT-1 (cat no 7649S), STAT-3 (9139S) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH: cat no 5174S) - proteins were analyzed by western blotting with specific antibodies (Cell Signaling Technology, Danvers, MA).

2.14 Statistical Analysis

All of the data were processed in Prism (GraphPad prism 6, 2014). Means and standard deviations summarized the levels of cytokines and cell counts with and without PM and stimulants (*M.tb*, PPD, PHA, and LPS). To examine the effects of PM and stimulant on cytokine levels/cell counts, we used mixed linear regression models. Random intercepts for subjects accounted for correlation between measurements of samples from the same individuals. We considered each factor (PM and stimulant) separately, stratified by levels of all other factors, and also examined whether PM modified the effect of stimulant by adding an interaction. F-tests were used to assess significance overall while t-tests were used to examine pairwise differences. For these analyses, counts were log-transformed counts because their distribution were right-skewed. All data are presented as means ± Standard error of the mean (SEM). ($_P < 0.05$) was considered statistically significant. In 5-95 percentile box and whisker plots, the center represents the 50^{th} percentile, the upper hinge is the 75^{th} percentile, and the lower hinge is the 25^{th} percentile. In the western blot data, fold changes in protein expression and phosphorylation were expressed relative to the change from the unexposed/uninfected cells results.

3.0 SPECIFIC AIMS AND RESULTS

3.1 Aim 1: To assess $PM_{2.5}$ and *M.tb* uptake by monocytes, assess $PM_{2.5}$-induced cellular toxicity and investigate the role of $PM_{2.5}$ in altering growth control of *M.tb* by human PBMC.

3.1.1 Aim 1a: To examine the uptake of $PM_{2.5}$ and *M.tb* in human monocytes

3.1.1.1 Rationale: The initial events during *M.tb* infection involve phagocytosis of the bacteria by phagocytes [93] and antigen presenting cells (monocytes, macrophages and dendritic cells). Also, the initiation of innate and subsequent adaptive host immune responses depend on the uptake of the invading pathogen. To the best of our knowledge, no data is available on the concurrent uptake of *M.tb* and real life air pollution PM by human monocytes.

3.1.1.2 Results: After pre-exposure to 5µg/ml of $PM_{2.5}$ for 20h followed by infection with *M.tb* MOI 5 for 18hours, uptake of *M.tb* and PM by monocytes was observed by TEM. Multiple *M.tb* bacteria were observed within membrane-enclosed vesicles (Fig 5A, B, E, and F). We observed clusters of free, non-membrane bound $PM_{2.5}$ in the cytoplasm of $PM_{2.5}$-exposed monocytes (Fig 5C and 5D) as well as extracellularly (circular insets in Fig 5E and 5F), and concurrent uptake of *M.tb* and $PM_{2.5}$ in monocytes (Fig 5E and 5F). No PM clusters were noted in $PM_{2.5}$-unexposed monocytes (not shown) or monocytes infected with *M.tb* only (Fig 5A and 5B). $PM_{2.5}$ clusters were never observed in nuclei of the cells.

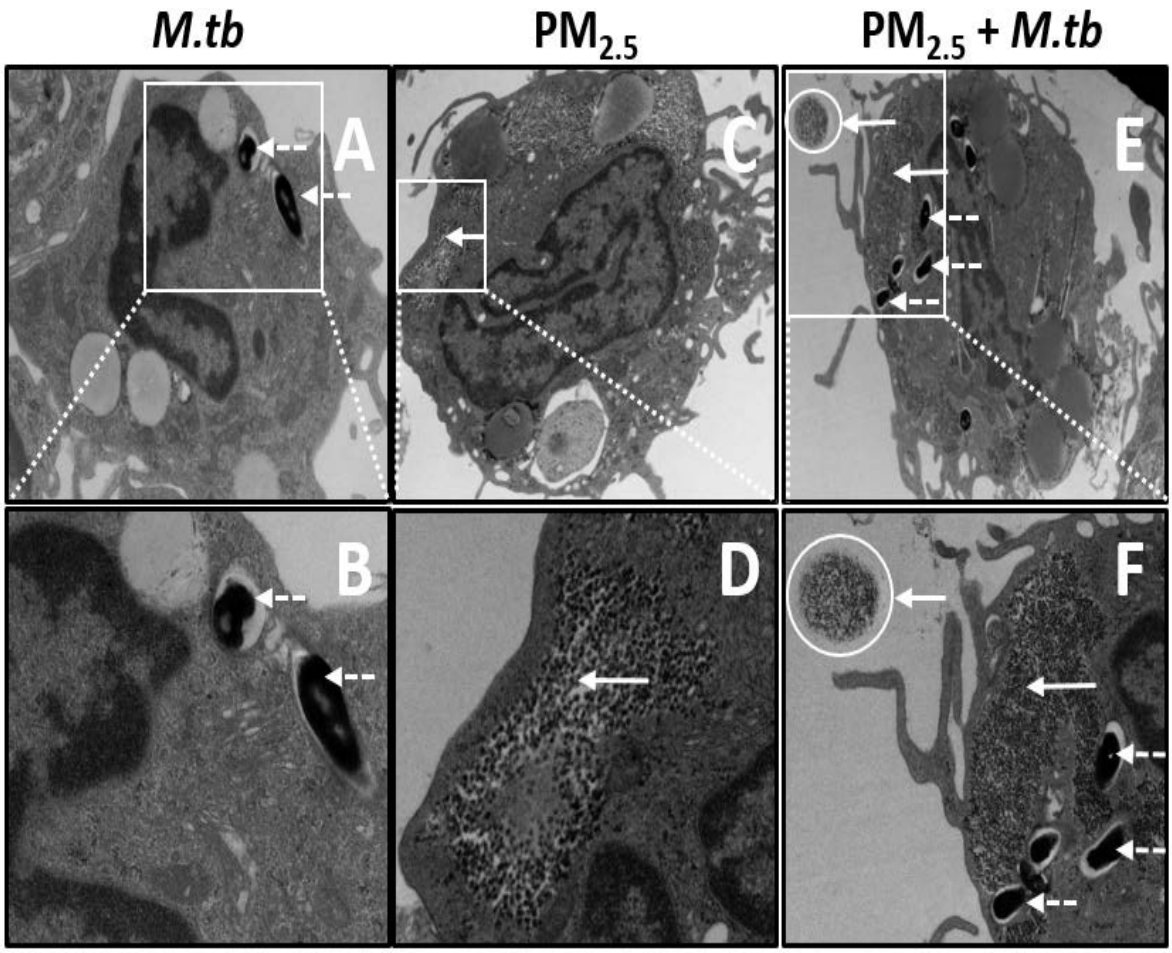


Figure 5. Transmission electron microscopy (TEM) of $PM_{2.5}$ and M.tb uptake in peripheral blood monocyte.

(A, B) Endocytic vacuoles containing M.tb (white dotted arrows) showing uptake of multiple M.tb by a monocyte. (C, D) Monocyte containing air pollution $PM_{2.5}$ (20 h exposure to 5µg/ml $PM_{2.5}$) (white solid arrows). (E, F) Monocyte showing endocytic vacuoles containing M.tb (white dotted arrows) and clusters of $PM_{2.5}$ (white solid arrows) 20 h exposure to 5µg/ml $PM_{2.5}$, followed by M.tb infection (MOI 5) for additional 18 h.

Free aggregate of $PM_{2.5}$ outside the cell is shown in E & F (white circular inserts). Magnification X 8000 (A, C, E) and zoomed images (B, D, F).

3.1.2 Aim 1b: To examine the cytotoxic effects of $PM_{2.5}$ and PM_{10} in human PBMC

3.1.2.1 Rationale: PM-induced damage to cells would lead to defective functional activity. Lactate Dehydrogenase (LDH) is a stable enzyme found inside every living cell and released into the surrounding extracellular space when cell membranes are damaged [94]. LDH assay quantitates the relative amounts of live and dead cells in cell culture supernatants by measuring the amount of released LDH using a colorimetric method. Release of high levels of LDH indicates cellular damage and loss of viability. To ascertain that any observed PM-induced alterations to the *M.tb*-induced functional responses of the PBMC did not result from cellular death (reduced cellular **v**iability) upon exposure to PM, we assessed the viability of cells exposed to PM alone and in the context of *M.tb* infection by LDH assay.

3.1.2.2 Results: To assess the cellular cytotoxicity resulting from PM exposures, PBMC from six study subjects were exposed to $PM_{2.5}$ or PM_{10} at final concentrations of 0 (negative control), 1, or 5µg/ml for 2h, 1, 4, and 7 days and cell viabilities were determined by measuring cellular leakage (i.e. concentrations) of LDH into culture supernatants. The average of OD_{490nm} of replicate wells of each LDH standard, PM exposed samples, PM unexposed samples (controls) and blank (media only) were obtained, and average OD_{490nm} value of the blank was subtracted from the average OD_{490nm} values obtained with all other samples. Based on the calibrated OD_{490nm} of the LDH standard, a standard curve was made by plotting OD_{490nm} as a function of LDH

concentration. The equation of the trend line of the standard curve (Y=Ax + B) and R^2 value of the trend line were determined. The LDH concentration of the PM exposed samples and controls were calculated using the formula (LDH) = (OD_{490nm} – B) /A X 100. Exposure of PBMC to 1 and 5 µg $PM_{2.5}$ (Fig 6A) or PM_{10} (Fig 6B) for 2 h, 1, 4, or 7 days did not cause significant increases in LDH release. Cellular cytotoxicity was also assessed in PBMC from ten study subjects following 1 and 5 µg of PM pre-exposures (final concentration of 0, 1, or 5 µg/ml) for 20 h and infections with *M.tb* MOI1 for 2 h, 1, 4, and 7 days. For each day, PBMC incubated in complete culture medium alone served as negative controls. No statistically significant difference between control PBMC and *M.tb*-infected or $PM_{2.5}$-exposed (1 and 5 µg/ml) and –*M.tb*-infected PBMC on days 0, 1, 4, and 7 among the donors were observed (Fig 7A). Also, no statistically significant differences between control PBMC and *M.tb*-infected or PM_{10}-exposed (1 and 5 µg/ml) and –*M.tb*-infected PBMC on days 0 and 1 were observed. However, on days 4 and 7, significant death in *M.tb*-infected PBMC pre-exposed to 1 and 5 µg/ml of PM_{10} (Fig 7B) was noted. Based on these observations as well as the generally acknowledged greater importance of $PM_{2.5}$ than PM_{10} for air pollution health effects, we used $PM_{2.5}$ for subsequent studies.

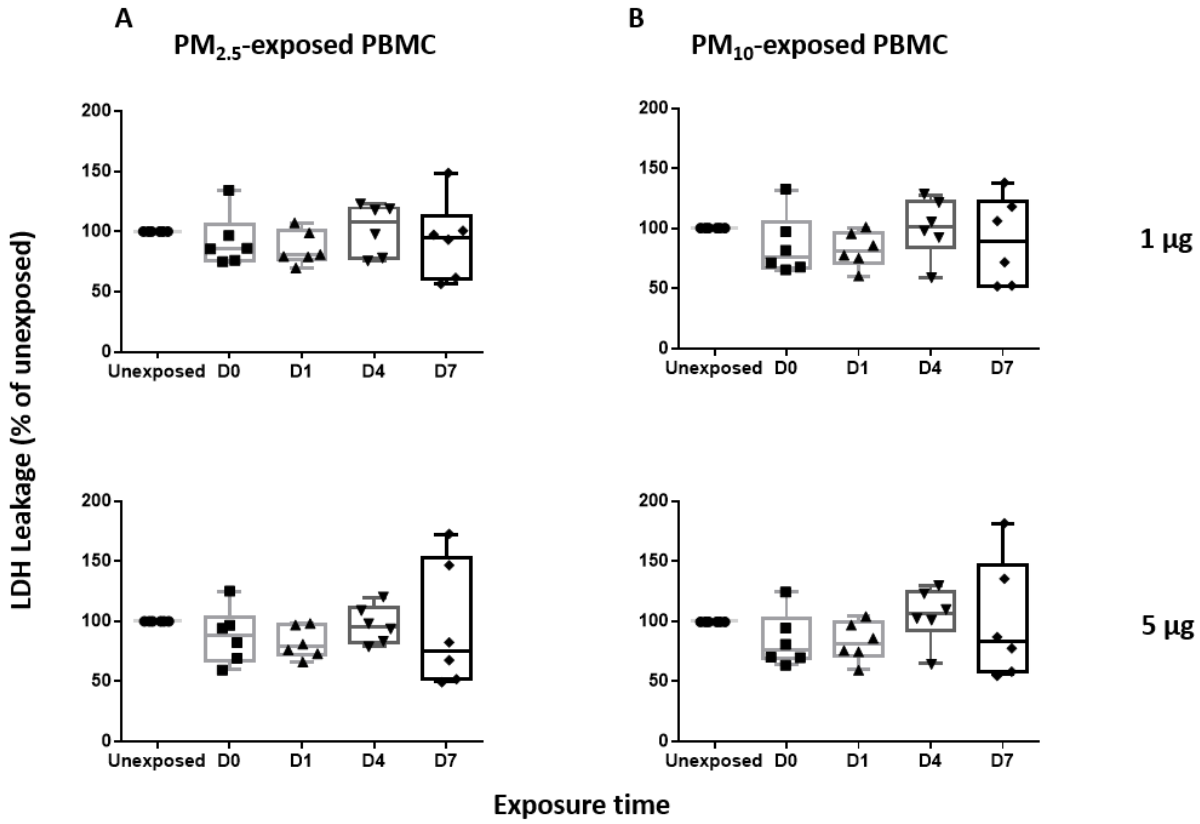


Figure 6. Cytotoxic Effects of $PM_{2.5}$ and PM_{10} (1µg and 5µg) in Human PBMC.

PBMC from 6 study subjects were exposed to $PM_{2.5}$ (A) and PM_{10} (B) at final concentrations of 0, 1 and 5µg/ml for 0, 1, 4, and 7 days. Leakage of LDH into culture supernatant, as a measure of cell viability, was determined. Figures are scatter plots of individual donors superimposed on box plots and expressed as the mean +/- SEM of six independent experiments.

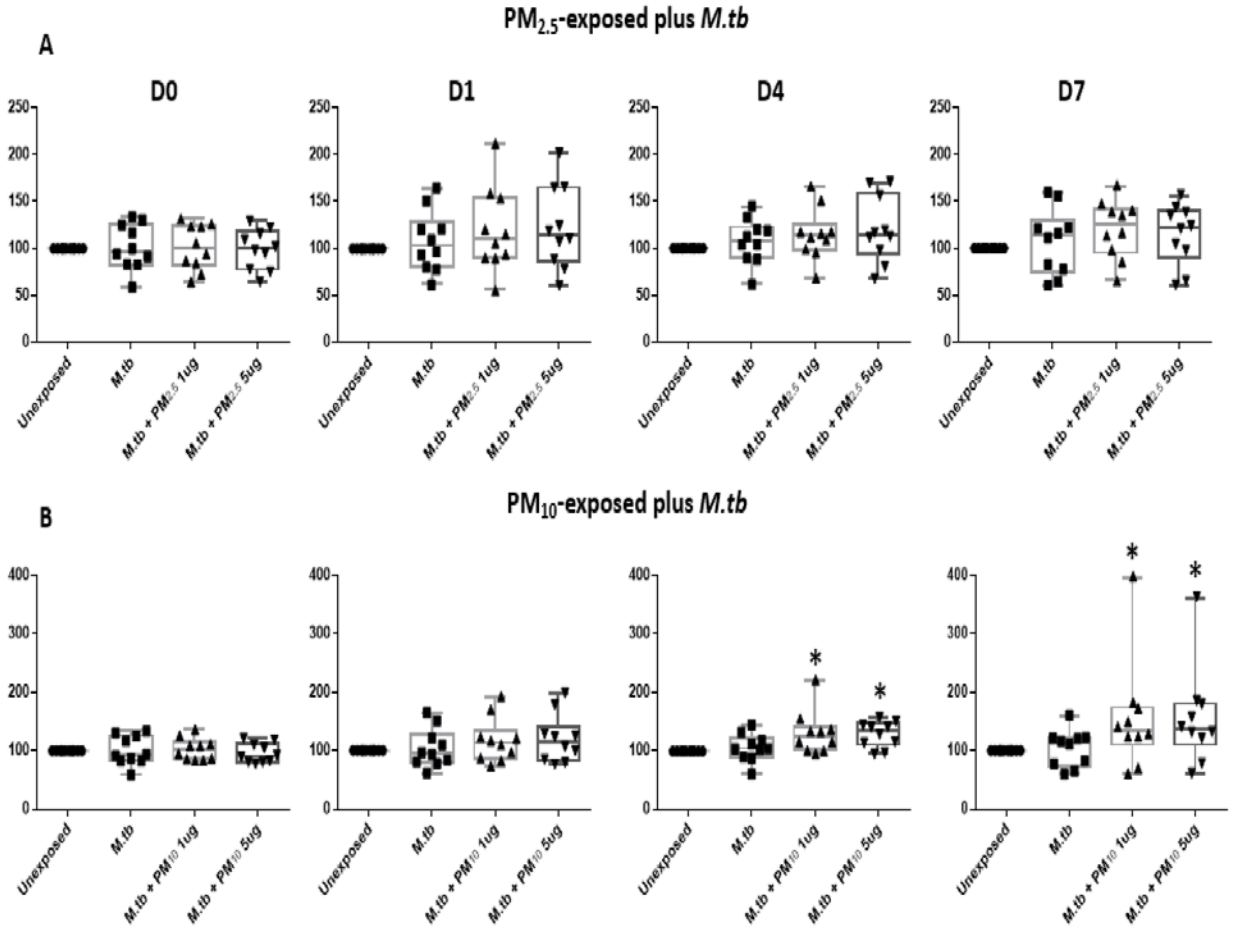


Figure 7. Cytotoxic Effects of $PM_{2.5}$ and PM_{10} (1 µg and 5 µg) in M.tb-infected PBMC.

Cytotoxic effects of $PM_{2.5}$ (A) and PM_{10} (B) on the viability of PBMC from 10 study subjects pre-exposed to $PM_{2.5}$ or PM_{10} for 20 h followed by infection with M.tb at multiplicities of infection (MOI) 1 for 0 (2 h), 1, 4, and 7 days were determined by measuring leakage of LDH. Figures are scatter plots of individual donors superimposed on box plots and expressed as the mean +/- SEM of ten independent experiments. Statistically significant differences between results for unexposed and PM-exposed- M.tb-infected-PBMC are shown with single ($P< 0.05$) asterisks.

3.1.3 Aim 1c: To examine the effects of $PM_{2.5}$ exposure on apoptosis and necrosis of mononuclear cells.

3.1.3.1 Rationale: In our studies, we used a PBMC model which allows lymphocyte-monocyte interactions required for T-cell activation and subsequent cytotoxic T cell functions. To further examine the extent of PM-induced toxicity on monocytes and lymphocytes in PBMC, we assessed the induction of apoptosis (programmed cell death) and necrosis (cell death due to injury) by flow cytometry in monocytes and lymphocytes, in addition to our LDH leakage measurements (discussed above).

3.1.3.2 Results: PBMC from three study subjects were pre-exposed to $PM_{2.5}$ (5 µg/ml) for 24 and 48 hours, corresponding to the cell culture periods during functional immune response experiments. Detection of phosphatidylserine residue exposure at the outer plasma membrane leaflet by Annexin-V FITC/propidium iodide double-staining was performed according to manufacturer's protocols to assess the proportion of lymphocytes and monocytes simultaneously undergoing apoptosis (annexin V-positive) and necrosis (propidium iodide-positive). Exposure of PBMC to 5 µg $PM_{2.5}$ for 24 and 48 hours (see Fig 8A & 8B: exemplary dot plots) did not cause any significant increases in proportions of monocytes (90.5% viable and 3.2% apoptotic monocytes in unexposed PBMC compared to 87.9% viable and 4.0% apoptotic monocytes in $PM_{2.5}$-exposed PBMC at 24 h); and (87.4% viable and 4.2% apoptotic monocytes in unexposed PBMC compared to 87.2% viable and 4.3% apoptotic monocytes in $PM_{2.5}$-exposed PBMC at 48 h) (Fig 8C) or lymphocytes (97.6% viable and 0.8% apoptotic lymphocytes in unexposed PBMC compared to 97.4% viable and 0.9% apoptotic lymphocytes in $PM_{2.5}$-exposed PBMC at 24 h); and (95.5% viable and 1.5% apoptotic lymphocytes in unexposed PBMC compared to

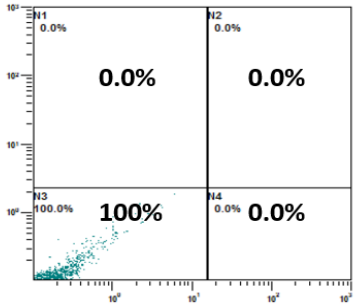
95.5% viable and 1.5% apoptotic lymphocytes in $PM_{2.5}$-exposed PBMC at 48 h) (Fig 8D) undergoing apoptosis and necrosis compared to unexposed PBMC. We also assessed if PM exposure (20 h) combined with *M.tb* infection at MOI1 and 5 for 18 h would induce apoptosis and necrosis in PBMC (Fig 9A & 9B: exemplary dot plots) . No statistically significant difference in the proportions of monocytes and lymphocytes undergoing apoptosis and necrosis were observed between control PBMC (89.8% viable, 3.4% apoptotic monocytes; and 97.8% viable, 0.7% apoptotic lymphocytes) and *M.tb*-infected MOI 1 (88.8% viable, 3.7% apoptotic monocytes and 98.2% viable and 0.6% apoptotic lymphocytes) (Fig 9C) and MOI5 (88.2% viable, 3.7% apoptotic monocytes and 98.0% viable and 0.6% apoptotic lymphocytes) (Fig 9D) or PM-exposed (87.6% viable, 4.1% apoptotic monocytes and 97.4% viable and 0.9% apoptotic lymphocytes) and PM-exposed–*M.tb*-infected (88% viable, 4% apoptotic monocytes and 97.6% viable and 0.8% apoptotic lymphocytes) MOI 1 (Fig 9C) and MOI 5 (87.5% viable, 4% apoptotic monocytes and 97.3% viable and 0.9% apoptotic lymphocytes) (Fig 9D) PBMC.

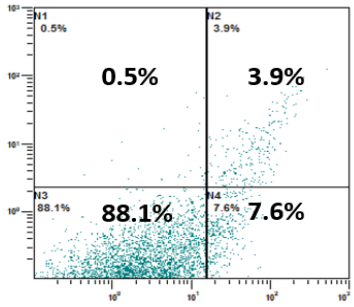
A

24 h

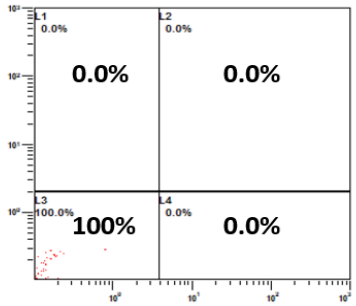
Untreated | **$PM_{2.5}$**

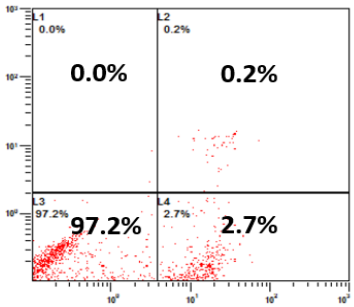
Monocyte





Lymphocyte





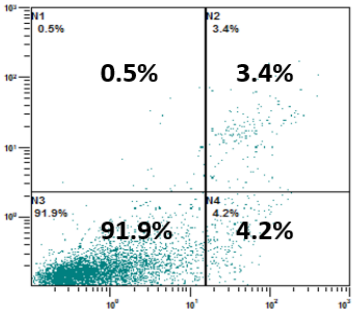
N1/L1: Necrosis
N2/L2: Late apoptosis
N3/L3: Viable
N4/L4: Early apoptosis

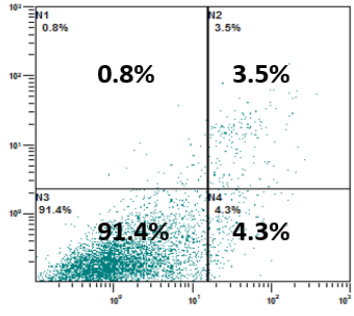
B

48 h

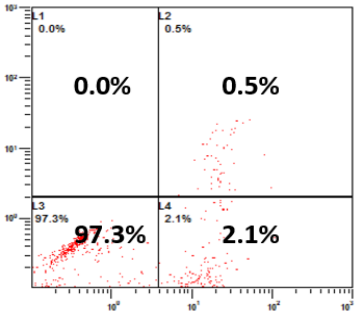
Untreated | **$PM_{2.5}$**

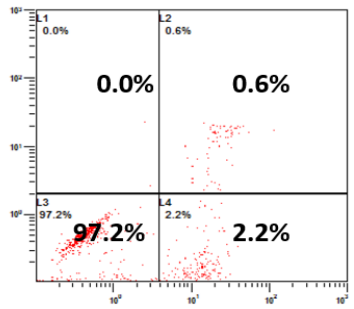
Monocyte





Lymphocyte





N1/L1: Necrosis
N2/L2: Late apoptosis
N3/L3: Viable
N4/L4: Early apoptosis

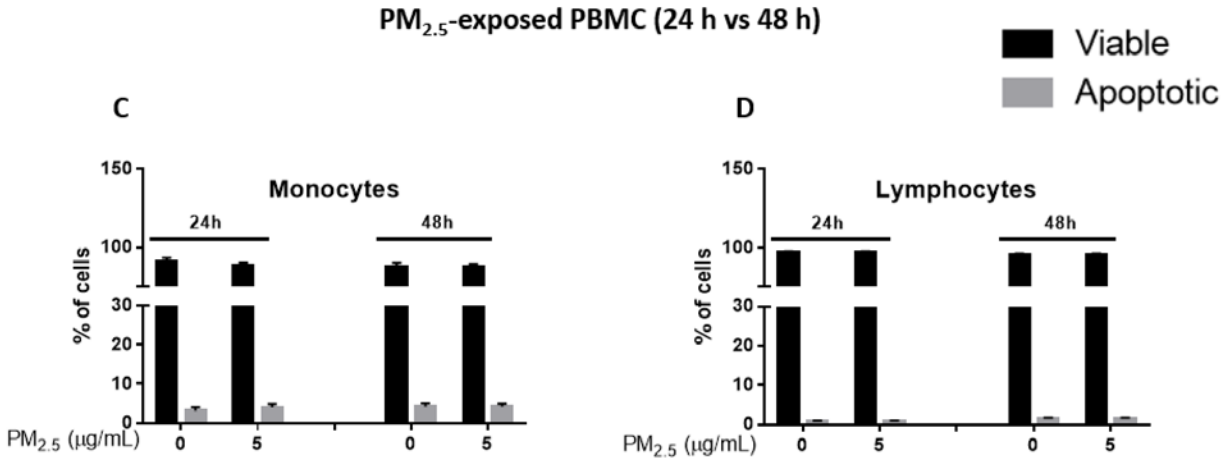
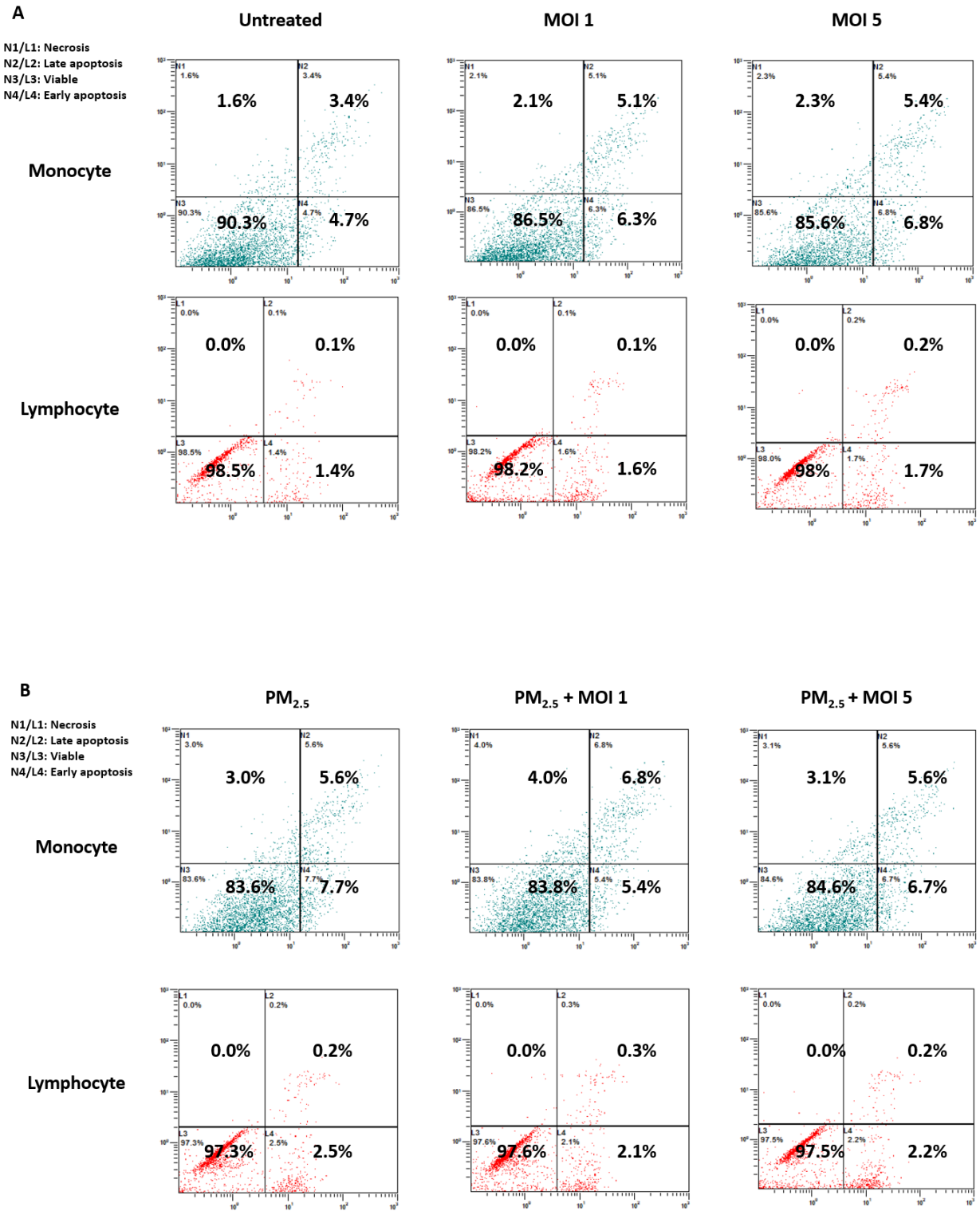


Figure 8. Effects *of $PM_{2.5}$ exposure on apoptosis and necrosis (24 h vs 48 h) of peripheral blood monocytes and peripheral blood lymphocytes.*

Exemplary dot plots showing percentages of monocytes and lymphocytes undergoing apoptosis and necrosis upon PM exposure for 24 h (A) vs 48 h (B). PBMC from 3 study subjects were pre-exposed to $PM_{2.5}$ (5µg/ml) for 24 and 48 hours. Detection of phosphatidylserine exposure by Annexin-V FITC/propidium iodide double-staining was performed to assess the proportion of monocytes(C) and lymphocytes (D) that were simultaneously undergoing apoptosis (Annexin V positive) and necrosis (Propidium Iodide positive). Figures are expressed as the mean +/- SEM of three independent experiments.


A
N1/L1: Necrosis
N2/L2: Late apoptosis
N3/L3: Viable
N4/L4: Early apoptosis
Untreated
MOI 1
MOI 5
Monocyte
1.6%
3.4%
90.3%
4.7%
2.1%
5.1%
86.5%
6.3%
2.3%
5.4%
85.6%
6.8%
Lymphocyte
0.0%
0.1%
98.5%
1.4%
0.0%
0.1%
98.2%
1.6%
0.0%
0.2%
98%
1.7%
B
N1/L1: Necrosis
N2/L2: Late apoptosis
N3/L3: Viable
N4/L4: Early apoptosis
PM2.5
PM2.5 + MOI 1
PM2.5 + MOI 5
Monocyte
3.0%
5.6%
83.6%
7.7%
4.0%
6.8%
83.8%
5.4%
3.1%
5.6%
84.6%
6.7%
Lymphocyte
0.0%
0.2%
97.3%
2.5%
0.0%
0.3%
97.6%
2.1%
0.0%
0.2%
97.5%
2.2%

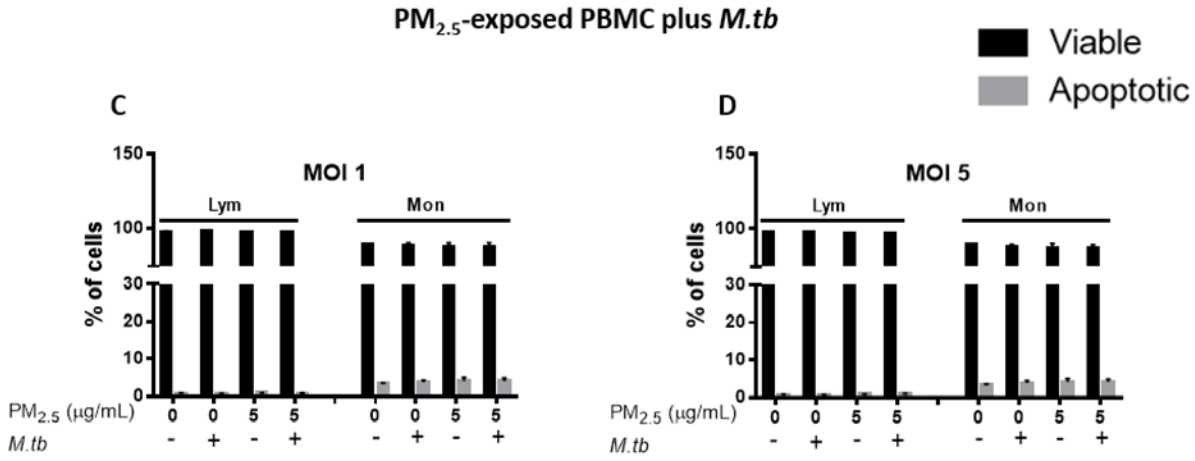


Figure 9. Effects of $PM_{2.5}$ exposure on apoptosis and necrosis of M.tb-infected mononuclear cells (MOI 1 vs MOI 5).

Exemplary dot plots showing percentages of monocytes and lymphocytes undergoing apoptosis and necrosis in PBMC infected with M.tb MOI 1 and 5 (A) and PM exposure for 20 h combined with M.tb infection at MOI 1 and 5 for 18 h (B). $PM_{2.5}$ effects on apoptosis and necrosis in PBMC from 3 study subjects pre-exposed to $PM_{2.5}$ for 20h followed by infection with M.tb MOI 1 (C) and 5 (D) for 18 hours were determined by detecting phosphatidylserine exposure. Figures are expressed as the mean +/- SEMs of three independent experiments.

3.1.4 Aim 1d: To examine the effects of $PM_{2.5}$ exposure on intracellular growth control of *M.tb* by PBMC.

3.1.4.1 Rationale: Effector functions of cytotoxic T cells during *M.tb* infection are represented by their ability to lyse infected target cells [95, 96] thereby controlling intracellular growth of *M.tb*. To assess whether PM exposure alters the intracellular growth control of *M.tb*, we designed an *M.tb* growth control assay (Fig 10A). We then assessed *M.tb* growth control in PBMC following PM exposure and *M.tb* infection by colony forming unit (cfu) assays. Cfu assays allow determination of numbers of viable *M.tb.* Each colony that develops on 7H10 agar plates represents an aggregate of *M.tb* that is derived from a single *M.tb* progenitor bacterium. Numbers of cfu thus represent numbers of *M.tb* bacteria that were present initially in a particular bacterial suspension or cell lysate at the point of the inoculation of the agar plates. The higher the number of *M.tb* cfu the weaker the growth controlling capacity of the human cell cultures and vice versa.

3.1.4.2 Results: PBMC from eleven study subjects were pre-exposed to $PM_{2.5}$ (final concentrations of 0, 1, and 5µg/ml) for 20 hours followed by infection with *M.tb* MOI 1 for 0 (2 hours), 1, 4 and 7 days and cfu assays were performed. To ensure that cfu counts represent intracellular *M.tb* growth, PBMC were washed 2 times with 1x PBS prior to cell lysis and plating of the cell lysates on agar plates to remove extracellular bacteria. *M.tb* cfu numbers were significantly higher in PBMC pre-exposed to 5µg/ml of $PM_{2.5}$ on days 1, 4, and 7 and in PBMC pre-exposed to 1µg/ml of $PM_{2.5}$ on days 4 and 7 (Fig 10B) ($p < 0.05$) than in PM-unexposed *M.tb*–infected PBMC. These observations indicated loss of intracellular growth control of *M.tb* by PBMC upon $PM_{2.5}$ exposures. Interestingly, while

the observed $PM_{2.5}$-induced loss of growth control of *M.tb* was dose-independent on days 1 and 4, on day 7, significantly higher *M.tb* cfu numbers were found in PBMC pre-exposed to 5µg/ml compared to 1µg/ml of $PM_{2.5}$ ($p < 0.05$). No significant differences in *M.tb* uptake (cfu numbers) were noted on day 0 (2hours after infection) at both $PM_{2.5}$ concentrations.

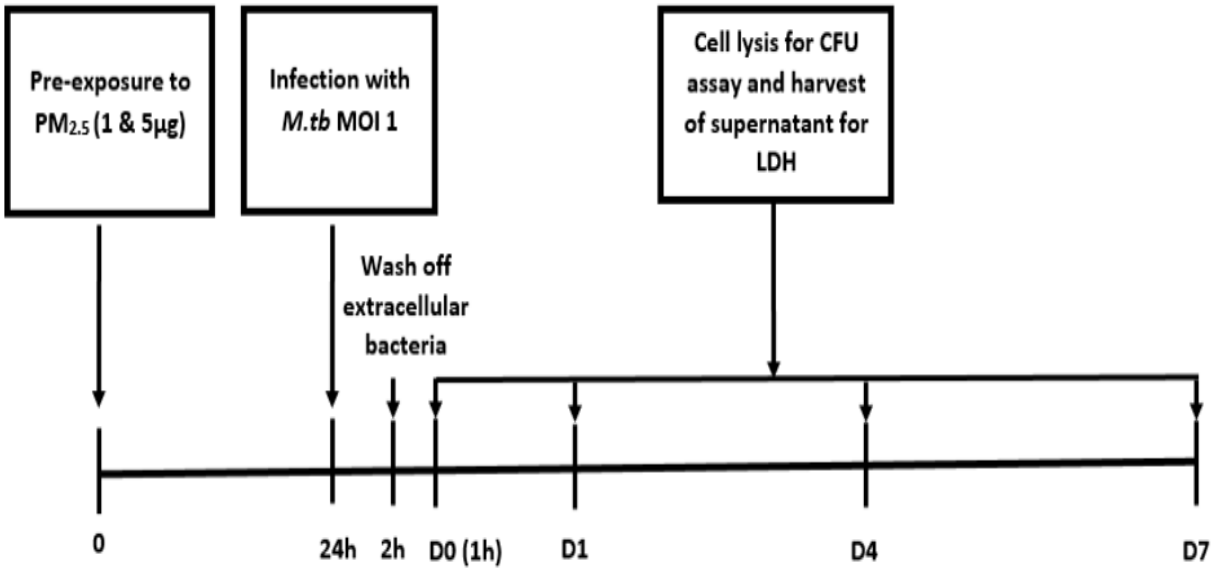


Figure 10A. Schematic diagram of M.tb growth control assay design.

PBMC from study subjects were pre-exposed to 1 & 5µg/ml of $PM_{2.5}$ for 24 hours followed by infection with M.tb MOI 1 for 2 hours. Cells were then washed three times to remove extracellular bacteria. On days 0 (2 hours), 1, 4 and 7, supernatants were collected for LDH analysis and cells were lysed for CFU assays.

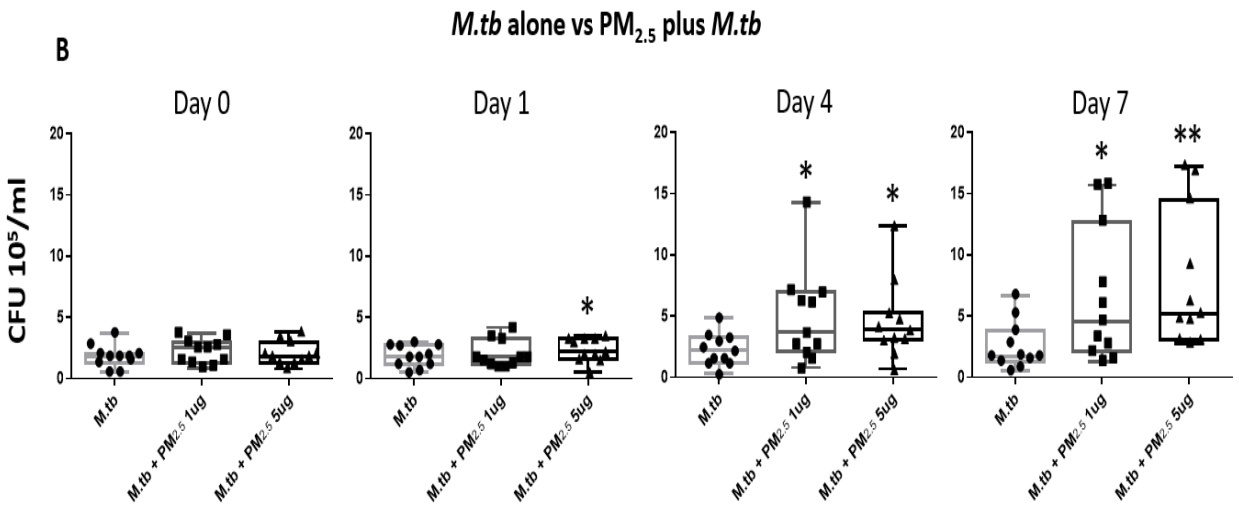


Figure 10B. Effects of PM exposure on M.tb growth control in PBMC.

PBMC from 11 study subjects were pre-exposed to $PM_{2.5}$ (final concentrations of 0, 1, and 5µg/ml) for 20 hours followed by infection with M.tb MOI 1 for 0 (2 hours), 1, 4 and 7 days. CFU assays were performed to determine the effects of PM exposure on M.tb growth control of PBMC. CFU numbers are expressed as mean results +/- SEMs from 11 independent experiments. Statistically significant differences between results for M.tb-infected and PM-exposed- M.tb-infected-PBMC are shown with single ($P< 0.05$) or double ($P< 0.01$) asterisks.

3.2 Aim 2: To investigate $PM_{2.5}$ effects on the markers of antigen -presenting cell activation, co-stimulation, and T-cell activation during *M.tb* infection

3.2.1 Aim 2a: To examine the effects of $PM_{2.5}$ exposure on the expression of CD54, HLA-DR and other costimulatory molecules in PBMC.

3.2.1.1 Rationale: Our *M.tb* growth control assay results (discussed above) suggest an underlying defect in the responsiveness of T- cells to *M.tb* upon PM exposure. T- cell immune responses to pathogens rely upon the ability of APC to activate antigen-specific T cells [97]. This process is achieved by two pathways working in concert: (1) processing and presentation of antigenic fragments via MHC I/II on the membranes of APC and (2) co-stimulation via costimulatory molecules such as CD28 and ICOS (inducible costimulatory) on T cells, and CD80 (B7.1) and CD86 (B7.2) on the membranes of APC [98].

As defective antigen presentation by APC (as a result of PM exposure) would impair T-cell mediated responses to *M.tb*, we examined T-cell responses to *M.tb* in the presence of PM by assessing the activation of APC in PBMC by flow cytometry examining CD54, a surrogate marker of APC cellular activation. CD54 was identified by Sheikh et al. as a surrogate marker of APC activation since antigen uptake, processing and presentation as well as costimulatory activity were restricted to CD54+ cell populations in human PBMC [99]. CD54, a member of the B7 family, by virtue of its biological role, has been reported as an appropriate candidate phenotypic marker of APC and indicator of their activation state. The primary biological role of CD54 is to promote cell to -cell contact [100], thereby strengthening the interface of the immunological synapse between an APC and a T cell

[101]. In addition, the direct role of CD54 for APC functionality, in terms of activating or priming T cells, is demonstrated in murine studies that show that absence of CD54 expression on APC leads to lowered activation of naïve T cells [102] or functionally impaired memory T cells [103].

We also examined the expression of HLA (human leukocyte antigen)-DR in *M.tb*-infected PBMC exposed to PM. HLA-DR is one of the most prominent (other than HLA-DQ) MHC class II molecules on the surface of APC [104]. The HLA-DR –microbial antigen peptide complex is recognized by the T cell receptor during antigen presentation. Specifically, development of *M.tb*-specific T cells and IFN-γ involves binding of MHC class II molecule/mycobacterial antigen peptide complexes to T cell receptors, along with CD80/86 coupling to CD28, and CD40 coupling to CD40L [105]. Furthermore, we examined the expression of costimulatory molecules CD80 and CD86 in *M.tb*-infected PBMC exposed to PM. CD80, expressed on APC, works in tandem with CD86 to provide a costimulatory signal for T cell activation [106].

3.2.1.2 Results: To examine the effects of PM exposure on antigen presentation and the activation of APC during *M.tb* infection, we compared the expression of stimulation markers from the following PBMC samples: untreated controls (n = 3), PM-exposed (5 µg/ml), *M.tb* MOI 5-infected, PPD -stimulated (10 µg/ml), PM-exposed (5 µg/ml) and *M.tb* MOI 5-infected or PM-exposed (5 µg/ml) and PPD -stimulated (10 µg/ml). Samples were stained with monoclonal antibodies against CD14 (monocytes), CD54, CD80, CD86 and HLA-DR. Fixable viability dye eFluor 780 was used to exclude dead cells and live cells were gated and analyzed using flow cytometry. We observed constitutive expression of CD54 (Fig 11A and Fig 11C) and HLA-DR (Fig 11B and Fig 11C) on monocytes in

PBMC. Almost all $CD14^+$ monocytes expressed CD54 (Fig 11A), however, a great percentage of $CD14^+$ monocytes did not express HLA-DR (Fig 11B). HLA-DR was expressed on a fraction (70%) of $CD54^+$ cells only (Fig 11C). PM exposure did not cause any significant change in the expression of CD54 (Fig 12A) and HLA-DR (Fig 12B) when control PBMC was compared to PM-exposed (5 µg/ml) and PM-exposed (5 µg/ml) and *M.tb* MOI 5-infected or PM-exposed (5 µg/ml) and PPD 10 µg-stimulated PBMC. Also, there was no significant difference observed in the percentage of monocytes co-expressing CD54 and HLA-DR ($CD54^+HLA\text{-}DR^+$) in PM-exposed (5 µg/ml) and PM-exposed (5 µg/ml) and *M.tb* MOI 5-infected or PM-exposed (5 µg/ml) and PPD -stimulated (10µg/ml) PBMC as compared to control PBMC (Fig 12C). PM exposure increased the expression of CD80 while decreasing the constitutively expressed CD86 on monocytes (Fig 13A). PM exposure significantly increased the co-expression of CD80 and CD86 (CD80/86) in PM-exposed (5µg/ml) and PM-exposed (5µg/ml)-*M.tb* MOI 5-infected, and PM-exposed (5µg/ml)-PPD 10µg-stimulated PBMC (Fig 13B). Taken together with our CD54 and HLA-DR results, the data suggest that while PM exposure did not alter APC activation, it increased the expression of costimulatory molecules in *M.tb* infection.

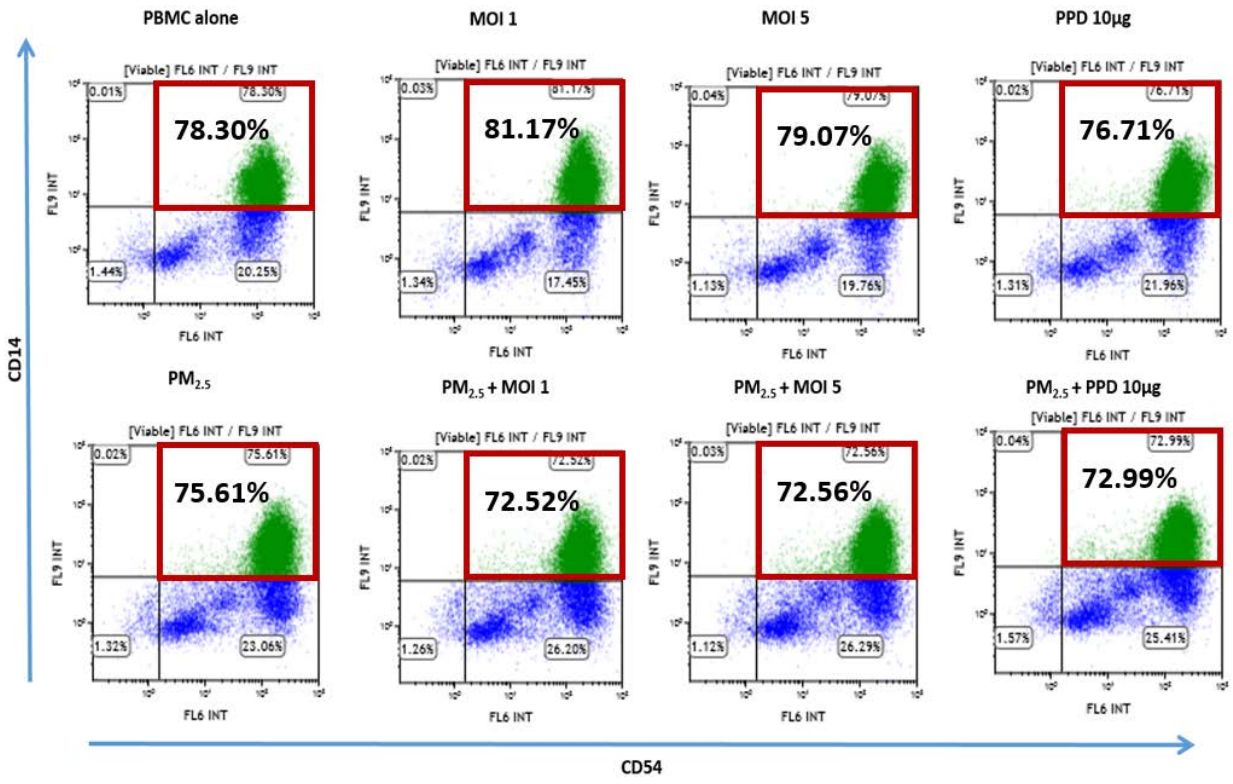


Figure 11A. Effects of PM exposure on the expression of CD54 on monocytes (CD14) in M.tb-infected PBMC.

PBMC were pre-exposed to $PM_{2.5}$ (5µg/ml) for 20 hours followed by infection with M.tb MOI 1 and 5 or PPD 10 µg for 18 h. Surface staining with anti-CD14 and CD54 monoclonal antibodies and viability dye eFluor780 was performed for detection by flow cytometry. A representative plot of 3 independent experiments is shown.

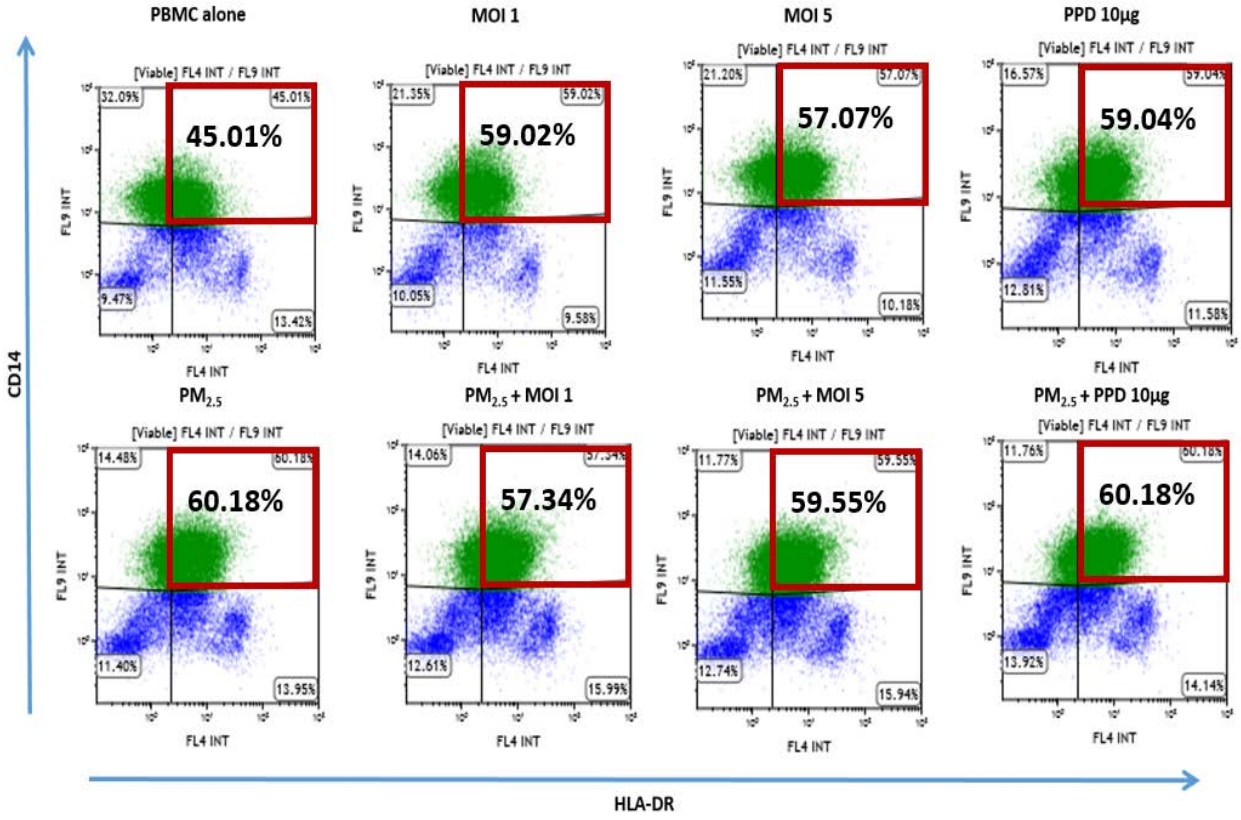


Figure 11B. Effects of PM exposure on the expression of HLA-DR on monocytes (CD14) in M.tb-infected PBMC.

PBMC were pre-exposed to $PM_{2.5}$ (5µg/ml) for 20 hours followed by infection with M.tb MOI 1 and 5 or PPD 10 µg for 18 h. Surface staining with anti-CD14 and HLA-DR monoclonal antibodies and viability dye eFluor780 was performed for detection by flow cytometry. A representative plot of 3 independent experiments is shown.

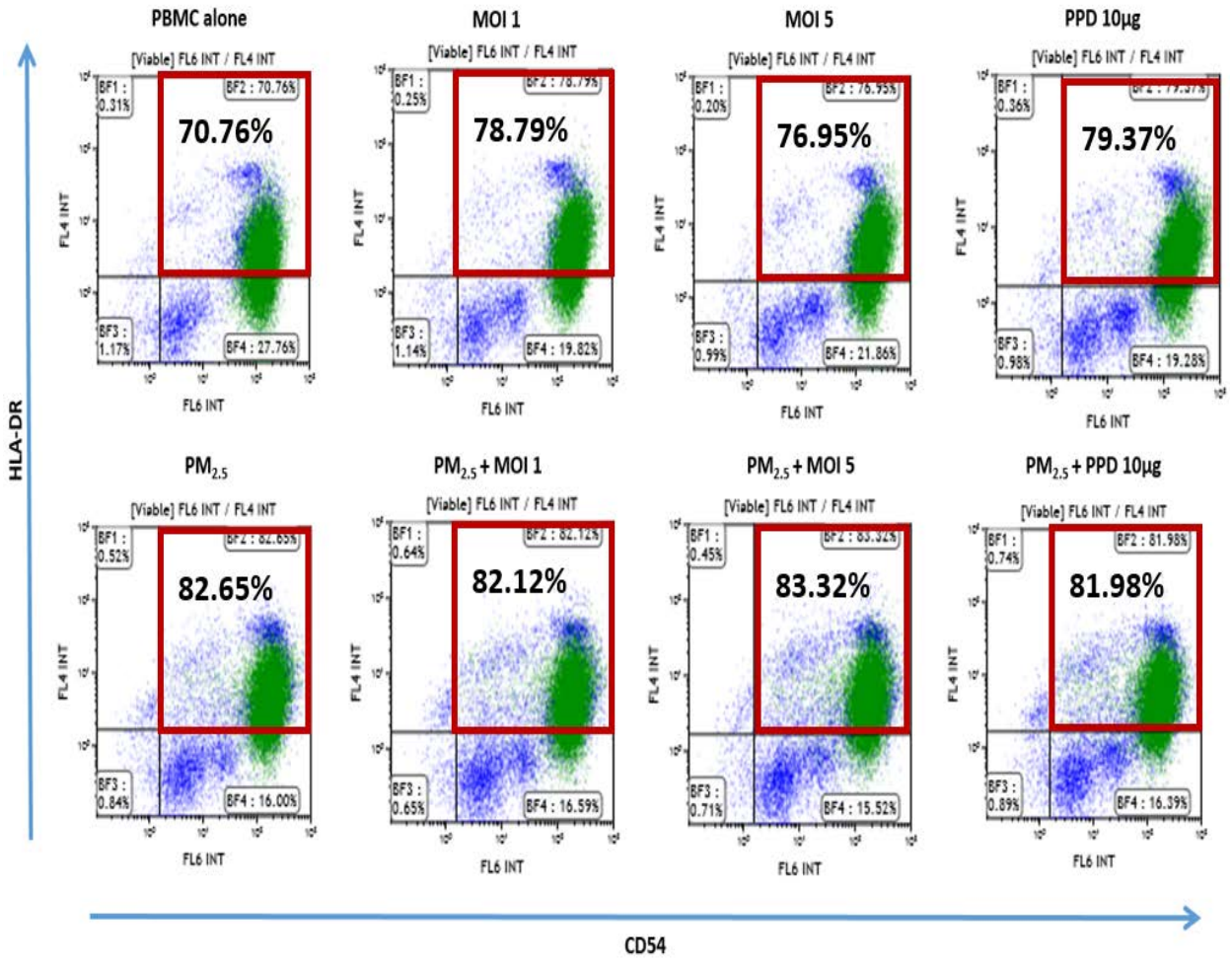


Figure 11C. Effects of PM exposure on the co-expression of HLA-DR and CD54 on monocytes in M.tb-infected PBMC.

PBMC were pre-exposed to $PM_{2.5}$ (5µg/ml) for 20 hours followed by infection with M.tb MOI 1 and 5 or PPD 10 µg for 18 h. Surface staining with anti-CD54 and HLA-DR monoclonal antibodies and viability dye eFluor780 was performed for detection by flow cytometry. A representative plot of 3 independent experiments is shown.

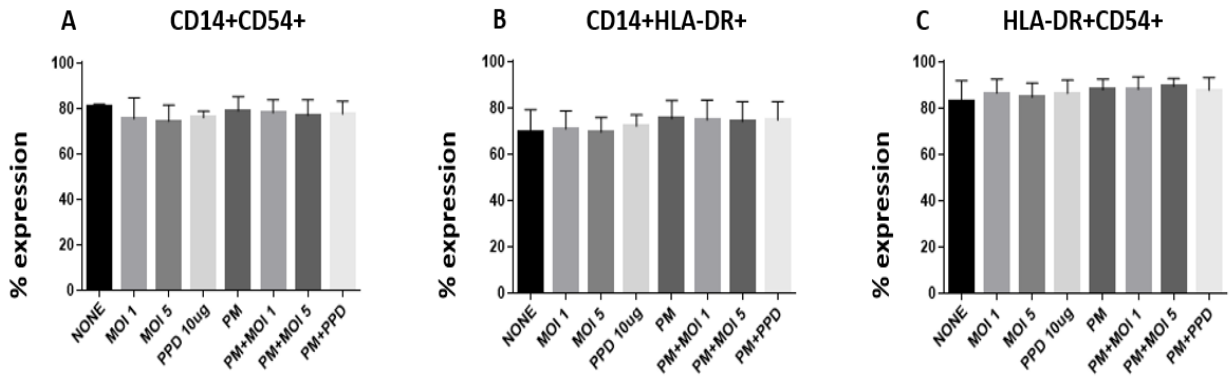


Figure 12. Effects of PM exposure on the expression of HLA-DR and CD54 on monocytes (CD14) in M.tb-infected PBMC.

PBMC were pre-exposed to $PM_{2.5}$ (5µg/ml) for 20 hours followed by infection with M.tb MOI 1 and 5 or PPD 10µg for 18h. Surface staining with anti-CD14, anti-CD54 and anti-HLA-DR monoclonal antibodies and viability dye eFluor780 was performed for detection by flow cytometry. The values represent the percentage expression of CD54 (A), HLA-DR (B), and percentage co-expression of CD54 and HLA-DR (C) on monocytes (CD14) from three independent experiments expressed as mean+/- SD.

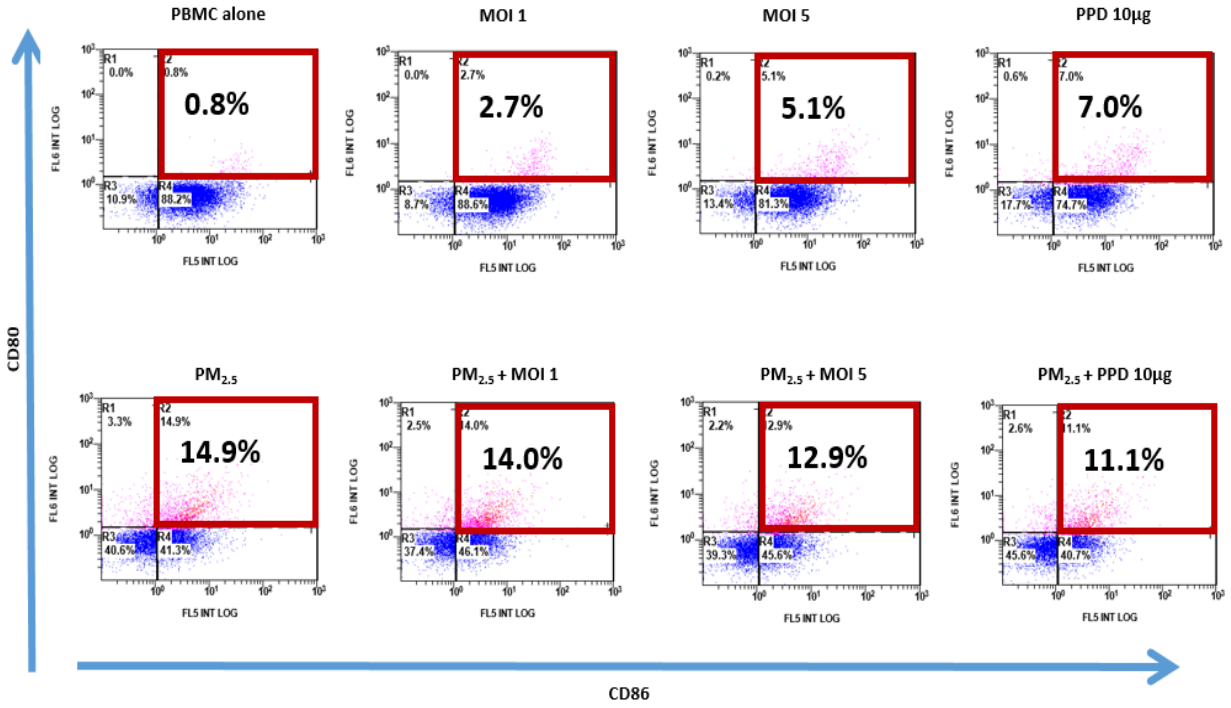


Figure 13A. Effects of PM exposure on the expression of CD80 and CD86 in M.tb-infected PBMC.

PBMC were pre-exposed to $PM_{2.5}$ (5µg/ml) for 20 hours followed by infection with M.tb MOI 1 and 5 or PPD 10 µg for 18 h. Surface staining with anti-CD80 and CD86 monoclonal antibodies and viability dye eFluor780 was performed for detection by flow cytometry. A representative plot of 3 independent experiments is shown.

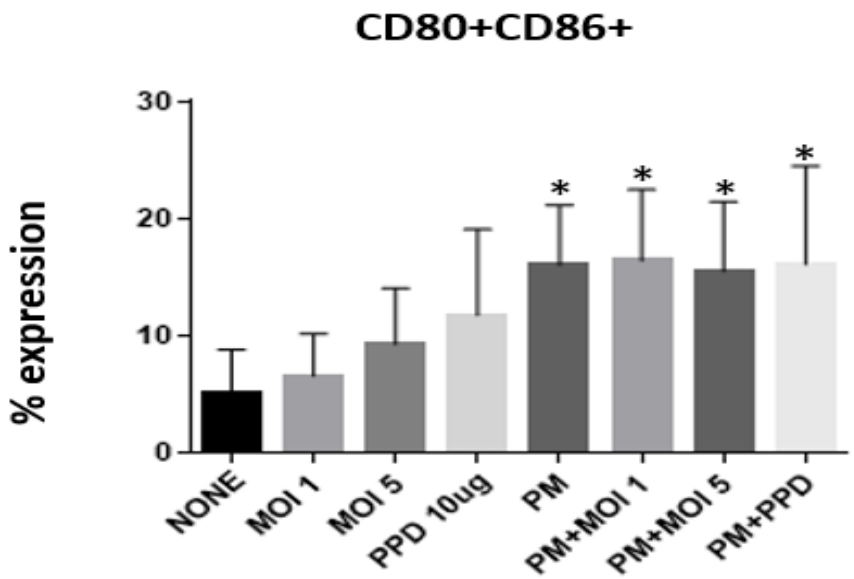


Figure 13B. Effects of PM exposure on the co-expression of CD80 and CD86 on monocytes in M.tb-infected PBMC.

PBMC were pre-exposed to $PM_{2.5}$ (5µg/ml) for 20 hours followed by infection with M.tb MOI 1 and 5 or PPD 10µg for 18h. Surface staining with anti-CD14, -CD80 and CD86 monoclonal antibodies and viability dye eFluor780 was performed for detection by flow cytometry. The values represent the percentage co-expression of CD80 and CD86 on monocytes from three independent experiments expressed as mean+/- SD. Statistically significant differences between results for unexposed and PM-exposed or PM-exposed-M.tb-infected or PM-exposed-PPD-stimulated-PBMC are shown with single ($P < 0.05$) asterisks.

3.2.2 Aim 2b: To examine the effects of $PM_{2.5}$ exposure on the expression of early T-cell activation markers CD69 and CD25 on T-cell subsets in PBMC.

3.2.2.1 Rationale: Activation of APC leads to T cell activation in co-culture systems, physiologically in blood and in tissue environments. T cell activation is a key event in host immunity against *M.tb* infection. Should PM exposure interfere with T cell activation, this would affect the T cell effector functions required for *M.tb* control. Assessing the expression of early activation markers on T cells during *M.tb* infection is a reliable measure of T cell activation [107] and critical to *M.tb* host immunity [81, 108]. CD69, a cell surface glycoprotein expressed upon activation via CD25, was identified as the earliest stimulation/activation marker on the surfaces of antigen-specific, activated lymphocytes *in vitro* [109] and acts as a costimulatory molecule for T-cell activation and proliferation [110, 111]. We studied T cell activation markers on T cells derived from PBMC as T cells derived from PBMC were in interaction with APC.

3.2.2.2 Results: To determine if PM exposure reduces the expression of CD69 and CD25 on T cell subsets upon *M.tb* infection, several treated PBMC samples were compared. Control (untreated); PM-exposed (5 µg/ml); *M.tb*-infected (MOI 1and MOI 5); PPD stimulated (10µg/ml); PM-exposed (5 µg/ml) and *M.tb*-infected (MOI 1 and MOI 5) or PM-exposed (5 µg/ml) and PPD-stimulated (10 µg/ml). These were stained with monoclonal antibodies against CD3, CD4, CD8, CD25 and CD69 and analyzed by flow cytometry. Infection with *M.tb* and PM exposure increased the expression of both CD25 and CD69. However, $PM_{2.5}$ pre-exposure decreased the expression of both CD25 and CD69 in *M.tb* MOI 1-infected PBMC (Fig 14). PM exposure significantly downregulated the *M.tb* and PPD-induced CD69 expression in $CD3^+$, $CD4^+$, and $CD8^+$ T cells in *M.tb* MOI 1(Fig

15A), MOI 5 (Fig 15B) infected and PPD 10µg stimulated PBMC ($p < 0.05$ (Fig 15C)). CD69 expression levels in all T cell subsets were lower in cells exposed to PM 5µg/ml than in cells infected with *M.tb* alone. $PM_{2.5}$ pre-exposure of PBMC, however, decreased CD69 expression upon *M.tb*–infection. Interestingly, in the aggregate of seven experiments, CD25 which was constitutively expressed on T cell subsets (Table 2), did not show any further upregulation upon *M.tb* infection following PM exposure. This suggests together with our CD69 findings, that PM exposure alters the activation of T cells during *M.tb* infection. The observed effects of PM exposure on the expression of CD69 and CD25 on T cells were independent of PM-induced changes to the viability of the T cells as shown in our Annexin-V FITC/propidium iodide double-staining experiment (described above).

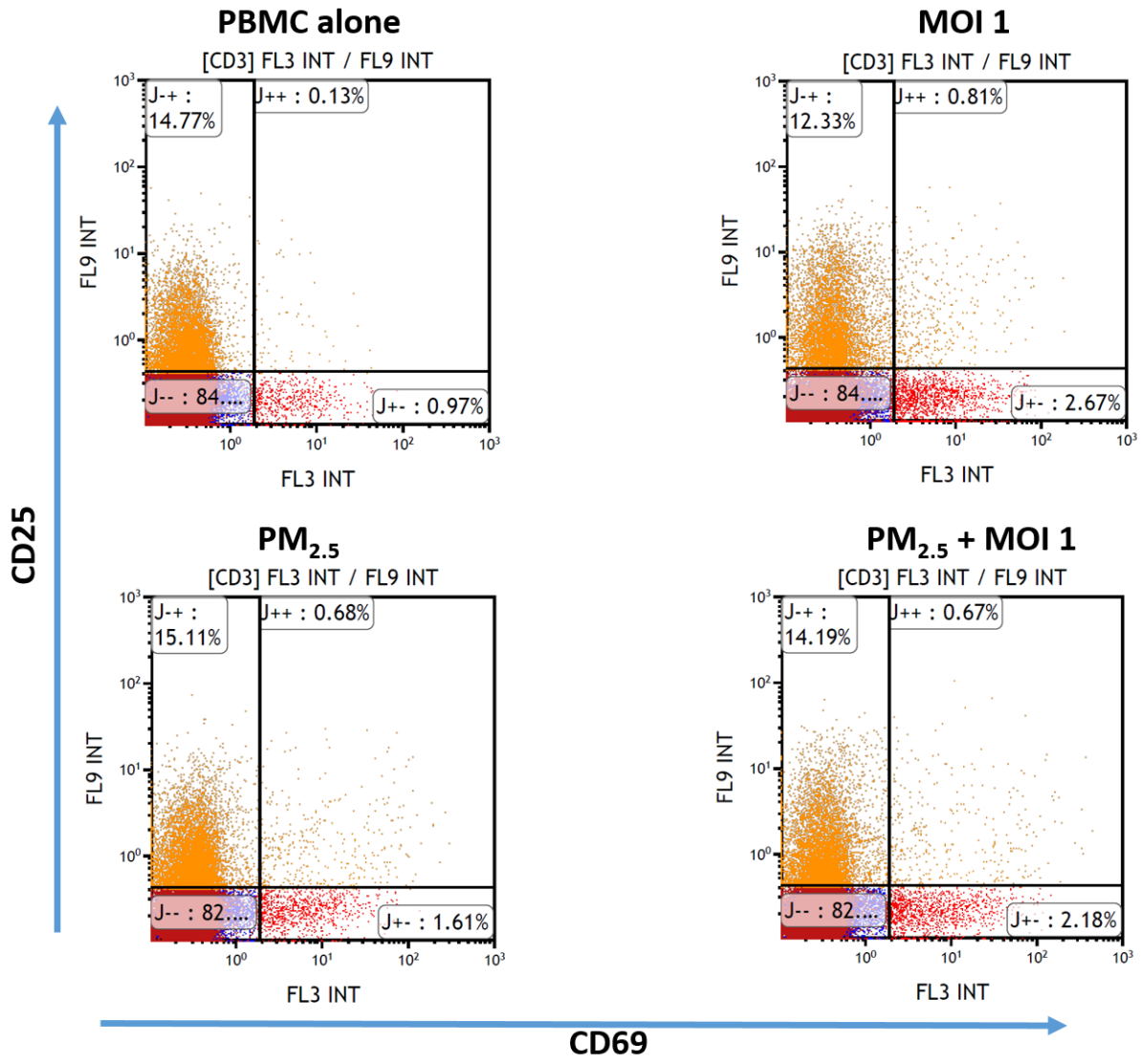


Figure 14. Effects of PM exposure on the expression of CD69 and CD25 on CD3 in M.tb-infected PBMC.

PBMC were pre-exposed to $PM_{2.5}$ (5µg/ml) for 20 hours followed by infection with M.tb MOI 1 for 18 h. Surface staining with anti-CD3, CD25 and CD69 monoclonal antibodies and viability dye eFluor780 was performed for detection by flow cytometry. A representative plot of 7 independent experiments is shown.

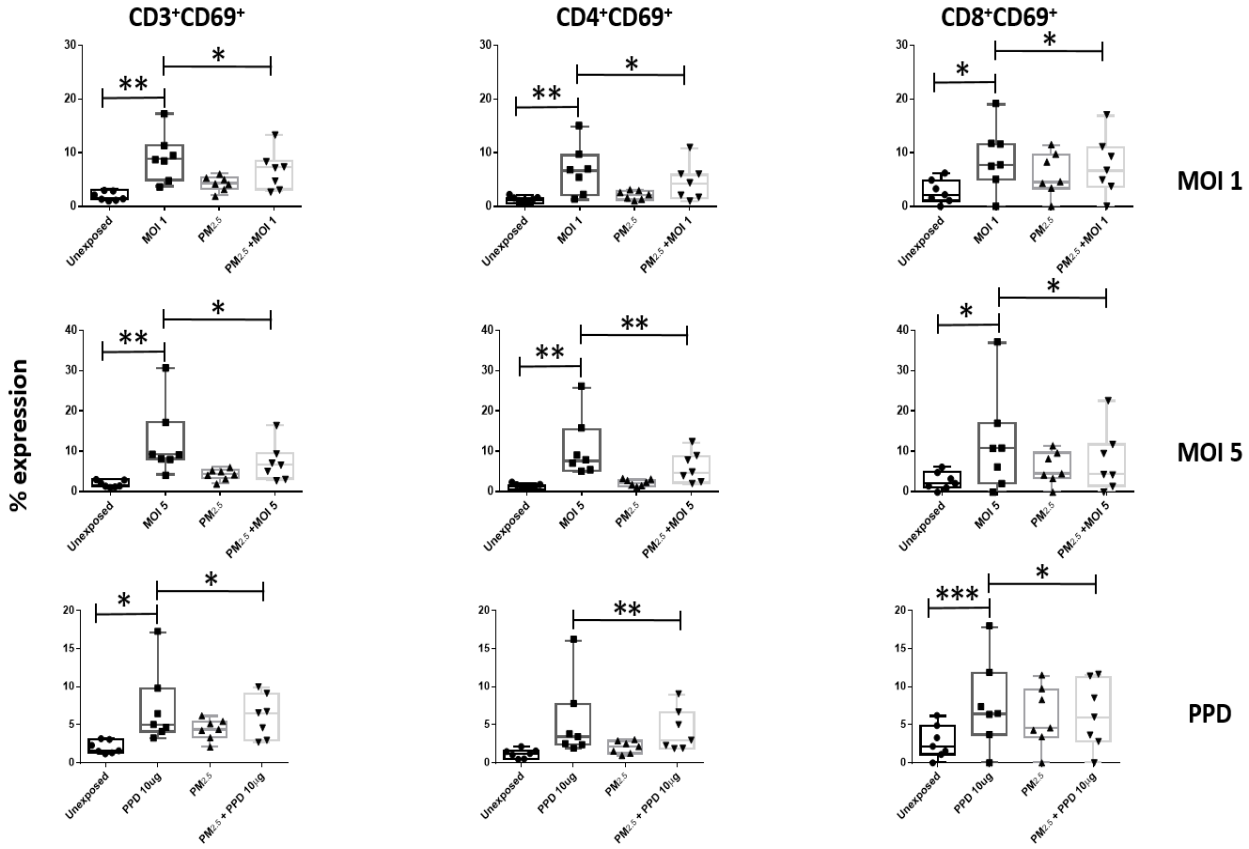


Figure 15. Effects of PM exposure on the expression of CD69 on T cell subsets in M.tb-infected and PPD-stimulated PBMC.

PBMC were pre-exposed to $PM_{2.5}$ (5µg/ml) for 20 hours followed by infection with M.tb MOI 1(A) and 5 (B) or stimulation by PPD 10µg (C) for 18h. Surface staining with anti-CD3, -CD4, -CD8, and -CD69 monoclonal antibodies and viability dye eFluor780 was performed for detection by flow cytometry. The values represent the percentage expression of CD69 on CD3+, CD4+, and CD8+ cells from seven independent experiments. Percent

expressions of CD69 are expressed as scatter plots of seven independent experiments superimposed on 5-95 percentile box-and-whiskers where the center represents "the 50^{th} percentile", the upper hinge is "the 75^{th} percentile", and the lower hinge is "the 25^{th} percentile". Statistically significant (mean +/- SEM) increases between results for uninfected and M.tb-infected or PPD-stimulated PBMC are shown with single ($P< 0.05$), double ($P< 0.01$) or triple $P< 0.001$) asterisks, while statistically significant decreases between results for M.tb-infected or PPD-stimulated and PM-exposed-M.tb-infected or PM-exposed-PPD-stimulated PBMC are shown with single ($P< 0.05$) or double ($P< 0.01$) asterisks.

	PBMC only	MOI 1	MOI 5	PPD	$PM_{2.5}$	$PM_{2.5}$ + MOI 1	$PM_{2.5}$ + MOI 5	$PM_{2.5}$ + PPD
$CD3^+CD25^+$(% expression)	18.01	16.94	22.69	16.57	19.74	18.66 (0.4)	17.63 (0.2)	17.94 (0.5)
$CD4^+CD25^+$(% expression)	26.59	21.75	23.40	22.17	24.66	23.09 (0.8)	21.36 (0.7)	22.62 (0.9)
$CD8^+CD25$(% $expression)^+$	3.55	2.63	6.10	1.98	9.02	4.72 (0.3)	4.80 (0.6)	3.94 (0.4)

Table 2. Effects of PM exposure on the expression of CD25 on T cell subsets in M.tb-infected and PPD-stimulated PBMC.

PBMC were pre-exposed to $PM_{2.5}$ (5µg/ml) for 20 hours followed by infection with M.tb MOI 1(A) and 5 (B) or stimulation by PPD 10 µg (C) for 18 h. Surface staining with anti-CD3, -CD4, -CD8, and –CD25 monoclonal antibodies and viability dye eFluor780 was performed for detection by flow cytometry. The values represent the percentage expression of CD25 on CD3+, CD4+, and CD8+ cells in PBMC. Figures are expressed as the mean of seven independent experiments with p-values comparing $PM_{2.5}$ only to $PM_{2.5}$-preexposure followed by MOI 1, 5, or PPD in parenthesis.

3.2.3 Aim 2c: To examine the effects of $PM_{2.5}$ exposure on the expression of T cell inactivation molecules CTLA-4 and PD-1 on T-cells in PBMC.

3.2.3.1 Rationale: Cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), a surface molecule of activated T cells, is a protein receptor that acts as an 'off' switch of T cell activation when bound to CD80 or CD86 on the surface of APC [112-114]. We examined the intracellular expression of CTLA-4 in peripheral blood CD3 T cells by flow cytometry. As a regulatory mechanism, programmed cell death protein 1 (PD-1), also expressed on the surface of T cells, prevents activation of T cells by promoting apoptosis in antigen-specific T cells while reducing apoptosis in regulatory T cells [115, 116]. Finally, we also examined the expression of PD-1 on CD3 T cells in *M.tb*-infected and PPD-stimulated PBMC exposed to PM by flow cytometry.

3.2.3.2 Results: To further examine the effects of PM exposure on T cell activation during *M.tb* infection, PBMC from three control, PM-exposed (5µg/ml), *M.tb* MOI 5-infected, PPD-stimulated (10µg/ml), PM-exposed (5µg/ml) and *M.tb* MOI 5-infected or PM-exposed (5µg/ml) and PPD -stimulated (10 µg/ml) were stained with monoclonal antibodies against CD3, CTLA-4 and PD-1. Fixable viability dye eFluor 780 was used to exclude dead cells, while live cells were gated and analyzed using flow cytometry. Infection with M.tb MOI 1 and MOI 5, as well as stimulation with PPD (10 µg/ml) increased intracellular expression of CTLA-4 in CD3 T cells. However, PM exposure did not cause any further increase in CTLA-4 expression (Fig 17). Interestingly, PD-1, which was constitutively expressed in CD3 T cells (Fig 18), did not show any further upregulation upon *M.tb* infection, PPD stimulation, or following PM exposure. This suggests, together

with our CTLA-4 findings, that PM exposure did not alter the expression of inactivation molecules in T cells during *M.tb* infection.

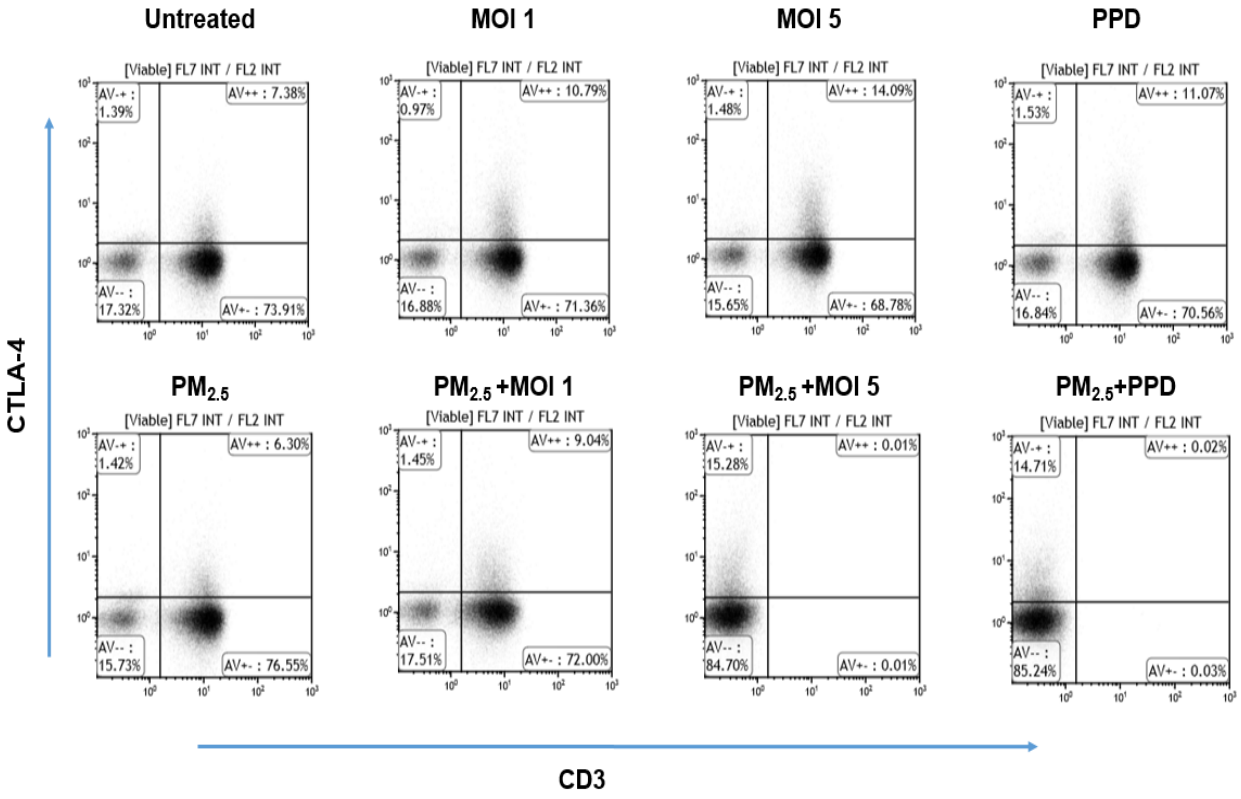


Figure 17. Effects of PM exposure on the expression of CTLA-4 on CD3+ T cell in M.tb-infected and PPD-stimulated PBMC.

PBMC were pre-exposed to $PM_{2.5}$ (5µg/ml) for 20 hours followed by infection with M.tb MOI 1 and 5 or stimulation with PPD 10µg for 18 hours. Surface staining with monoclonal antibodies against CD3 and viability dye eFluor780 was performed followed by permeabilization and intracellular staining with CTLA-4 antibody for flow cytometry analysis.

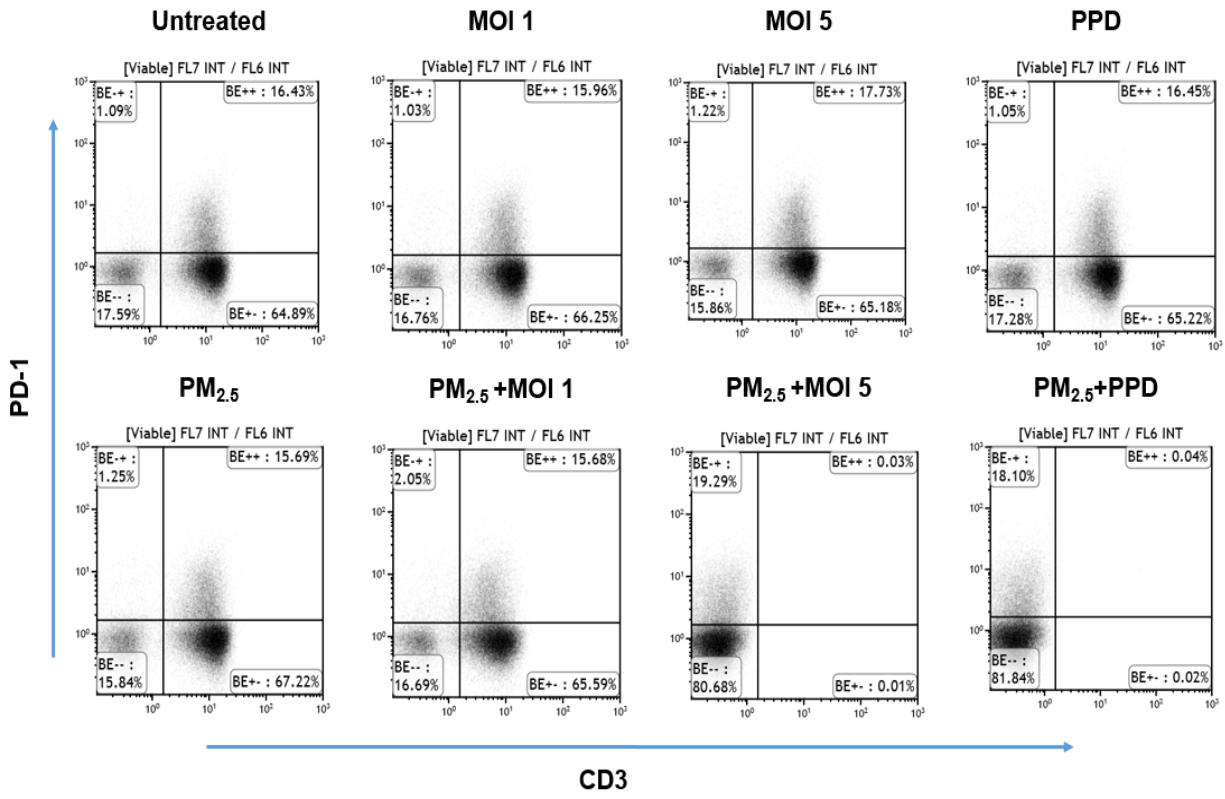


Figure 18. Effects of PM exposure on the expression of PD-1 on CD3+ T cell in M.tb-infected and PPD-stimulated PBMC.

PBMC were pre-exposed to $PM_{2.5}$ (5µg/ml) for 20 hours followed by infection with M.tb MOI 1 and 5 or stimulation with PPD 10µg for 18 hours. Surface staining with monoclonal antibodies against CD3 and viability dye eFluor780 was performed followed by permeabilization and intracellular staining with PD-1 antibody for flow cytometry analysis.

3.3 Aim 3: To examine the effects of $PM_{2.5}$ on *M.tb*-induced cytokine production.

3.3.1 Aim 3a: To examine the effects of $PM_{2.5}$ exposure on *M.tb*-induced IFN-γ expression in PBMC.

3.3.1.1 Rationale: The ability of T-cells to produce IFN-γ is an integral part of T cell effector functions during *M.tb* infection and leads to activation of macrophages and lysis of *M.tb*-infected phagocytes [28, 31]. As shown by our *M.tb* growth control data, $PM_{2.5}$ exposure increases *M.tb* growth in PBMC. To further explore the $PM_{2.5}$ effects on *M.tb* growth control, we next assessed if $PM_{2.5}$ interferes with cell-mediated immunity by altering the production of the pro-inflammatory cytokine IFN-γ. We examined the intracellular expression of IFN-γ in peripheral blood T cells in the context of *in vitro* $PM_{2.5}$ exposures, by flow cytometry.

3.3.1.2 Results: To determine if $PM_{2.5}$ exposure modifies the expression of IFN-γ in response to *M.tb* infection, PBMC from control, $PM_{2.5}$-exposed (5μg/ml), *M.tb*-infected (MOI 1and MOI 5), or $PM_{2.5}$-pre-exposed (5μg/ml) and *M.tb*-infected (MOI 1 and MOI 5) were stained with monoclonal antibodies against CD3, CD4, CD8, CD16, IFN-γ, and viability dye eFluor780 (Affymetrix eBioscience, San Diego CA). IFN-γ expression in $CD3^+$, $CD4^+$, $CD8^+$, and $CD16^+$ cells was measured by flow cytometry. While *M.tb* infection at both MOI 1 and MOI 5 induced IFN-γ expression by T cells, $PM_{2.5}$ pre-exposure significantly reduced the T cell expression of IFN-γ upon *M.tb* MOI 1 infection in $CD3^+$ and $CD8^+$ cells (Fig 6A) and upon MOI 5 infection in $CD3^+$, $CD4^+$, and $CD8^+$ T cells (Fig 6B) ($p < 0.05$). We also observed a significant increase in IFN-γ expression in

$CD8^+CD16^+$ T cells upon infection with *M.tb* MOI 1 and 5. However, $PM_{2.5}$ pre-exposure significantly reduced the expression of IFN-γ in *M.tb* MOI 1 (Fig 6A) and MOI 5 (Fig 6B) infected $CD8^+CD16^+$ T cells ($p < 0.05$). It is important to note, that none of the differences between the subpopulations and effects of $PM_{2.5}$ on the *M.tb*-induced IFN-γ expression were related to decreases in the cellular viability, as equal numbers of live cells (after exclusion of dead cells as per flow cytometry study design), were assessed in each experimental condition. In fact, our *in vitro* $PM_{2.5}$ exposures did not affect percentages of viable IFN-γ-expressing MOI 1 and MOI 5 *M.tb*-infected $CD3^+$, $CD4^+$, and $CD8^+$ T cells (data not shown). Together, the data described above shows that PM exposure decreases T-cell immune response to *M.tb*.

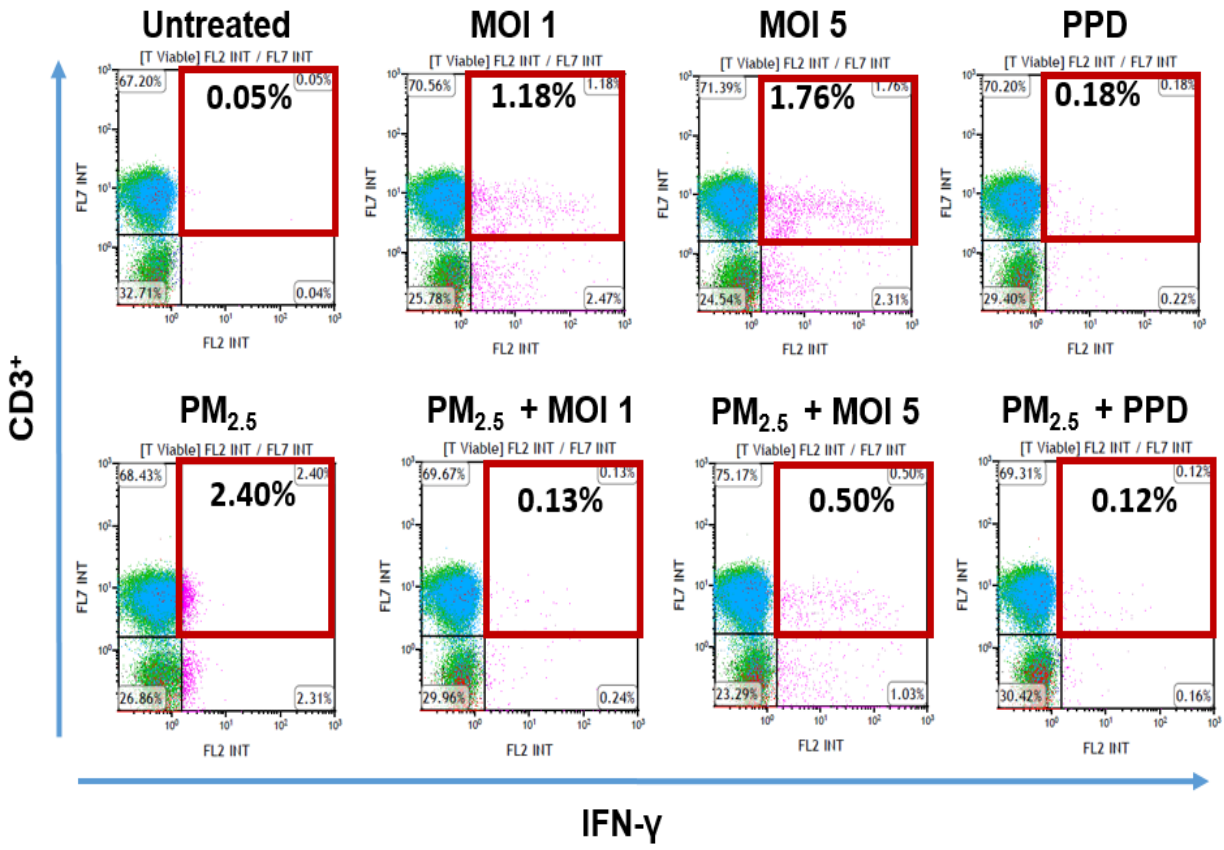


Figure 19. Effects of PM exposure on M.tb-induced IFN-γ production by CD3 T cell in PBMC.

PBMC were pre-exposed to $PM_{2.5}$ (5µg/ml) for 20 hours followed by infection with M.tb MOI 1 and 5 or stimulation with PPD 10 µg for 18 hours. Surface staining with monoclonal antibodies against CD3 and viability dye eFluor780 was performed followed by permeabilization and intracellular staining with IFN-γ antibody for flow cytometry analysis. A representative plot of 9 independent experiments is shown.

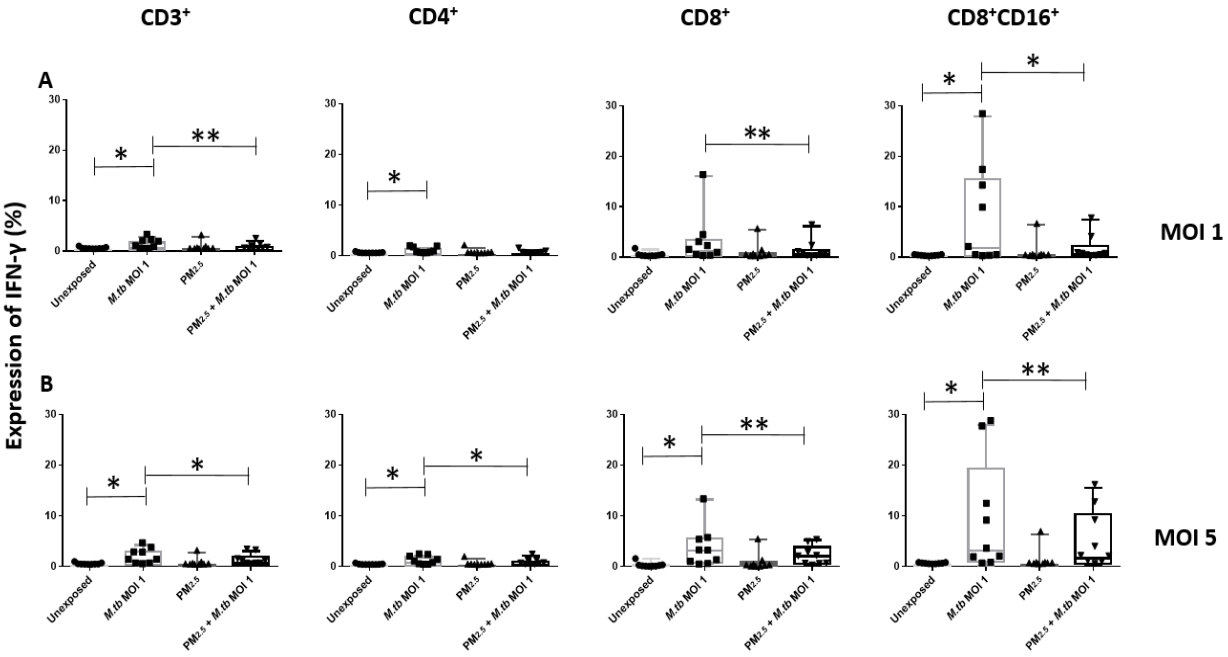


Figure 20. Effects of PM exposure on M.tb-induced IFN-γ production in peripheral blood T cell subpopulations in PBMC.

PBMC from 9 study subjects were pre-exposed to $PM_{2.5}$ (5µg/ml) for 20 hours followed by infection with M.tb MOI 1 (A) and 5 (B) for 18 hours. Surface staining with monoclonal antibodies against CD3, CD4, CD8, CD16, and viability dye eFluor780 was performed followed by permeabilization and intracellular staining with IFN-γ antibody for flow cytometry analysis. The values represent the percentage expression of IFN-γ in various phenotypic markers on T-cells. Percent expressions of IFN-γ are expressed as scatter plots of nine independent experiments superimposed on 5-95 percentile box-and-whiskers where the center represents "the 50^{th} percentile", the upper hinge is "the 75^{th} percentile", and the lower hinge is "the 25^{th} percentile". Statistically significant (expressed as the mean +/- SEM) increases between results for uninfected and M.tb-infected PBMC are shown with single ($P < 0.05$) asterisks while statistically significant decreases between results for M.tb-

infected and PM-exposed-M.tb-infected PBMC are shown with single ($P< 0.05$) or double ($P< 0.01$) asterisks.

3.3.2 Aim 3b: To examine the effects of $PM_{2.5}$ exposure on *M.tb*-induced TNF-α production in PBMC.

3.3.2.1 Rationale: Another critical cytokine in the host control of *M.tb* is TNF-α. Stimulation of phagocytes through *M.tb* results in secretion of TNF-α, a cytokine that is required for cell activation and inhibition of mycobacterial growth [33, 35]. Considering earlier reports that optimal macrophage activation and full inhibition of mycobacterial growth are acquired through synergistic effects of TNF-α and IFN-γ produced by T cells [24], we confirmed PM-induced inhibition of pro-inflammatory cytokine production by assessing the effects of PM exposure on TNF-α expression during *M.tb* infection. In PBMC, TNF-α is produced by $CD3^+$ T cells and monocytes in response to *M.tb* [117, 118], however, monocytes are greater TNF-α producers than T cells.

3.3.2.2 Results: To explore if PM exposures alter TNF-α production upon *M.tb* infection, PBMC were pre-exposed to $PM_{2.5}$ (5μg/ml) for 20 h and then infected with *M.tb* MOI 1 and 5, or stimulated with PPD (10 μg/ml), phytohemagglutinin (PHA 5 μg) (a potent T cell activator and positive control for T cell activation), or lipopolysaccharide (LPS 100 ng) (a potent NF-kB activator and positive control for monocyte activation) for an additional 8 h. TNF-α expression increased significantly with *M.tb* MOI 1, MOI 5, PPD, PHA, and LPS in $CD3^+$ T cells (Fig 22A) and with *M.tb* MOI 1, MOI 5, PPD (see representative flow cytometry contour with density plots, Fig 21), PHA, and LPS in $CD14^+$ cells (Fig 22B). As

expected, monocytes produced more TNF-α than T cells and expression levels of TNF-α were higher after stimulation with PHA than with LPS in $CD3^+$ T cells and after stimulation with LPS than with PHA in $CD14^+$ cells. The expression of TNF-α in response to *M.tb* infection and stimulation with PPD, PHA and LPS was significantly decreased in PM-exposed PBMC compared to PBMC infected with *M.tb* MOI 1 and 5, or stimulated with PPD, PHA or LPS ($p < 0.05$). Although there were decreases in PPD-induced TNF-α expression in the $CD3^+$ T cells upon PM exposure in each of the six experiments, we did not observe a significant decrease in PPD-induced TNF-α expression upon PM exposure in the aggregate of six experiments (Fig 22A, $p < 0.08$). These data show that PM exposure downregulates the expression of TNF-α in both $CD3^+$ and $CD14^+$ cells in response to *M.tb* infection. This confirmed our earlier observation that exposure to PM inhibits the production of pro-inflammatory cytokines during *M.tb* infection.

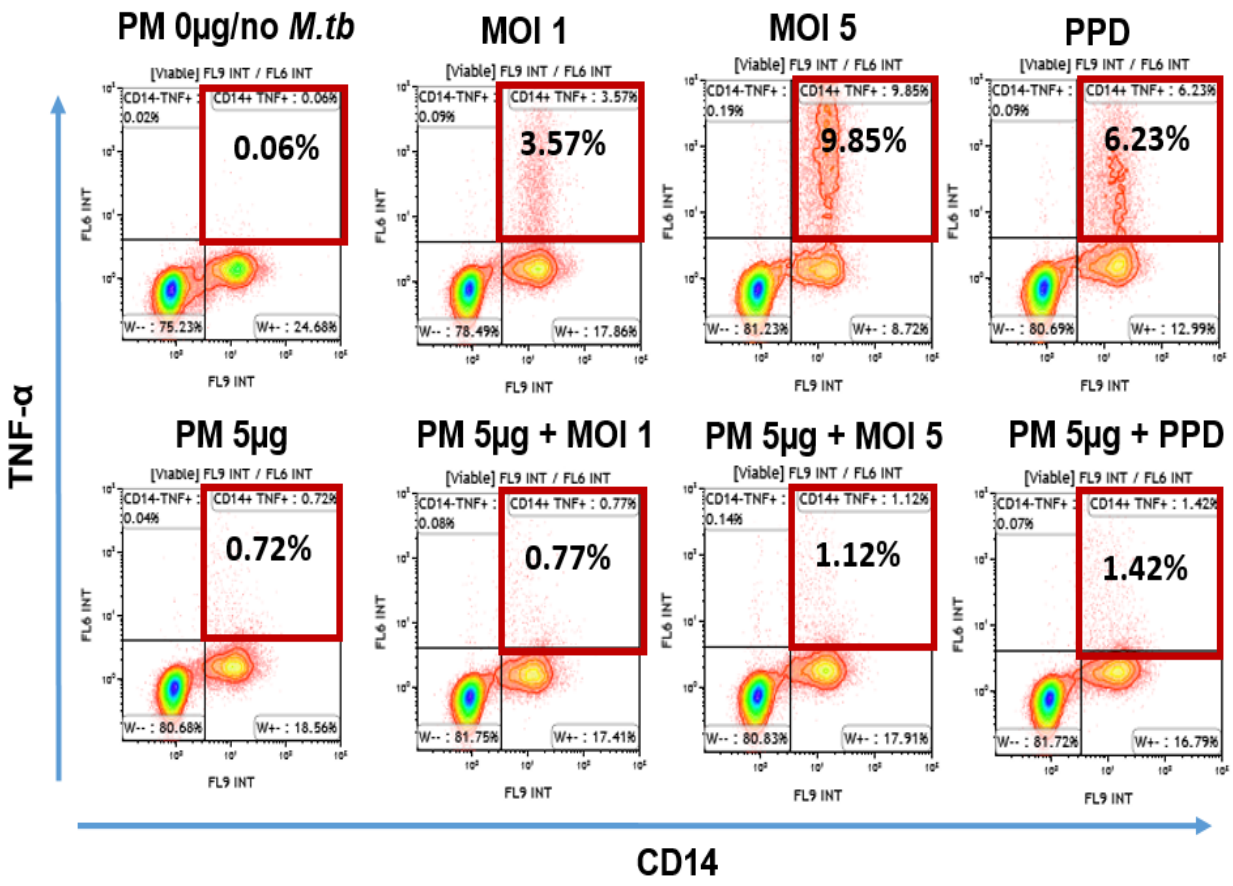


Figure 21. ***Effects of PM exposure on M.tb and PPD-induced TNF-α production by CD14+monocytes in PBMC.***

PBMC were pre-exposed to $PM_{2.5}$ (5µg/ml) for 20 hours followed by infection with M.tb MOI 1 and 5 or stimulation with PPD (10 µg) for 8 hours. Surface staining with anti-CD14 (X-axis) and viability dye eFluor780 monoclonal antibodies was performed followed by permeabilization and intracellular staining for detection of TNF-α (Y-axis) by flow cytometry analysis. A representative flow cytometry contour with density plots of 6 independent experiments is shown.

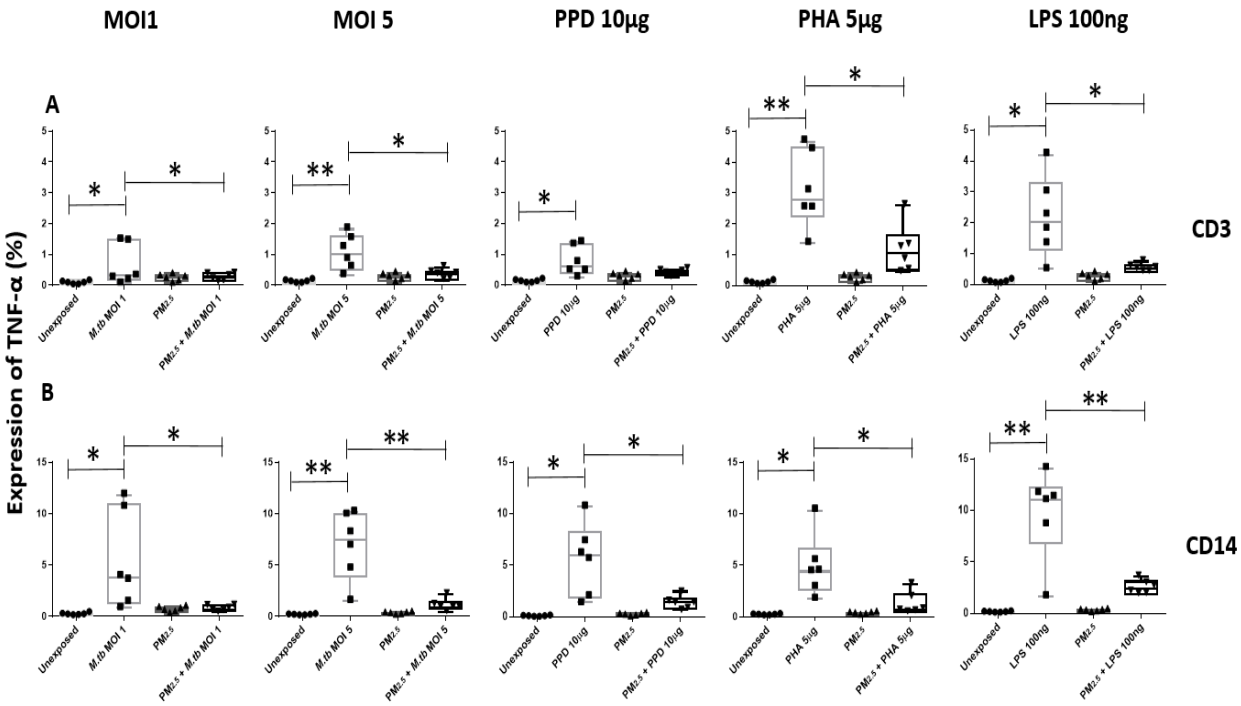


Figure 22. Effects of PM exposure on TNF-α production in CD3+ T cell and CD14+ monocytes in PBMC.

PBMC were pre-exposed to $PM_{2.5}$ (5µg/ml) for 20 hours followed by infection with M.tb MOI 1 and 5 or stimulation with PPD (10 µg), PHA (5 µg) or LPS (100 ng) for 8h. Surface staining with anti-CD3 and anti-CD14 monoclonal antibodies was performed followed by permeabilization and intracellular staining for detection of TNF-α by flow cytometry. The values represent the percentage expression of TNF-α on CD3+ and CD14+ cells from 6 independent experiments. Percent expressions of TNF-α are expressed as scatter plots superimposed on 5-95 percentile box-and-whiskers where the center represents the 50^{th} percentile, the upper hinge is the 75^{th} percentile, and the lower hinge is the 25^{th} percentile. Statistically significant increases between results for uninfected and M.tb-infected and

PPD, PHA or LPS-stimulated PBMC are shown with single (P< 0.05) or double (P< 0.01) asterisks while statistically significant decreases between results for M.tb-infected, PPD, PHA or LPS-stimulated and PM-exposed-M.tb-infected, - PPD, PHA or LPS-stimulated PBMC are shown with single (P< 0.05) or double (P< 0.01) asterisks.

3.3.3 Aim 3c: To examine the effects of $PM_{2.5}$ exposure on the production of anti-inflammatory cytokine IL-10 in PBMC.

3.3.3.1 Rationale: The control and clearance of *M.tb* rely on the regulated induction of pro-inflammatory cytokines IFN-γ and TNF-α during infection [119-121]. IL-10 inhibits immune responses to *M.tb* by blocking the production of pro-inflammatory cytokines [43, 122-124] and can be produced by both T cells and monocytes [41]. T cells are a major source of IL-10 during experimental *M.tb* infection (shown in the murine model in which IL-10 contributes to increased host susceptibility to *M.tb*) [123]. We examined the effects of PM exposure on the production of IL-10 in *M.tb*-infected PBMC.

3.3.3.2 Results: PBMC were pre-exposed to $PM_{2.5}$ (5μg/ml) for 20 h and then infected with *M.tb* MOI 1 and 5 or stimulated with PPD (10 μg/ml), PHA (5 μg/ml), or LPS (100 ng/ml) for an additional 18h. The protein expression of IL-10 by $CD3^+$ T cells and monocytes (CD14) was assessed by flow cytometry. IL-10 was produced by both T cells and monocytes in response to *M.tb* infection and stimulation with PPD, PHA, or LPS. However, IL-10 expression levels were higher in $CD14^+$ than $CD3^+$ cells. Interestingly, exposure to PM significantly increased IL-10 expression in $CD3^+$ T cells (Fig 23: representative flow cytometry dot plots and Fig 24A) and monocytes (Fig 24B) compared to unexposed controls and in PM-exposed- and *M.tb*-infected PBMC compared to PBMC infected with

M.tb alone (Fig 24). Taken together, unlike decreases noted in IFN-γ and TNF-α production by PM-exposed PBMC, PM exposure upregulates the expression of IL-10, thus probably dampening T cell immune responses to *M.tb.*

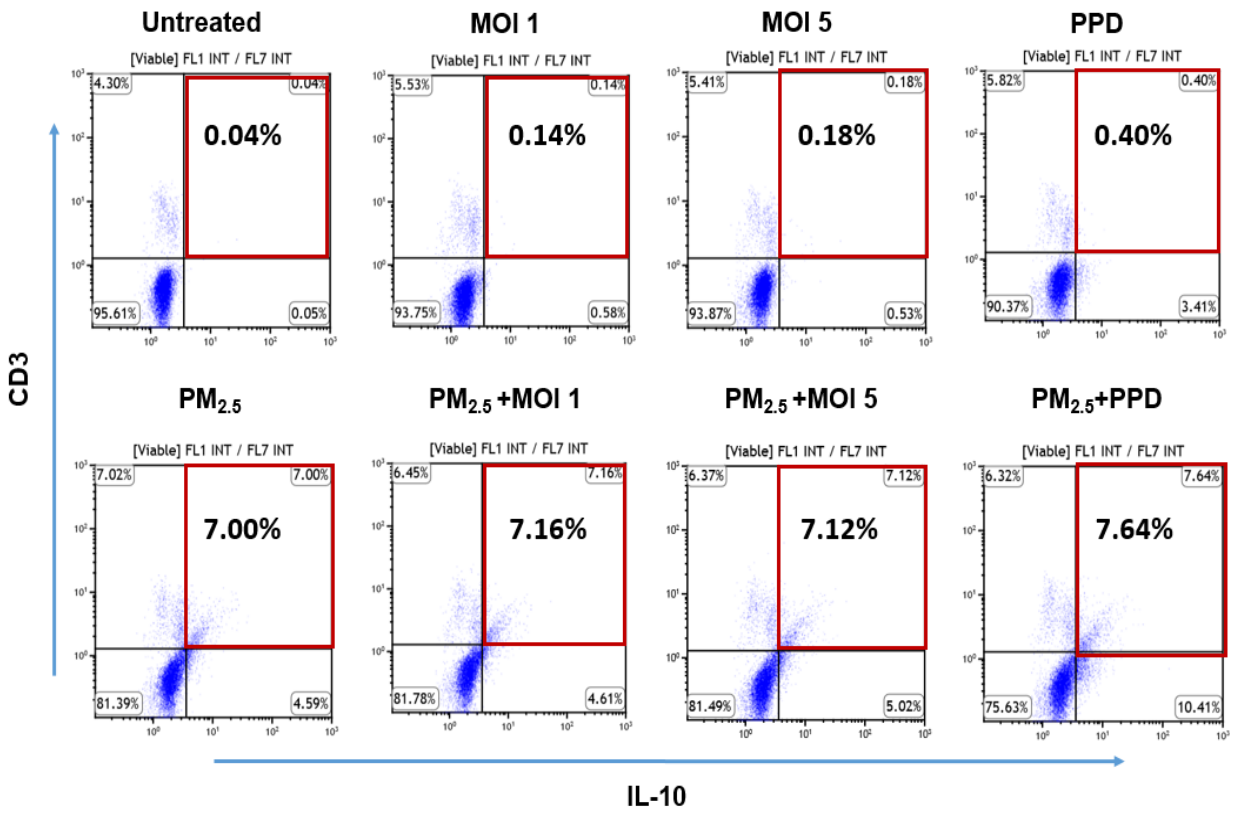


Figure 23. Effects of PM exposure on IL-10 production by CD3+T cells in PBMC.

PBMC were pre-exposed to $PM_{2.5}$ (5 µg/ml) for 20 hours followed by infection with M.tb MOI 1 and 5 or stimulation with PPD 10 µg for 18 hours. Surface staining with anti-CD3 (Y-axis) and viability dye eFluor780 monoclonal antibodies was performed followed by permeabilization and intracellular staining for detection of IL-10 (X-axis) by flow

cytometry analysis. A representative flow cytometry dot plots of 6 independent experiments is shown.

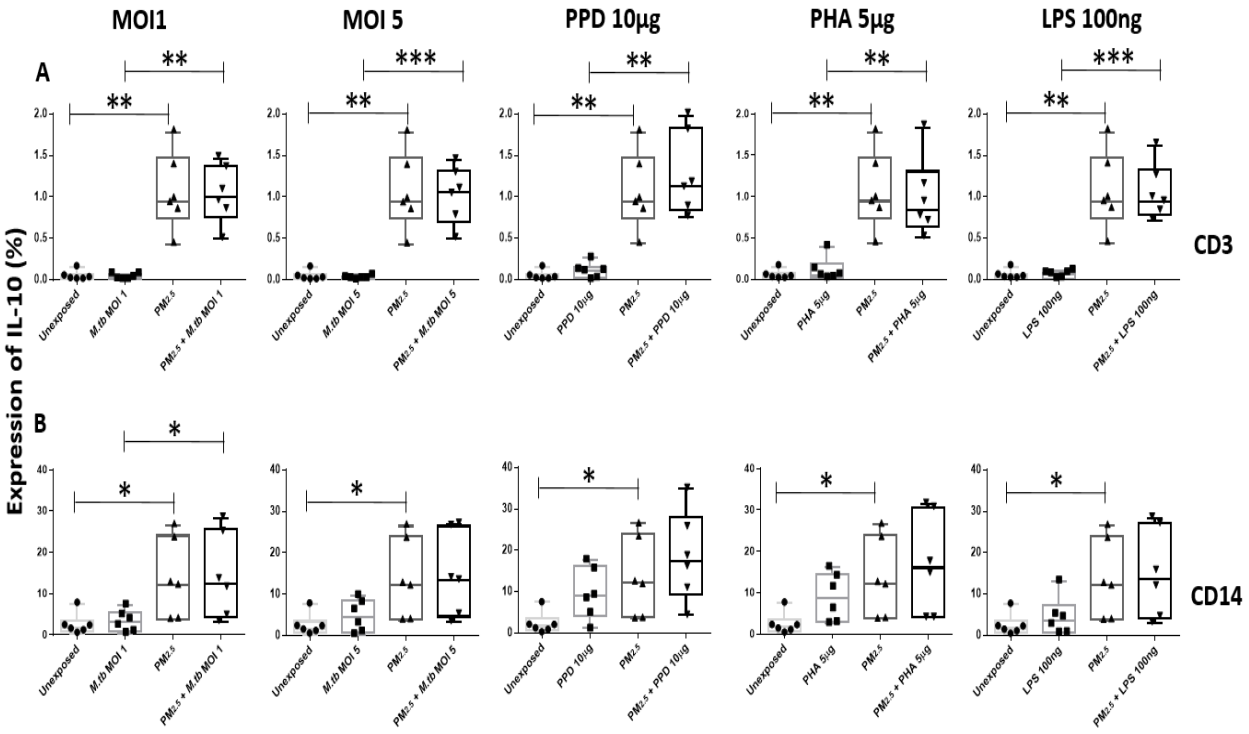


Figure 24. Effects of PM exposure on IL-10 production in CD3+ T cell and CD14+ monocytes in PBMC.

PBMC were pre-exposed to $PM_{2.5}$ (5µg/ml) for 20 hours followed by infection with M.tb MOI 1 and 5 or stimulation with PPD 10µg, PHA 5µg or LPS 10µg for 18h. Surface staining with anti-CD3 and anti-CD14 monoclonal antibodies was performed followed by permeabilization and intracellular staining for detection of IL-10 by flow cytometry. The values represent the percentage expression of IL-10 on CD3+ (A) and CD14+ cells (B)

from 6 independent experiments. Percent expressions of IL-10 are expressed as scatter plots superimposed on 5-95 percentile box-and-whiskers where the center represents the 50^{th} percentile, the upper hinge is the 75^{th} percentile, and the lower hinge is the 25^{th} percentile. Statistically significant increases between results for uninfected and M.tb-infected and PPD, PHA or LPS-stimulated PBMC are shown with single ($P< 0.05$) or double ($P< 0.01$) asterisks while statistically significant decreases between results for M.tb-infected, PPD, PHA or LPS-stimulated and PM-exposed-M.tb-infected, - PPD, PHA or LPS-stimulated PBMC are shown with single ($P< 0.05$), double ($P< 0.01$), or triple ($P< 0.001$) asterisks.

3.4 Aim 4: To study $PM_{2.5}$ effects on cellular activation processes involved in IFN-γ production.

3.4.1 Aim 4a: To examine the effects of $PM_{2.5}$ exposure on the expression of transcription factors (T-bet and STAT 1) involved in the control of IFN-γ in PBMC.

3.4.1.1 Rationale: To further investigate the underlying mechanisms of altered IFN-γ expression, we examined the expression of relevant transcription factors involved in the expression of IFN-γ.

T-bet, a T-cell associated transcription factor, is known to directly activate the expression of IFN-γ through the activation of signal transducer and activator of transcription 1 (STAT1) [125]. STAT 1 becomes activated and directly regulates T-bet when phosphorylated at tyrosine 701 (Y701). We prepared cell extracts from *M.tb*-infected or PPD-stimulated PBMC stimulated with or without $PM_{2.5}$ and analyzed by SDS PAGE.

Western blotting using specific antibodies was used to determine whether PM reduces the level of T-bet expression and anti-phospho antibodies were used to examine the phosphorylation of STAT1.

3.4.1.2 Results: To investigate the effect of PM exposure on the expression of T-bet and phosphorylation of STAT 1, cell extracts prepared from PBMC pre-exposed to $PM_{2.5}$ (5µg/ml) for 20 h and then infected with *M.tb* at MOI 1 and 5 or stimulated with PPD 10µg for an additional 18 h were subjected to SDS-PAGE and western blot analysis with anti-T-bet, -phospho-STAT 1 (P-stat 1), -total-STAT 1(T-stat 1)and GAPDH antibodies was performed. The expression of T-bet in *M.tb*-infected and PPD-stimulated PBMC was markedly increased compared to unexposed controls (Fig 25A & B). While the expression of T-bet was slightly increased in PBMC exposed to PM alone, its expression was significantly reduced in $PM_{2.5}$ -exposed- and *M.tb*-infected PBMC as well as in PM-exposed- and PPD-stimulated PBMC (Fig 25A & B). Furthermore, infection with *M.tb* MOI 1 and 5 increased the phosphorylation of STAT 1 at Y701. However, pre-exposure to $PM_{2.5}$ reduced STAT 1 phosphorylation in *M.tb*-infected PBMC (Fig 26). This observation is consistent with our IFN-γ findings (see above) showing increased levels upon infection with *M.tb* and decreased levels with PM exposure. This data suggests that PM exposure inhibits the production of IFN-γ upon *M.tb* infection and PPD stimulation by modifying the expression and phosphorylation of transcription factors T-bet and STAT 1 (which positively regulate IFN-γ expression) respectively.

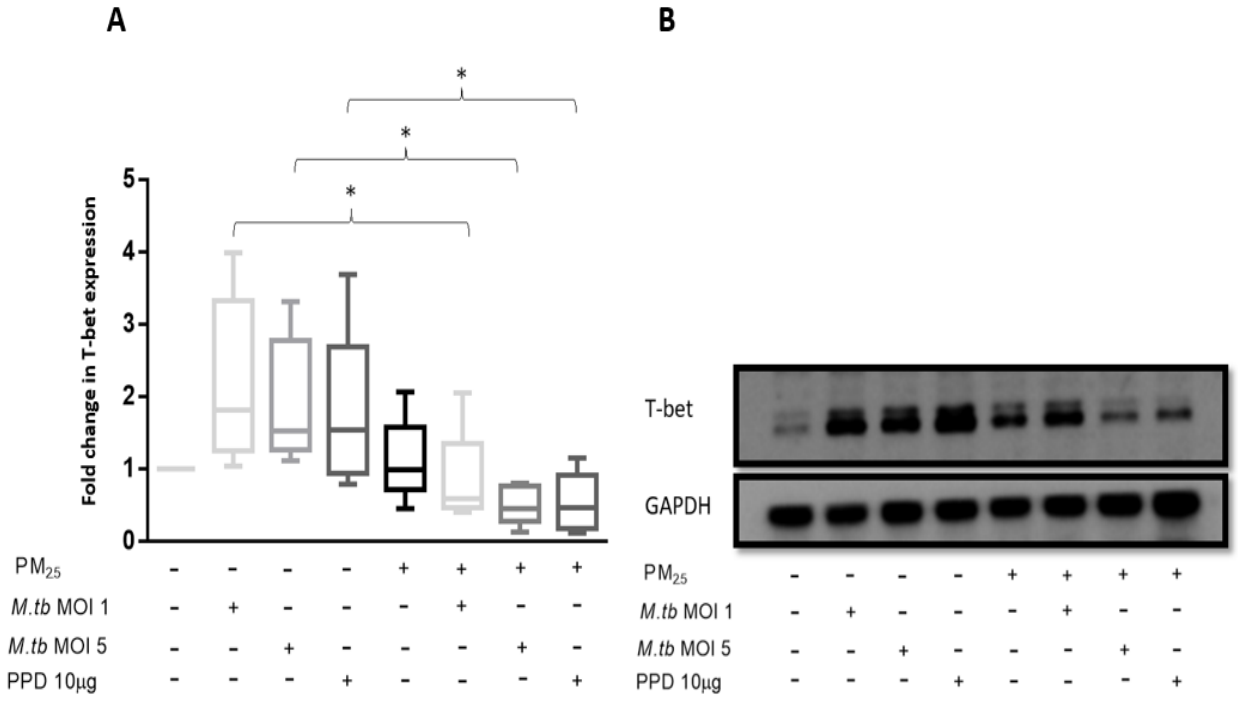


Figure 25. Effects of PM exposure on the expression of T-bet in PBMC.

PBMC were pre-exposed to $PM_{2.5}$ (5µg/ml) for 20 hours followed by infection with M.tb MOI 1, MOI 5 or stimulation with PPD 10 µg for 18 hours. Cell were lysed, cellular protein extracts were prepared and resolved by SDS-PAGE, and western blotting was performed with specific antibodies for T-bet and GAPDH (as loading control). (A) Representative western blot result showing the effects of PM-exposure on the expression of T-bet. (B) Densitometric results were obtained and normalized for GAPDH. Data represent mean results +/- SEM of 5 independent experiments. Statistically significant decreases between results for M.tb-infected- or PPD-stimulated- and PM-exposed-M.tb-infected or PM-exposed- PPD-stimulated PBMC are shown with single ($P< 0.05$) asterisks.

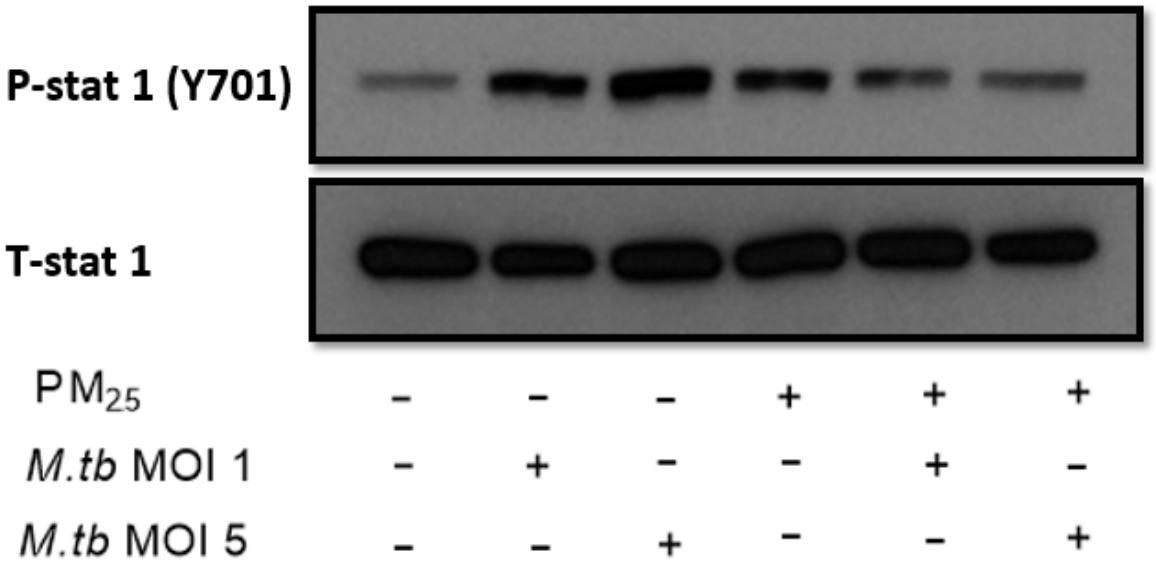


Figure 26. Effects of PM exposure on the phosphorylation of STAT 1 in PBMC.

PBMC were pre-exposed to $PM_{2.5}$ (5µg/ml) for 20 hours followed by infection with M.tb MOI 1 and MOI 5 for 18 hours. Cell were lysed, cellular protein extracts were prepared and resolved by SDS-PAGE, and western blotting was performed with specific antibodies for P-STAT 1 and T-STAT 1 (as internal control). Western blot result shows the effects of PM-exposure on the phosphorylation of stat 1.

3.4.2 Aim 4b: To examine the effects of $PM_{2.5}$ exposure on the expression of transcription factor STAT 3 that negatively regulates IFN-γ production.

3.4.2.1 Rationale: Activation of STAT3 via phosphorylation at tyrosine 705 (Y705) causes its translocation into the nucleus to upregulate IL-10 and represses the pro-inflammatory genes including IFN-γ. Finally, we examined the expression STAT3 to further investigate the mechanism underlying the altered expression of pro-inflammatory cytokines. We prepared cell extracts from unexposed and $PM_{2.5}$-exposed PBMC and analyzed by SDS PAGE. Western blotting using anti-human phospho STAT 3 antibodies was employed to examine the phosphorylation of STAT3.

3.4.2.2 Results: To investigate the effect of PM exposure on the phosphorylation of STAT 3, cell extracts prepared from unexposed PBMC or PBMC exposed to $PM_{2.5}$ (5 µg/ml) for 20 h were subjected to SDS-PAGE and western blot analysis with phospho-STAT 3 (P-stat 3) and GAPDH antibodies was performed. The phosphorylation of STAT3 at Y705 was markedly increased in $PM_{2.5}$-exposed compared to unexposed controls (Fig 27A & B). This data suggests that PM inhibits production of pro-inflammatory cytokines by modifying the phosphorylation of transcription factor STAT 3 (which positively regulates IL-10 expression).

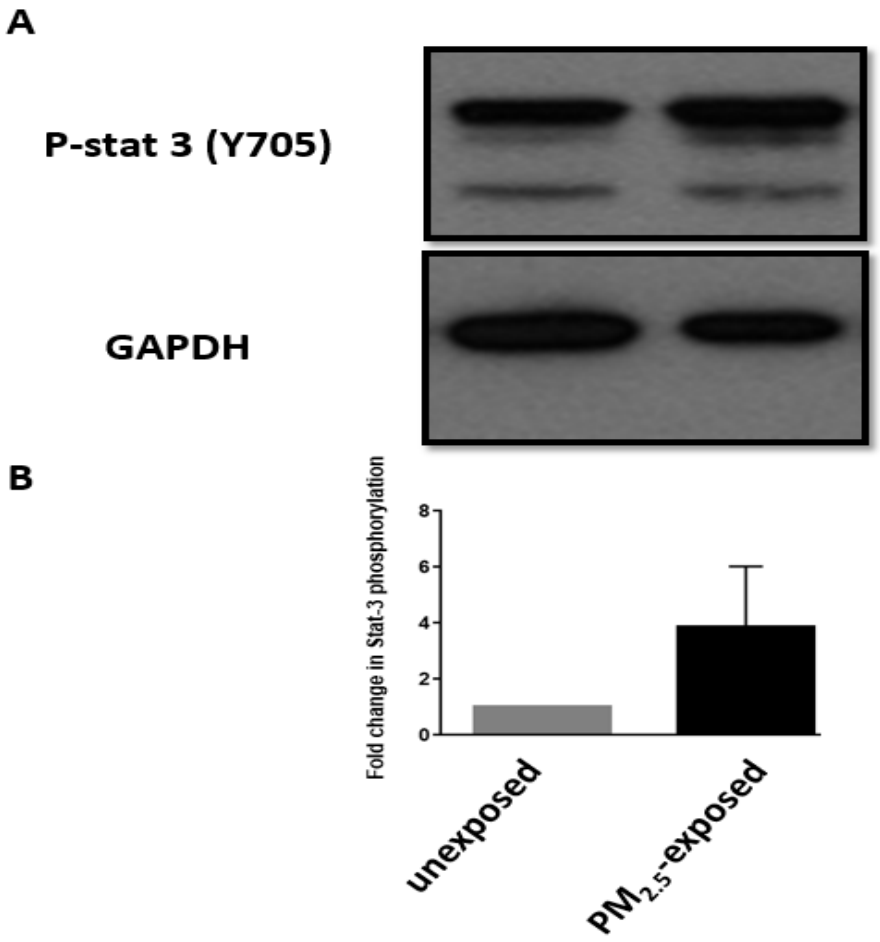


Figure 27. Effects of PM exposure on the phosphorylation of stat 3 in PBMC.

PBMC were pre-exposed to $PM_{2.5}$ (5µg/ml) for 20 hours. Cell were lysed, cellular protein extracts were prepared and resolved by SDS-PAGE, and western blotting was performed with specific antibodies for P-stat 3 and GAPDH (as loading control). (A) Representative western blot result shows the effects of PM-exposure on the phosphorylation of stat 3. (B) Densitometric results were obtained and normalized for GAPDH. Data represent mean results +/- SEM of 4 independent experiments.

4.1 DISCUSSION

Epidemiological evidence of significantly increased risk of TB development after exposure to cigarette smoking [7-9] indoor pollution [8, 9, 14, 17] or outdoor pollution [16, 126] suggests that air pollutants are potentially detrimental to antimicrobial immune effector functions. There is evidence that exposure to ambient air pollution PM increases the risk of progression from latent *M.tb* infection to TB [16, 19, 20].

We have shown that air pollution PM modifies innate ant mycobacterial immune responses in the human respiratory epithelium [47] and that DEP exposure of PBMC alters *M.tb*-induced NF-κB and IRF pathway target gene expression [46]. Studies assessing the effects of PM on T-cell responses have been lacking to date. The current study therefore explored the impact of $PM_{2.5}$ exposure on human peripheral blood T cell responses to *M.tb.*

In a first step, to assess cellular uptake and localization of PM and *M.tb* in human PBMC by TEM, we observed clusters of free, non-membrane-bound, PM in the cytoplasms of PM-exposed monocytes as well as extracellularly, and concurrent uptake of *M.tb*. This observation coincided with preceding observations by our group of co-uptake of DEP and *M.tb* in enriched peripheral $CD14^+$ monocytes [46]. Our observations differ from findings in human T cells, which showed cytoplasmic localization of DEP in membrane-surrounded vesicles [127] or vacuoles of monocyte-derived macrophages [128]. The differences in cellular localization and uptake between our studies and that of others may be due to the differences in the particle and the cell type used. Co-uptake of $PM_{2.5}$ and *M.tb* in the same

cell, as observed in the current study, may result in intracellular PM-*M.tb* interactions leading to potential interference with host anti-mycobacterial immune responses.

Intracellular growth control of *M.tb* is a critical component of the host response to *M.tb [129]* and involves cytotoxic T cell-mediated lysis of *M.tb*-infected target cells [95, 96]. In the current study, we assessed whether urban $PM_{2.5}$ exposure alters the intracellular growth control of *M.tb* in human PBMC. $PM_{2.5}$ exposure resulted in loss of intracellular growth control of *M.tb* in PBMC. Whereas $PM_{2.5}$-induced loss of growth control of *M.tb* was dose-independent on days 1 and 4, we observed significantly higher *M.tb* cfu numbers in PBMC pre-exposed to 5 µg/ml compared to 1 µg/ml of $PM_{2.5}$ on day 7, ($p < 0.05$). To explore if cell death due to cellular toxicity of PM could have contributed to the observed lack of *M.tb* growth control upon $PM_{2.5}$ exposure, we assessed the impact of $PM_{2.5}$ on cell viability microscopically, by LDH release assay and assessing induction of apoptosis and necrosis in monocytes and lymphocytes by flow cytometry. Although microscopically some cell death was observed on day 7 upon $PM_{2.5}$, numbers of viable PBMC allowed for appropriate assessment of growth control by PBMC. No statistically significant $PM_{2.5}$-induced cytotoxicity was noted by LDH assay on day 7 (or the earlier days 1 and 4). Pre-exposure to 1 and 5 µg of PM_{10} followed by infection with M.tb caused cell death, therefore we assessed growth control by PBMC using $PM_{2.5.}$ No significant increases in the proportions of monocytes and lymphocytes undergoing apoptosis and necrosis were noted in any of the experimental conditions employed for all of the immune functional studies. Furthermore, significantly higher *M.tb* cfu numbers following $PM_{2.5}$ exposure were already observed on days 1 and 4. We can also exclude the possibility that any differences in *M.tb* phagocytosis would underlie the observed $PM_{2.5}$ effects on *M.tb* growth control, as the *M.tb*

uptake on day 0 (2 h after infection) was comparable between either of the $PM_{2.5}$ concentrations *M.tb*. The observed suppressive effect of $PM_{2.5}$ on *M.tb* growth control is consistent with findings in the murine model studies which reported a correlation between DEP exposures and higher lung BCG load [130] and another in which continued inhalation of diesel exhaust reduced the capacity of murine alveolar macrophages to kill *M.tb*, and decreased the expression levels of pro-inflammatory cytokines thereby enhancing susceptibility to murine mycobacterial infections [131]. Taken together, our observation of PM-induced decrease of the capacity of PBMC to control *M.tb* growth is likely due to defects in effector mechanisms for the killing of *M.tb*, supporting the hypothesis that air pollution exposure to $PM_{2.5}$ increases susceptibility to *M.tb.*

During T cell-mediated immune responses, activated antigen-presenting cells present antigens to naïve T cells to cause their activation. Several receptor molecules are required in this interaction, which we studied next to begin exploring how $PM_{2.5}$ exposures weaken *M.tb* growth control capacity of PBMC. First, we wanted to examine the effects of $PM_{2.5}$ exposures on the activation of APC by examining the expression of CD54 and HLA-DR on *M.tb*-infected PBMC exposed to $PM_{2.5.}$ $PM_{2.5}$ exposure did not alter the expression of CD54 and HLA-DR, markers of APC activation. However, exposure to $PM_{2.5}$ significantly increased the expression of co-stimulatory molecule CD80.

Evidence from both animal and human models suggests an important role for both CD4 and CD8 T cells in successful immune control of *M.tb* infection [76-81]. Activated CD4 and CD8 T cells produce pro-inflammatory cytokines for macrophage activation. To further explore the $PM_{2.5}$ effects on *M.tb* growth control, we next assessed if $PM_{2.5}$ interferes with cell-mediated immunity at the level of T cell activation [132] and production

of pro-inflammatory cytokines (IFN-γ and TNF-α) for macrophage activation [133]. Assessing the expression of early activation markers CD69 and CD25 molecules on T cells during *M.tb* infection is a reliable measure of T cell activation [107]. Earlier studies had shown that DEP exposure reduces the expression of T cell activation marker CD25 in human peripheral blood T cells [127]. In the current study, $PM_{2.5}$ pre-exposure downregulated the *M.tb* MOI 1 and MOI 5- and PPD-induced CD69 expression in $CD3^+$, $CD4^+$, and $CD8^+$ T cells in PBMC while the constitutive expression of CD25 remained unchanged on T cell subsets during *M.tb* infection. Interestingly, earlier studies reported that exposure to DEP significantly reduces the expression of CD25 without affecting the expression of CD69 in $CD4^+$, and $CD8^+$ T cells [127]. Differences in activation marker expression profiles observed between our studies and that of others may be due to differences in the composition of the PM or the cell culture systems used to study PM effects. We also examined the expression of T-cell inactivation markers PD-1 and CTLA-4. $PM_{2.5}$ pre-exposure did not alter the expression of PD-1 and CTLA-4. Our data suggest that PM exposure decreases the activation of T cells during *M.tb* infection. This suggests that modulation of T cell activation by $PM_{2.5}$ could affect the production of cytotoxic molecules required for lysis of mycobacteria-infected phagocytes by T cells.

As T cell activation precedes cytokine responses, we next focused on IFN-γ and TNF-α as key Th1 type cytokines in human immunity during *M.tb* infection [30, 38, 40, 134-136] and how air pollution $PM_{2.5}$ may alter *M.tb*-induced IFN-γ and TNF-α production in PBMC. In our previous study, DEP suppressed the production of *M.tb* and PPD-induced IFN-γ in a dose-dependent manner in PBMC [46]. In the current study we aimed at identifying if $PM_{2.5}$ alters the production of IFN-γ and TNF-α by T cell subpopulations

($CD3^+$, $CD4^+$, $CD8^+$), and $CD16^+$ cells. We found that $PM_{2.5}$ exposure decreases *M.tb* MOI 1 and MOI 5-induced IFN-γ levels ($p < 0.05$). Interestingly, the highest production of IFN-γ was noted in both CD8 and CD16 cells ($CD8^+CD16^+$) during infection with both MOI 1 and MOI 5 (Fig 4C and 4D). Both $CD8^+$T cells (adaptive) [137] and NK cells (innate) [138] are host protective killer cells against bacterial infection, and $CD8^+CD16^+$ cells mediate cytotoxicity [139]. Our findings therefore suggest suppression of cytotoxic responses to *M.tb* as a consequence of $PM_{2.5}$-induced decrease of IFN-γ production by $CD8^+CD16^+$ cells. This observation is also supported by the murine study in which DEP exposure decreased LPS-induced secretion of IFN-γ in antigen-specific T cells. [140]. Together, this suggests that the observed loss of growth control by PBMC upon PM exposure (Fig 3 above) may have been impacted by defective IFN-γ production by dysfunctional T cells.

TNF-α is another pro-inflammatory cytokine crucial for the control of mycobacterial infection [24, 29, 141-145]. Here, we examined the effects of $PM_{2.5}$ exposures on TNF-α production by $CD3^+$ T cells and $CD14^+$ monocytes in PBMC infected with *M.tb* or stimulated with PPD, PHA, and LPS. Infection with *M.tb* and stimulation with PPD, PHA or LPS significantly increased TNF-α expression ($p < 0.05$). $PM_{2.5}$ exposures decreased TNF-α expression in both T cells and monocytes in response to *M.tb* infection as well as in PPD-, PHA-, and LPS-stimulated PBMC ($p < 0.05$). These findings show that PM exposure decreases the production of protective human host pro-inflammatory cytokines during *M.tb* infection.

Anti-inflammatory cytokine IL-10 is an inhibitor of pro-inflammatory cytokines and it impairs immune responses to *M.tb* [146-149]. In human TB, high production of IL-10 is associated with an increase in the disease incidence in TB patients [40]. In the current

study, PM exposure significantly increased IL-10 production in both $CD3^+$ T cells and monocytes compared to un-exposed cells. It is not surprising that we observed higher levels of IL-10 expression in monocytes than in T cells given that IL-10 is primarily produced by monocytes in humans. Interestingly, in our study, IL-10 levels were significantly higher in T cells pre-exposed to PM followed by infection with *M.tb* or stimulated with PPD, LPS, and PHA compared to T cells infected with *M.tb* alone or stimulated with PPD, LPS, and PHA alone. As IL-10 produced by T cells during *M.tb* infection contributes most to increased host susceptibility [123] and suppresses immune responses to TB [150], our findings indicate that $PM_{2.5}$ exposure promotes anti-inflammatory capacity of T cells in response to *M.tb* and may be underlying the reduced expression of IFN-γ and TNF-α.

To further explore the observed reduction in IFN-γ production, we assessed the expression of transcription factor T-bet and phosphorylation of STAT1 which are required for IFN-γ expression and generation of type 1 immunity [151, 152]. Mice lacking T-bet, for example, are susceptible to virulent *M.tb* infection with increased systemic bacterial burden, diminished IFN-γ production and increased IL-10 production [153]. Interestingly, although very different in its composition, cigarette smoke has been shown to reduce T-bet expression in murine lung T cells [10]. In the current study, one may speculate that decreases in *M.tb*-induced IFN-γ production upon $PM_{2.5}$ exposure were mediated via reduced expression of T-bet and decreased phosphorylation of STAT1 thus providing a mechanism by which exposure to 'real-world'-derived urban ambient $PM_{2.5}$ may adversely affects *M.tb*-specific human host T cell functions. The study of other transcription factors such as CREB, ATF-2, and cJun, also known to regulate IFN-γ expression in primary human T cells in response to mycobacterial antigen [154] was beyond the scope of the

current study. Finally, increases in phosphorylation of STAT3 in our study may suggest that IL-10 was upregulated upon $PM_{2.5}$ exposure via phosphorylation of STAT3 to downregulate the production of pro-inflammatory cytokines IFN-γ and TNF-α during *M.tb* infection.

In summary, our study data show coexistence of *M.tb* and $PM_{2.5}$ in human monocytes, PM-induced loss of intracellular growth control of *M.tb*, PM-induced decrease of T cell activation marker CD69 in *M.tb* infection, reduced production of key cytokines (IFN-γ and TNF-α) in protective immune response to *M.tb* upon PM exposures, PM-induced increase of anti-inflammatory cytokine IL-10 and PM-induced downregulation of transcription factor T-bet in *M.tb* infection. We hypothesize that PM-induced loss of intracellular growth control resulted from decreased production of IFN-γ and TNF-α by T cells mediated through the increased production of IL-10 and decreased expression of T-bet (**Fig 28**). To our knowledge, this is the first report of the mechanisms underlying ambient air pollution PM-related susceptibility to *M.tb* infection by elucidating the effects of PM on T cell immune responses to *M.tb*. Our findings strongly suggest PM-induced alterations of *M.tb*-specific T cell functions.

These findings are significant because weakening of *M.tb*-specific T cell immunity by interactions of air pollutant PM with human immune cells would increase the susceptibility to *M.tb* infection and progression to active TB. These findings may also have implications for TB vaccine development as currently used TB vaccine candidates in clinical trials [155] are designed to induce T-cell mediated immune responses. PM-induced alteration of *M.tb*-specific T cell function will affect the efficacy of these vaccines. Furthermore, the widely used diagnostic IFN-γ release assay (IGRA) tests [156] rely on

the induction of T-cell-mediated immune responses. PM-induced alterations of *M.tb*-specific T cell functions will have implications for the performance of the diagnostic tests. In conclusion, our study shows that air pollution PM exposure mitigates the expression of protective human host immune cell responses against *M.tb*, interfering with the multitude of host immune mechanisms required for the immunological control of *M.tb* **(Fig 28).** This provides mechanistic evidence for increasing concerns about the adverse impact of air pollution on global TB control efforts.

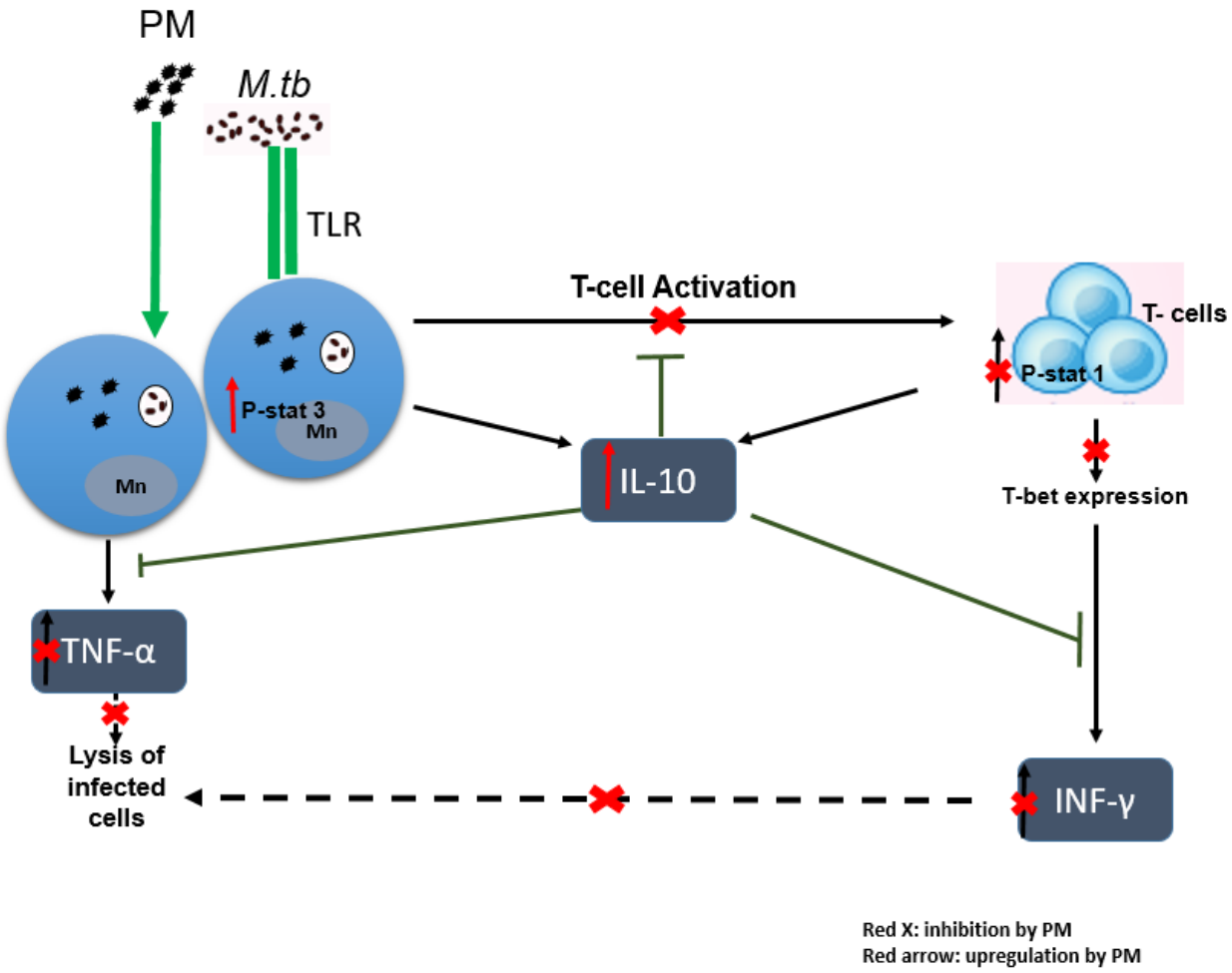


Figure 28. **A hypothetical representation of PM-mediated suppression of M.tb-specific T cell functions.**

Air pollution PM exposures dampen anti-mycobacterial T cell immune responses by downregulating T cell activation and inducing loss of intracellular growth control of M.tb probably by reducing the production of protective cytokines (IFN-γ and TNF-α) via downregulation of transcription factors (T-bet and Stat 1) that positively regulate IFN-γ production and/or by increasing the production of anti-inflammatory cytokine IL-10 through the upregulation of stat-3.

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7.1 **APPENDICES**

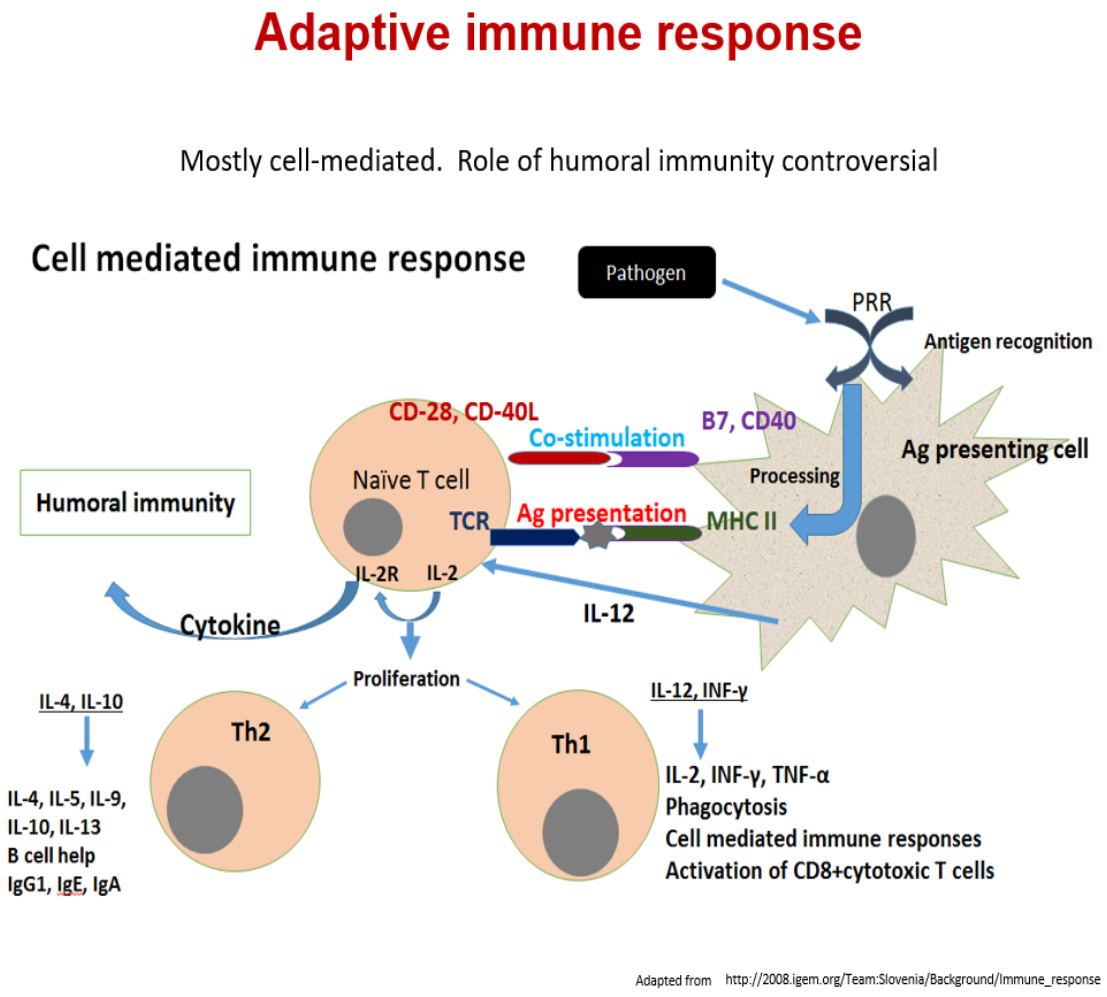

Figure 29. **T cell-mediated immunity against *M.tb***

T cell-mediated immunity is considered the central element of antimycobacterial immunity and includes the development of antigen-specific T cells to eliminate M.tb. When stimulated by an antigen, the receptors of APC cause the stimulating antigen to be endocytosed and display the antigen on their surface via major histocompatibility complex (MHC) molecules. Activated APC then display those antigens to groups of naïve lymphocytes to cause their activation. Activated lymphocytes divide and mature, producing effector cells that cause the secretion of macrophage-activating cytokines, lysis of host infected cells and direct death or elimination of the microbe.

Figure 30. **PM collection site.**

$PM_{2.5}$ and PM_{10} were collected with high volume air filters in direct vicinity of the Iztapalapa municipality. PM were collected during the course of one year.