PM2.5 Mediated Alterations In The In Vitro Human Granuloma Resulting In Reactivation Of Mycobacteria

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Research Article

Keywords: Mycobacteria bovis BCG, Particulate matter, Granuloma, Dormancy, Reactivation, Cytokines

DOI: https://doi.org/10.21203/rs.3.rs-568825/v1

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Abstract

Exposure to pollutants diminishes the immune response to mycobacterial antigens relevant to contain the infection in the granuloma, thus leading to reactivation of latent bacilli. Present study was therefore designed based on the hypothesis that exposure to particulate matter pollutant PM$_{2.5}$ affects the granuloma formation and reactivation of latent mycobacterial bacilli contained in the granuloma. For the extraction of PM$_{2.5}$, based on initial standardizations, teflon filter was selected over the quartz filter. Two different approaches were used to study the effect of PM$_{2.5}$ on the human PBMCs granuloma formed by *Mycobacterium bovis* BCG at MOI 0.1. In the first approach, granuloma formed in the presence of PM$_{2.5}$ was loosely packed and ill-defined with significant downregulation of dormancy associated mycobacterial genes, upregulation of reactivation associated rpfB gene along with a significant increase in TNF$\alpha$ level without any change in the bacterial load in terms of CFUs. In the second approach, PM$_{2.5}$ treatment of already established human PBMCs granuloma formed with *M. bovis* BCG also led to its disruption. Although, in these conditions, downregulation of dormancy associated genes was observed but there was also a decrease in the expression of reactivation associated rpfB gene without any change in the cytokine levels. Therefore, it can be inferred that in the presence of PM$_{2.5}$, there is poor granuloma formation along with a change in mycobacterial gene expression characteristics of active bacilli and alteration in host immune response without any significant changes following treatment of already established granuloma with the pollutant.

Introduction

Recently, the rapid increase in urbanization and modernization has led to increase in air pollution, which adversely affect human health. A significant correlation between PM$_{2.5}$ (particulate matter with aerodynamic diameters of less than 2.5 µm) and respiratory diseases has been reported in numerous studies (Xing et al. 2016). WHO recommends mean PM$_{2.5}$ concentration of 25 µg/m$^3$ and level above this is reported to induce deterioration in lung function (Nhung et al. 2017). The increased risk of tuberculosis (TB) is also associated with ambient PM$_{2.5}$ (You et al. 2016). As per WHO reports, most TB deaths occur in countries where the people breathe smoggy air over a long time (Rajaei et al. 2018). Thus, ambient particulate matter (PM) is of great concern for public health, particularly in the case of socioeconomically and environmentally compromised communities, where TB is endemic. A positive association has been demonstrated between tuberculosis reactivation and prolonged exposure to PM$_{2.5}$ (Rajaei et al. 2018; Wong et al. 2017). Following inhalation of *Mycobacterium tuberculosis* (M.tb), only 10% of individuals develop the clinically symptomatic disease known as active TB due to their immune system's inability to control the infection. A larger percentage of infected individuals can contain the infection and pathogen in these individuals remains quiescent, a stage known as “Latent TB” characterized by granuloma formation, which is a focal collection of inflammatory immune cells at the site of infection. The latent bacilli may persist and remain contained in granuloma even for a life time unless reactivation to active TB occurs, due to certain conditions leading to host immune compromisation.
Pollutants which have been found to be associated with reactivation of TB are also known to affect the host immune system. Cigarette smoke extracts, diesel exhaust products and indoor pollutants are some of the known factors associated with the diminished inflammatory response in host following exposure to antigens (Ding et al. 2017; Lin et al. 2014; Rivas-Santiago et al. 2015). They have been reported to induce epigenetic changes in the host immune cells, leading to a decline in immune response (Weinhold 2006). Using various cell lines, it has been demonstrated that exposure to pollutants also decreases the immune response to mycobacterial antigens, resulting in the downregulation of pro-inflammatory markers relevant to contain the infection in the granuloma (Ding et al. 2017; Lin et al. 2014; Rivas-Santiago et al. 2015). Thus, exposure to pollutants might have detrimental effects on the formation and maintenance of granuloma following mycobacterial infection thereby leading to reactivation of latent bacilli and active disease. However, to date it is not investigated that how exposure to pollutants impacts the granuloma formation in the individuals infected with TB as well as reactivation of latent bacilli contained in the granuloma.

The present study attempts to address these questions by assessing the ability of PM$_{2.5}$ in disruption of in-vitro peripheral blood mononuclear cells (PBMCs) granuloma formation using $M$. bovis BCG ($Mycobacterium bovis$ Bacille Calmette-Guerin) as well as reactivation of latent bacilli in the granuloma.

**Methods**

**Study population**

Ten healthy individuals greater than 18 years of age without any heavy exposure to the particulate matter pollutant PM$_{2.5}$ were recruited for the present study. Study participants with a history of any chronic conditions (Type 2 Diabetes Mellitus, hypertension, renal failure, leprosy and respiratory disorders), HIV infection or any other immunodeficiency disorders, history of transplantation and under immunosuppression treatment and history of autoimmune disease were excluded. 15ml of blood was collected from each subject in a heparinised vial after obtaining the written informed consent.

**Culture**

*Mycobacterium bovis* BCG initially obtained from Microbial Type Culture Collection and Gene Bank (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh and maintained in our laboratory was used for this study. For various experiments, it was further subcultured in Middlebrook 7H9 broth.

**Pollutant extraction**

Particulate matter PM$_{2.5}$ was collected by placing the filter (quartz/teflon) in Fine Particulate Sampler Envirotech APM–550 installed at the Department of Community Medicine, PGIMER during January/February 2019. It allows the air to flow at a rate of 1m$^3$/hr through it and thus, PM$_{2.5}$ gets collected in the filter. PM$_{2.5}$ was initially collected in both teflon (PTFE) and quartz filters, followed by extraction using a specific protocol from each filter (Roper et al. 2015; Zhou et al. 2015). Briefly, a quartz
filter containing the pollutant was initially sonicated with 75% ethanol for 30 min at 35KHz followed by three further sonication steps at 35KHz for 15 min each. The resultant suspension was filtered through a 20µm nylon filter to avoid larger filter particles. The filtrate was centrifuged at 4800 rpm for 60 min.

On the contrary Teflon (PTFE) filter was sonicated at 35 KHz for 5 min with methanol and sterile water at a ratio of 9:1 v/v by placing the filter upside down in such a way that the pollutant containing surface was in contact with the solvent. The suspension was centrifuged at 8000g for 15 min. The supernatant obtained with both the filters was finally filtered through a 0.45µm nylon filter to eliminate the quartz fibers. The resulting filtrate was frozen at -80°C in pre-weighed small centrifuge tubes followed by lyophilization. Thus, the formed lyophilized extract was weighed and resuspended to 1mg/ml concentration and stored at -20°C till further use.

**Cell viability assay (MTT assay)**

Peripheral blood mononuclear cells (PBMCs) isolated from blood samples of recruited subjects were treated with different concentrations (0.1µg/ml, 1.0µg/ml, 10µg/ml & 50µg/ml) of PM₂.₅ in RPMI complete media with 10% heat-inactivated autologous serum. The cytotoxic effect of the pollutant on PBMCs was assessed using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (Sharma et al. 2017).

**Development of in-vitro human granuloma model with M. bovis BCG**

PBMCs were isolated from heparinised blood and viable cells were counted. Extracellular matrix (ECM) was prepared with 0.95 ml collagen I (bovine, 3 mg/ml), 50 µl 10X PBS, 10 µl 1N NaOH, 4µl human fibronectin per ml of ECM. The isolated PBMCs were mixed with ECM at a concentration of 5x10⁵ PBMCs / 50 µl of ECM. 50 µl of this mixture was added to each well of 96 well tissue culture plate followed by the addition of M. bovis BCG at two different MOIs (0.1;1 bacilli / 10 PBMCs and 0.2; 2 bacilli / 10 PBMCs) and incubation at 37°C for 1hr in 5% CO₂. Afterwards, RPMI 1640 media containing 20% heat-inactivated (56°C for 1 hour) autologous serum was added over the ECM and plates were again kept at 37°C for 7 days in 5% CO₂ incubator with the change of media on day 7 and harvesting of a granuloma on day 10.

**Effect of PM₂.₅ on granuloma**

To evaluate the effect of PM₂.₅ on the formation of PBMC granuloma using Mycobacterium bovis, PM₂.₅ at selected concentrations (0.1µg/ml and 10µg/ml) was added along with media on day 0 and media was changed on day 7 and after day 10 it was harvested. Further to assess the effect of PM₂.₅ on already formed PBMC granuloma, PM₂.₅ was added on day 10 after the granuloma establishment. The granuloma was harvested after 72 hr of treatment or 13 days of granuloma formation. During the harvesting of granuloma, the supernatant media was taken out and stored at -80°C for cytokines assay. To evaluate the effect of PM₂.₅ on the immune status of granuloma, cytokines of Th1, Th2 and Th17 cells were measured using BD™ Cytometric Bead Array kit. The reaction mixture was prepared as per the
manufacturer’s instruction. 50µl of culture supernatant stored during granuloma harvesting was used to estimate the levels of IL-2, IL-4, IL-6, IL-10, TNF, IFN-γ and IL-17 using a dual-laser flow cytometer capable of acquiring the fluorescence from phycoerythrin (PE) and allophycocyanin (APC) channels. The data analysis was done using BD FCAP Array software.

For CFU enumeration and gene expression studies, 50µl collagenase IV (2 mg/ml in 50mM TES buffer) was added to each well and the plate was kept at 37°C for 1 hour. After incubation, the cells in 4 wells were pooled and 200 µl 0.1% triton x-100 was added, followed by incubation at room temperature for 5 min. The cell suspension thus obtained was centrifuged at 3000g for 12 min and pellet was resuspended in trizol for gene expression studies. Pellet obtained from other sets of four wells was suspended in 7H9 media for CFU enumeration by plating on 7H11 agar as described earlier (Sharma et al. 2017). For histopathological examination, after removing the supernatant media as described above, 200µl of 10% buffered formalin was added to the wells and kept overnight at 37°C. The next day, formalin was removed and cells were processed for haematoxylin and eosin staining and acid-fast staining (Ziehl-Neelsen staining) using standard protocols.

**Gene expression studies**

For studying the expression of various mycobacterial genes, primers for dormancy genes tgs1 (triacylglycerol synthase1), icl (isocitrate lyase), hspX (heat shock protein X) and resuscitation gene rpfB (resuscitation promoting factor B) were designed using “Primer3web”.

RNA was isolated from the trizol stored samples using the bead beating method (Abhishek et al. 2018). Followed by DNase I (Thermo scientific) treatment. The DNase treated RNA was then reverse transcribed to cDNA using a cDNA synthesis kit as per the manufacturer’s instruction (iScript Biorad cDNA synthesis kit). A cDNA control without reverse transcriptase referred to as “no enzyme control (NEC)” was also prepared for each cDNA sample. The qRT-PCR was performed using SYBR green chemistry (Biorad, USA) on a Biorad machine (Maestro CFX 96) with primers mentioned in Online Resource 2 and relative quantification was done (Abhishek et al. 2018).

**Statistical analysis**

All the graphical representations and statistical analysis was done in SPSS software (version 22). The data was represented either as mean ±SD or mean ±SE and One-way ANOVA was performed to analyse the data obtained from CFU enumeration, gene expression studies and cytokine assay. p-value ≤0.05 was considered to be significant.

**Results**

In the present study, the effect of PM$_{2.5}$ on the formation of granuloma and the integrity of already established granuloma was studied in the in vitro model of human granuloma using PBMCs and *Mycobacterium bovis* BCG. An attempt was also made to examine the change in expression of the genes
responsible for maintaining dormancy and reactivation and to correlate these findings with CFU, histopathology, and finally with Th1, Th2 and Th17 cytokine levels.

**Extraction of PM2.5**

PM$_{2.5}$ was extracted from both teflon (PTFE) and quartz filters and suspended at a 1mg/ml concentration. This suspension was visualized under a light microscope to examine the contamination with filter material. The light microscope image of the lyophilized extracts of PM$_{2.5}$ extracted from quartz filter showed the presence of filter fiber materials; however, it was not observed in the case of teflon filter. The PM$_{2.5}$ extract from both quartz and teflon filters was then passed through a 0.45μm nylon filter, which led to the removal of fibre material. No loss of PM$_{2.5}$ mass was observed during filtering process based on the weight determination before and after filtration (data not shown). Based on the various parameters like the volume of the solvent required for sonication, duration of centrifugation and yield of the pollutant, the PTFE filter was found to be advantageous over quartz filter (Online Resource 1) and was selected for further studies.

**Effect of PM$_{2.5}$ on PBMCs viability**

To determine the maximum concentration of PM$_{2.5}$ that does not affect the in vitro PBMCs viability, MTT assay was performed. In this, PBMCs in RPMI media were treated with different concentrations of PM$_{2.5}$, followed by MTT assay at 24hr and 48 hr of treatment. Amongst three different concentrations used, the % viability of PBMCs was affected at the highest concentration of PM$_{2.5}$, i.e., 50μg/ml after both 24hr and 48h (Fig. 1). At 24 hr, the percent viability was observed to be 31% at 50 μg/ml of PM$_{2.5}$, 95% at 0.1μg/ml, 96% at 1 μg/ml and 94% at 10μg/ml of PM$_{2.5}$. A similar trend was also seen at 48 hr of PM$_{2.5}$ treatment. Therefore, the concentrations 0.1μg/ml (lowest) and 10μg/ml (highest) of PM$_{2.5}$ were used for further experiments.

**Development of granuloma**

To obtain a well-defined granuloma, optimal MOI of *M. bovis* BCG was required and was determined based on histopathological examination (H&E and ZN staining). Two MOIs (0.1 and 0.2 comprising of 1 bacillus per 10 PBMCs and 2 bacilli per 10 PBMCs, respectively) were used. At MOI 0.1, a well-defined granuloma was formed, however at MOI 0.2, the lymphoid cells were scattered and scanty clusters resembling granuloma were observed as compared to MOI 0.1. Further, ZN staining for AFB showed that at MOI 0.2, the bacilli were observed in large clumps indicating an excess of bacilli (data not shown). Thus, well-defined granuloma formation at MOI 0.1, it was used for further experiments (Online Resource 3).

**Effect of PM$_{2.5}$ on the formation of PBMC granuloma using Mycobacterium bovis BCG**

i) Effect on granuloma structure
After day 10 of granuloma induction in the presence and absence of two different concentrations of PM$_{2.5}$ (0.1 and 10μg/ml), the ECM containing PBMCs and bacilli were subjected to H&E and ZN staining. The histopathology showed images of granuloma induction in the presence and absence of PM$_{2.5}$ (Fig. 2). In the presence of 0.1μg/ml PM$_{2.5}$ concentration, granuloma's appearance was similar to that of the control group with a cluster of lymphoid cells. However, in the presence of 10μg/ml PM$_{2.5}$ concentration, histopathology revealed loosely clustered macrophages and lymphocytes, suggestive of ill-defined granuloma (Fig. 2). Thus, it was observed that as the concentration of PM$_{2.5}$ increases, the granuloma formation by \textit{M. bovis} BCG gets disrupted, forming a loose ill-defined granuloma.

Further, the treatment of already established granuloma with PM$_{2.5}$ demonstrated that at 0.1μg/ml PM$_{2.5}$ concentration, granuloma's appearance was similar to that of the control group with a cluster of cells comprising lymphocytes and macrophages (Fig. 2). However, treatment with 10μg/ml PM$_{2.5}$ concentration resulted in the disruption of granuloma as indicated by loosely packed macrophages and lymphocytes (Fig. 2). Therefore, it was observed that in in-vitro experiments, as the concentration of PM$_{2.5}$ increases, the granuloma formed by \textit{M. bovis} BCG gets disrupted. Further, acid-fast staining of control and PM$_{2.5}$ treated granulomas, although showed the presence of acid-fast bacilli, however, no evident changes were observed in the bacterial load in the stained granulomas (Fig. 3).

\textbf{ii) Effect of pollutant on bacterial load in the granuloma}

To see the effect of the pollutant on the bacterial load in the granuloma formed in PM$_{2.5}$, pellets obtained from granuloma were plated on 7H11 agar and observed for the appearance of colonies. There was no significant difference in the CFUs in control granulomas and those formed in the presence of 0.1μg/ml and 10 μg/ml of PM$_{2.5}$, respectively (Fig. 4a). Further the treatment of already formed granuloma with PM$_{2.5}$ also did not show any significant difference in the CFUs in control and pollutant treated granulomas (Fig. 4b)

\textbf{iii) Effect of PM$_{2.5}$ on the expression of dormancy and reactivation associated mycobacterial genes}

The PBMCs granuloma formed with \textit{M.bovis} BCG in the presence and absence of PM$_{2.5}$ were harvested and processed for qRT-PCR to study the gene expression changes. Relative expression of four mycobacterial genes related to dormancy (tgs1, icl, hspX) and reactivation (rpfB) was studied. As it is evident from Fig5a, there was a significant upregulation in the expression of icl gene (p<0.001) and rpfB gene(p<0.05) in granuloma formed in presence of PM$_{2.5}$ as compared to control granuloma. On the other hand, tgs1 along with hspX genes were significantly downregulated (p<0.001) in the mycobacterial cells recovered from granulomas formed in the presence of pollutant as compared to control granulomas. Overall, these results indicate downregulation of dormancy associated genes and upregulation of reactivation associated rpfB gene in the granuloma formed in the presence of PM$_{2.5}$. Alternatively, experiments were carried out to study the mycobacterial gene expression in the already established PBMCs granuloma using \textit{M. bovis} BCG following treatment with PM$_{2.5}$ for 72hr. As shown in Fig. 5b,
there was a significant upregulation in the expression of the icl gene (p<0.05) in PM$_{2.5}$ treated granuloma compared to untreated control granuloma. On the other hand, considerable downregulation was observed in tgs1 and hspx genes (p<0.01) in pollutant treated granulomas with respect to the control group. However, the rpfB gene though was significantly downregulated at 0.1µg/ml of PM$_{2.5}$ (p<0.001) but was non-significantly upregulated (1.7-fold) at 10 µg/ml of PM$_{2.5}$. Overall, these results indicate the downregulation of dormancy associated genes along with reactivation associated rpfB gene in the granuloma treated with 0.1 µg/ml of PM$_{2.5}$ and a non significant upregulation of rpfB gene at 10 µg/ml of PM$_{2.5}$ as compared to control untreated granuloma (Fig. 5b).

iv) Effect on cytokines level

The levels of different cytokines were measured in the culture supernatants that were preserved during the time of granuloma harvesting. The cytokines of Th1 cells (TNF$_\alpha$, IFN$_\gamma$ and IL-2), Th2 cells (IL-10, IL-6 and IL-4) and Th17 cells (IL-17A) were measured in the supernatants from PBMCs granuloma formed with M. bovis BCG in the presence and absence of PM$_{2.5}$ using flow cytometry as well as in the already established granuloma treated with PM$_{2.5}$. Fig6 shows the levels above-mentioned cytokine in all the above mentioned three groups (control, 0.1µg/ml PM$_{2.5}$, 10µg/ml PM$_{2.5}$, respectively). Among all the cytokines tested, a significant increase in TNF-α level (p < 0.01) was obtained in the granuloma formed in the presence of pollutant only at a high concentration of 10 µg/ml compared to control granuloma, whereas, there was no significant difference in all other cytokines tested. On the other hand, in the case of treatment of established granuloma with PM$_{2.5}$, although there was an increase in IFN$_\gamma$, TNF$_\alpha$, IL-17 and IL-10, these changes were statistically non-significant compared to control untreated granuloma (data not shown).

Discussion

Tuberculosis is a multistage disease and following infection with M.tb, not every person develops the disease. Most of the individuals can control and limit the infection due to localization and containment of bacilli within an organized structure of an aggregation of immune cells known as a granuloma that is formed due to host immune response. This stage of TB is known as latent TB and the latently infected individuals are at risk of future reactivation following any condition leading to an immunocompromised state.

A number of studies have shown the effect of various pollutants on the antimycobacterial immunity (Abhishek et al. 2018; Ding et al. 2017; Lin et al. 2014; Rivas-Santiago et al. 2015), thus suggesting the role of pollution in determining the disease outcome following exposure to tubercle bacilli. With increased air pollution due to increased urbanization and industrialization, it becomes crucial to investigate that how exposure to pollutants impacts the containment of M.tb in the granuloma as latent TB and reactivation of latent bacilli contained in the granuloma. Thus, in the present study, PM$_{2.5}$ was investigated for its effect on granuloma formation. In the previous reports, PM$_{2.5}$ has been reported
to be most harmful to the respiratory system due to its small size and ability to reach the terminal airways (Yang, Li, and Tang 2020). Since there are many particulate filters available, so initially the study was carried out for the selection of appropriate filter for the extraction of PM$_{2.5}$. Two different types of filters, i.e., quartz and teflon were used for the extraction of PM$_{2.5}$ as these are the most commonly used filters in the literature (Perrino, Canepari, and Catrambone 2013). During the standardization of extraction of PM$_{2.5}$, it was observed that there was a lot of contamination with filter fibers in the extract of quartz filter. Hence, to avoid the interference of these fibres in the cell culture assay, PM$_{2.5}$ extract was filtered through a 0.45μm membrane filter. Although no loss of PM$_{2.5}$ was observed based on the weight determination of the extract before and after filtration (data not shown), earlier it has been reported that it can also lead to the removal of some insoluble PM species (Yang et al. 2014). Further, based on a better yield of pollutant (Online Resource 1), PM$_{2.5}$ was extracted only from the teflon filter for the present study.

As our study involved the treatment of PBMCs granuloma with PM$_{2.5}$, for selection of the optimal dose of pollutant, MTT assay was carried out to study the effect of PM$_{2.5}$ on PBMCs viability. Based on the earlier studies, four different concentrations of PM$_{2.5}$ were used for MTT assay (Rivas-Santiago et al. 2015). The viability of PBMCs was not affected up to a concentration of 10μg/ml of PM$_{2.5}$. Based on these results, we used a lower concentration of 0.1μg/ml and a higher tolerable dose of 10μg/ml in the present study. Another study also used the same concentration of PM$_{2.5}$ for studying its effect on the antimycobacterial immunity induced by respiratory epithelial cells (Torres et al. 2019).

Further, for PBMCs granuloma, considering the limitations of using M.tb in BSL3 labs, we used _M. bovis_ BCG. A recent study has reported the successful formation of PBMCs granuloma with _M. bovis_ BCG (Islamoglu et al. 2018). Experiments in granuloma formation in the presence of PM$_{2.5}$ indicated loosely packed granuloma only at 10μg/ml concentration, which suggests that granuloma formation is disrupted in the presence of PM$_{2.5}$. To further confirm the effect of PM$_{2.5}$ on the growth of bacilli inside the granuloma, CFU enumeration was carried out. Surprisingly, there was no change in the CFUs recovered from the granuloma formed in the presence and in the absence of PM$_{2.5}$. To further see if PM$_{2.5}$ has any direct effect on the actively growing _M. bovis_ BCG, invitro grown culture was treated with different concentrations of PM$_{2.5}$ that were used for the granuloma experiment. In the case of actively growing BCG, even the higher concentration of PM$_{2.5}$ (10μg/ml) didn't affect the growth of bacilli as measured in terms of CFU (data not shown). This finding was not supported by earlier reports where PM$_{2.5}$ has been shown to affect the viability of active _M. tb_ grown inside the airway epithelial cells (Rivas-Santiago et al. 2015).

Although no change in CFU was observed by PM$_{2.5}$, we were further interested in studying the effect of PM$_{2.5}$ on the BCG inside the granuloma at the molecular level by investigating the expression of genes involved in dormancy and reactivation. Several mycobacterial genes implicated in the dormancy include gene encoding 16-kDa alpha crystalline homolog protein, also known as hspX gene (Sherman et al. 2001), devR/devS genes from Dos regulon (Kinger and Tyagi 1993), a family of novel TG synthase genes
(tgs) expressed under stress conditions (Sirakova et al. 2006). In addition to genes involved in mycobacterial dormancy, studies have identified a group of proteins called resuscitation promoting factors (RPF) involved in the reactivation of dormant mycobacteria under experimental conditions (Kana et al. 2008; Tufariello et al. 2006). In the present study, we investigated expression levels of four genes comprising both dormancy (tgs1, icl and hspX) and reactivation (rpfB) in the *M. bovis* BCG recovered from PBMC granuloma formed in the presence of PM$_{2.5}$. Results indicated downregulation of tgs1, hspX and upregulation of rpfB, suggesting that the BCG bacilli inside the granuloma mimic those of active bacilli under the effect of PM$_{2.5}$. Earlier studies demonstrated the decreased expression of hspX as the bacilli transformed from a dormant to exponential phase (Hu et al. 2006) and upregulation of rpfB gene upon reactivation of bacilli (Kapoor et al. 2013). Though the molecular changes seen in current study pointed towards the activation of bacilli inside granuloma in the presence of PM$_{2.5}$, but as stated earlier these changes were not replicated with CFU changes. This may be due to a lag phase whereby the bacteria are preparing itself for active replication (Du, Sohaskey, and Shi 2016). According to this study, on reaeration of a hypoxic culture, there is a reaeration lag phase of two days associated with molecular changes like downregulation of DosRST regulon and upregulation of rpfA gene without any change in CFU (Du, Sohaskey, and Shi 2016).

Further, the role of host immune response was also investigated in containing the bacilli inside the granuloma in the presence of PM$_{2.5}$. The level of TNF$\alpha$ was highly upregulated in the granuloma formed in the presence of PM$_{2.5}$. TNF$\alpha$ is considered an important regulatory cytokine produced by a variety of immune cells. It plays an essential role in the pathogenesis of tuberculosis. Both TNF$\alpha$ and IFN-\(\gamma\) are considered crucial cytokines required for granuloma formation for the containment of TB. In contrast, the increased concentration of TNF$\alpha$ has been reported to result in tissue destruction (Moodley 2008). Increased TNF$\alpha$, as observed in the present study in the presence of PM$_{2.5}$ indicate an active inflammatory response in the microenvironment of granuloma, thus suggesting poor granuloma formation, which was also evident based on the histopathological observations (Fig. 2).

In addition to the effect of PM$_{2.5}$ on the formation of granuloma, the consequences of treatment of already established invitro developed granuloma using human PBMCs with PM$_{2.5}$ were also studied. Although, these experiments showed disruption of granuloma by PM$_{2.5}$, there was no change in the CFU of *M. bovis* BCG and in any of Th1/Th2/Th17 cytokines. Gene expression analysis of dormancy and reactivation associated genes although indicated downregulation of dormancy genes tgs1 and hspx, however significant downregulation of reactivation associated rpfB at 0.1$\mu$g/ml but a non-significant upregulation at 10$\mu$g/ml concentration was observed which indicates the trend towards reactivation of bacilli at higher PM$_{2.5}$ concentration. As the upregulation of rpf B gene was not significant, it is further required to see the expression of these genes by exposing the granuloma for a longer period of time in further studies.

PM$_{2.5}$ is a complex mixture of different components, including acids, organics, metals, soil or dust particles, and microorganisms. Moreover, the composition of PM$_{2.5}$ may alter according to sources, the
geographic area and also by the seasons. The present study’s limitations are the use of PM$_{2.5}$ from a single source and lack of information on the composition of PM$_{2.5}$. Although the results of the present study demonstrate that PM$_{2.5}$ interferes with the granuloma formation, however further studies are required with a greater number of samples using PM$_{2.5}$ from different sources to validate these findings and a better understanding of the molecular mechanism involved in pollutant mediated effects on tuberculosis granuloma and reactivation of latent TB.

Overall, the present study demonstrates the effect of ambient PM$_{2.5}$ on the mycobacterial granuloma formation and the reactivation of mycobacteria in the granuloma. Based on the results obtained, it can be inferred that in the presence of PM$_{2.5}$, there is poor granuloma formation with a decrease in the dormancy associated mycobacterial genes with upregulation of reactivation gene rpfB along with increased TNF-α production. However, treatment of already formed granuloma with PM$_{2.5}$ although lead to disruption of granuloma but had no effect on the replication or reactivation of bacilli in the granuloma.

**Declarations**

i) Ethics approval and consent to participate: The study was approved by Institute Ethics Committee, PGIMER, Chandigarh with reference no: NK/4978/MD/442 dated 11.02.2019 and subjects were recruited after taking written informed consent.

ii) Consent for publication: Not applicable

iii) Availability of data and materials: All data generated or analysed during this study are included in this published article [and its supplementary information files].

iv) Competing interest: The authors declare that they have no competing interests.

v) Funding: Partial funding from Special Research Grant, PGIMER, Chandigarh.

vi) Authors’ contribution: IV formulated the hypothesis and work plan, data analysis and manuscript writing, AP did the experimental work, analysis of the data and manuscript writing, SS1 helped with the molecular work and manuscript writing, KK contributed to the experimental work, SS2 helped in providing departmental facilities and formulating the study, UNS helped with the histological examination, RK supported for PM$_{2.5}$ collection. All authors read and approved the final version of the manuscript.

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Figure 1

Percent viability of PBMCs treated with different concentrations of PM2.5 for different periods (24hr/48hr). The % viability is the mean of 7 values from 2 independent experiments. The percent viability has been calculated as mean OD570 of test / mean OD570 of control X 100

Figure 2

Effect of PM2.5 on the integrity of PBMCs granuloma as demonstrated by H&E staining. a) Control granuloma b) Granuloma formed in the presence of 0.1µg/ml of PM2.5. c) Granuloma formed in the presence of 10 µg/ml of PM2.5 d) Already established granuloma treated with 0.1µg/ml of PM2.5. e) Already established granuloma treated with 10µg/ml of PM2.5
Figure 3

Images showing AFB staining of PBMCs granuloma. a) Control granuloma b) & c) Granuloma formed in the presence of 0.1µg/ml and in the presence of 10 µg/ml of PM2.5 respectively d) & e) Already established granuloma treated with 0.1µg/ml and 10ug/ml of PM2.5 respectively

Figure 4

Effect of PM2.5 on the bacterial load in the granuloma. a) CFUs obtained from PBMCs granuloma formed with M. bovis BCG in the presence and absence of PM2.5. b) CFUs obtained from preformed PBMCs granuloma treated with different concentrations of PM2.5 for 72hrs. Values are presented as mean + SD of Log10 CFUs/ml obtained from granulomas of PBMCs from 4 subjects put in duplicates at two different dilutions.
Figure 5

Effect of PM2.5 on the expression of dormancy and reactivation associated mycobacterial genes in PBMCs granuloma formed using M. bovis BCG. a) Log2 fold change in the expression of mycobacterial genes in the granuloma formed in the presence of PM2.5 as compared to control granuloma b) Log2 fold change in the expression of mycobacterial genes in the preformed granuloma treated with different concentrations of PM2.5 for 72hrs. Log2 fold change less than zero depicts the downregulation and more than zero depicts upregulation with respect to the control group. Values are mean + SE of values from granulomas of 4 samples run in duplicates. *p < 0.05, **p < 0.01 and ***p < 0.001
Figure 6

Concentration of various Th1, Th2 and Th17 cytokines in the supernatants from PBMCs granuloma formed with M. bovis BCG in the presence and absence of PM2.5. a) IFNγ b) IL-6 c) IL-17A and IL-10 and d) TNFα. Values presented as pg/ml are mean + SE of the concentrations of cytokines obtained from supernatant of granuloma from blood of 4 subjects put in duplicates. p** < 0.01