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Particulate matter (PM_{10}) enhances RNA virus infection through modulation of innate immune responses☆

Richa Mishra [a], Pandikannan Krishnamoorthy [a], S. Gangamma [b, c], Ashwin Ashok Raut [d], Himanshu Kumar [a, e, *]

[a] Laboratory of Immunology and Infectious Disease Biology, Department of Biological Sciences, Indian Institute of Science Education and Research (IISER) Bhopal, Bhopal, 462066, MP, India
[b] National Institute of Technology Karnataka (NITK), Surathkal, Mangaluru, 575025, Karnataka, India
[c] Centre for Water Food and Environment, IIT Ropar, Rupnagar, 140001, Punjab, India
[d] Pathogenomics Laboratory, ICAR – National Institute of High Security Animal Diseases (NIHSAD), OIE Reference Laboratory for Avian Influenza, Bhopal, 462021, MP, India
[e] WPI Immunology, Frontier Research Centre, Osaka University, Osaka, 5650871, Japan

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ABSTRACT

Sensing of pathogens by specialized receptors is the hallmark of the innate immunity. Innate immune response also mounts a defense response against various allergens and pollutants including particulate matter present in the atmosphere. Air pollution has been included as the top threat to global health declared by WHO which aims to cover more than three billion people against health emergencies from 2019 to 2023. Particulate matter (PM), one of the major components of air pollution, is a significant risk factor for many human diseases and its adverse effects include morbidity and premature deaths throughout the world. Several clinical and epidemiological studies have identified a key link between the PM existence and the prevalence of respiratory and inflammatory disorders. However, the underlying molecular mechanism is not well understood. Here, we investigated the influence of air pollutant, PM_{10} (particles with aerodynamic diameter less than 10 μm) during RNA virus infections using Highly Pathogenic Avian Influenza (HPAI) – H5N1 virus. We thus characterized the transcriptomic profile of lung epithelial cell line, A549 treated with PM_{10} prior to H5N1infection, which is known to cause severe lung damage and respiratory disease. We found that PM_{10} enhances vulnerability (by cellular damage) and regulates virus infectivity to enhance overall pathogenic burden in the lung cells. Additionally, the transcriptomic profile highlights the connection of host factors related to various metabolic pathways and immune responses which were dysregulated during virus infection. Collectively, our findings suggest a strong link between the prevalence of respiratory illness and its association with the air quality.

1. Introduction

Alarming rate of air pollution has been shown to be linked with enhanced morbidity and subsequent mortality rate which affected the global health and economy (Anderson et al., 2012; Cohen et al., 2017; Katsouyanni et al., 2009; Krewski et al., 2009; Landrigan et al., 2018; Lelieveld et al., 2015; Liu et al., 2019a; Romieu et al., 2012; Wong et al., 2008). One of the major components of air pollution is the particulate matter (PM) which are a complex mixture of particles of varied sizes and physiochemical properties, derived from both the natural and anthropogenic sources. Pollution by ambient PM has been reported to cause 2.9 million deaths and 69.7 million disability-adjusted life-years in 2013, according to a systematic analysis for the Global Burden of Disease Study (Collaborators et al., 2015). These particles are divided into three main categories on the basis of their diameter: coarse particles, or PM_{10}, (with an aerodynamic diameter between 10 and 2.5 μm); fine particles, or $PM_{2.5}$, (with diameters <2.5 μm); and ultrafine particles, or $PM_{0.1}$, (with diameters <0.1 μm) (Deng et al., 2019; Idani

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* Corresponding author. Laboratory of Immunology and Infectious Disease Biology, Department of Biological Sciences, Indian Institute of Science Education and Research (IISER) Bhopal, AB-3, Room No. 220, Bhopal By-pass Road, Bhauri, Bhopal, 462066, MP, India.
E-mail address: hkumar@iiserb.ac.in (H. Kumar).

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et al., 2018).

PM_{10} is generally deposited in the upper respiratory tract and found to be linked with many diseases other than respiratory damage like cardiovascular diseases (Dastoorpoor et al., 2020; Dastoorpoor et al., 2019; Momtazan et al., 2019), various types of cancers (Chu et al., 2019; Consonni et al., 2018; Hamra et al., 2014; Li and Gao, 2014; Reyes-Zarate et al., 2016), tuberculosis (Rivas-Santiago et al., 2015; Sarkar et al., 2019), Asthma (Donaldson et al., 2000; Saygin et al., 2017; Tecer et al., 2008). A large cohort epidemiology study of 39,054 participants showed that an increase in 10 $\mu g/m^3$ PM_{10} concentration is associated with the increase in 3.4%–6% of lung cancer mortality (Chen et al., 2016).

Numerous respiratory risk studies have shown the strong association of the PM with increased morbidity and mortality (Chen et al., 2017b; Dai et al., 2014; Daryanoosh et al., 2018; Goudarzi et al., 2018; Harrison and Yin, 2000; Idani et al., 2018; India State-Level Disease Burden Initiative Air Pollution, 2019; Landrigan et al., 2018; Lu et al., 2015; Samet et al., 2000; Valavanidis et al., 2008; Carugno et al., 2016; Lin et al., 2005). PM is readily associated with respiratory infections such as chronic obstructive pulmonary disease (COPD) (Khaefi et al., 2017; Ling and van Eeden, 2009; MacNee and Donaldson, 2003; Ni et al., 2015; Wen and Gao, 2018). The short-term exposure to PM including PM_{10} and $PM_{2.5}$ was shown to be linked with disease which was demonstrated from a large-scale modelling study comprising the data from 652 cities across the globe (Liu et al., 2019a). Meanwhile, reduction in the PM_{10} levels as recommended by World Health Organisation (WHO) can increase the health benefits (Marzouni et al., 2017) and may decrease the mortality by 8%–9% in highly polluted cities (Yorifuji et al., 2015). WHO has recommended the standard permissible level of air contaminants but nearly 80% of the urban cities are well above the standard permissible level to cause severe health problems.

India recorded the highest increase in the air pollution related death among the most populated countries in 2015 compared to 1990, according to Lancet commission report (Landrigan et al., 2018). Annual PM concentration in most of the Indian cities were higher than the National Ambient Air Quality Standards of India. Several studies have attempted to understand the link between isolated PM from the heavily populated regions of India and associated health concerns in term of occurrence of disease (Jain et al., 2017; Khafaie et al., 2017; Manojkumar and Srimuruganandam, 2019; Sharma et al., 2018; Sharma et al., 2004; Sharma et al., 1998). However, there were very few studies which aim to explore the mechanistic action of PM, collected from Indian subcontinent.

PM has been shown to induce innate immune responses by the production of pro-inflammatory cytokines through activation of toll-like receptor signalling pathway (Bauer et al., 2012; Becker et al., 2002). It has been known that PM can modulate the level of cytokines, upon its exposure to the humans airways (Bengalli et al., 2013; Hirota et al., 2015; Tang et al., 2019). PM were also reported to be associated with the virus infections such as Respiratory Syncytial Virus (RSV) (Karr et al., 2009; Vandini et al., 2013), Influenza virus, Dengue virus (Carneiro et al., 2017), Measles virus (Chen et al., 2017a; Peng et al., 2020) and Rhinovirus (Proud et al., 2012). PM concentration has been shown to be linked with the increased number of infection rates in most of the virus-related outbreaks in the past two decades caused by RNA viruses, such as Severe Acute Respiratory Syndrome (SARS) – CoV in 2003 (Cui et al., 2003), Dengue outbreak (Massad et al., 2010), Swine flu H1N1 (Influenza) pandemic in 2009 (Morales et al., 2017), H5N2 Influenza outbreak in United States (US), (Zhao et al., 2019), Measles outbreak in 2019 (Peng et al., 2020) and the recent ongoing COVID-19 outbreak (Conticini et al., 2020; Dutheil et al., 2020b; Fattorini and Regoli, 2020; Sciomer et al., 2020; Setti et al., 2020a; Zhu et al., 2020). Many studies, based on the epidemiological and modelling suggest that PM caused health severity both in the urban and remote locations (Cohen et al., 2017; Idani et al., 2018; Karambelas et al., 2018; Landrigan et al., 2018). However, air pollutant-mediated lung infections and respiratory damage are poorly understood. Therefore, our study aims to investigate the PM-based modulation of innate immune responses which may shed light on the cause of severity and mortality during respiratory infections in virus outbreaks.

In this study, we hypothesize that PM_{10} modulates the innate immune system upon virus infection and promotes viral replication. To support this hypothesis, we aimed to observe the effect of PM_{10} during different RNA virus infections, including H5N1 flu virus, in the human lung epithelial cell line, A549 and performed the RNA-Sequencing to determine the pathways which were dysregulated by PM_{10} upon virus infection. Our results demonstrated that PM_{10} modulates the innate immune system associated pathway upon viral infection and significantly enhanced the viral replication of the RNA viruses like New-Castle Disease Virus (NDV), Influenza virus – H1N1 (PR8) and H5N1, via downregulation of innate immune responses and upregulation of several metabolic pathways-related genes, which collectively facilitates the infectivity of virus to cause enhanced respiratory illness.

2. Methods

2.1. Cell culture experiments

A549 human alveolar basal epithelial cells (Cell Repository, NCCS, India) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% Antibiotic-Antimycotic solution. DMEM, FBS and Antibiotic-Antimycotic solution were purchased from Invitrogen. Ambient particulate matter (PM) of coarse particle size PM_{10} was obtained from Dr. Gangamma S. laboratory which was collected and isolated from the selected monitoring stations geographically distributed across Bengaluru city, at NITK, Surathkal, Mangaluru, Karnataka. The A549 cells were seeded in 12 well culture plate at a concentration of 3×10^5/well overnight (37 °C, 5% CO_2). Cells were treated with PM_{10} along with controls namely blank and/or LPS (100 ng) for 24 h prior to infection. Plasmids containing Firefly Luciferase gene under *IFNβ* (type- I interferon beta) and *ISRE* (interferon-sensitive response element) promoters, were obtained from Professor Shizuo Akira's (Osaka University, Japan). All short hairpin (*sh*)- clones, were obtained from the whole RNAi human library for shRNA mediating silencing (Sigma, Aldrich) maintained at IISER, Bhopal, India.

2.2. Virus infection

Airborne PM_{10} treated A549 cells were infected with New-castle Disease Virus (NDV) – LaSota strain, Highly Pathogenic Avian Influenza virus (H5N1) - A/duck/India/02CA10/2011 strain and vaccine strain PR8 virus (H1N1) – A/PR8/H1N1 virus was generated using the eight plasmid reverse genetics system (Kumar et al., 2018), at respective multiplicity of infection as mentioned in the figures and/or figure legends. PM_{10} treated A549 cells were washed by 1X PBS (phosphate-buffered saline) solution and infected with appropriate RNA viruses in serum-free media as per the subsequent experiment then after 60 min, virus containing media was removed from the cells and cells were washed once with 1X PBS solution. Then cells were again supplemented with new PM_{10} containing DMEM media for 24 h. Samples were then harvested and forwarded for respective quantitative analysis.

2.3. Airborne particulate matter sampling method

Bengaluru is an inland city (12°58′ N, 77°34′) situated on the south-central part of India at a height over 900m above sea level. General sources of airborne PM in the city include vehicular emissions, industrial emissions and re-suspended road dust (ARAI, 2018; CPCB, 2020). Air samples were collected from six ambient air quality monitoring sites of Karnataka State Pollution Control Board (KSPCB). PM with aerodynamic diameter less than 10 μm was collected using high volume samplers (Poll tech, India). The time duration of sampling corresponds to 8 h. The samples were collected on quartz fibre filter paper (GE healthcare, India). The filter papers were de-pyrogenated and conditioned prior to sampling (Gangamma et al., 2011). To ensure contamination free sampling, field blanks were included in the samples. After sampling, filter papers were sealed in de-pyrogenated aluminium foil and transported to the laboratory. The samples were stored at −20 °C until further processing. PM on the filter was extracted into methanol. Further, methanol was purged and samples were reconstituted with dimethyl sulfoxide (DMSO) (Bach et al., 2015; Totlandsdal et al., 2014). Samples were pooled and used for further experiments.

2.4. Particulate matter (PM_{10}) dose standardization

For all the preliminary experiments three different dosage form of PM_{10} was used in the ratios 1:1 (PM_{10}: DMEM) − (50% of PM_{10} in DMEM), 0.25:1 (PM_{10}: DMEM) - (25% of PM_{10} in DMEM) and 0.10:1 (PM_{10}: DMEM) - (10% of PM_{10} in DMEM) named as PM(I), PM(II) and PM(III) respectively. And after the standardization through different experiments PM(I), that is 1:1 (PM_{10}: DMEM) dosage of PM_{10} was used for subsequent experiments, labelled and/or described as PM.

2.5. Characterization of particulate matter (***PM_{10}***) by scanning electron microscopy − Energy dispersive X-ray spectrometer (SEM-EDS) analysis

PM_{10} dissolved in appropriate solvents was installed on the metallic stabs in the form of droplets and dried overnight in the desiccator for complete solvent dry process. Samples were then loaded on the high-resolution field emission scanning electron microscope (SEM) (HR FESEM) from Zeiss, model name ULTRA Plus at IISER Bhopal for PM_{10} morphological analysis. Then chemical composition of the PM_{10} was elucidated by the Energy Dispersive X-ray Spectrometer (EDS) component of the scanning electron microscope.

2.6. Quantitative real-time reverse transcription PCR − qRT-PCR analysis

Total RNA was extracted from the three and/or two independent experiments as mentioned in the figure legends with the Trizol reagent (Ambion/Invitrogen) and used to synthesize cDNA with the iScript cDNA Synthesis Kit (BioRad, Hercules, CA, USA) according to the manufacturer's protocol. Gene expression was measured by quantitative real-time polymerase chain reaction (PCR) using gene-specific primers and SYBR Green (Biorad, Hercules, CA, USA). cDNA obtained from each experiment was analysed in triplicate. Ct (cycle threshold) values − that determines the abundance and expression of the transcript within the cells was obtained for calculation, to determine the fold change or relative expression of a particular transcript. Ct levels are inversely proportional to the amount of target transcript in the sample (i.e. the lower the Ct level the greater is the amount of target transcript in the sample. The formula and concept on which complete calculation of obtaining fold change/relative expression is based, defines as the difference in the Ct values (ΔCt) for selected genes/transcripts, among the treated (PM_{10} and virus infected) and non-treated (control/mock) group, calculated by: 2^-ΔΔCt as the final fold change. The 18S gene was used as a reference control for normalization of Ct (critical threshold) values, obtained after qRT-pCR (of 40 cycles each), of the genes to determine the fold change of the selected gene/mRNA transcript in the treated group with respect to the control/mock group of the experiment. Real time quantification was done using StepOne Plus Real time PCR Systems by Applied BioSystems (Foster City, CA, USA).

2.7. Promoter activity estimation by luciferase reporter assay

A549 cells (5×10^4) were seeded into a 12-well plate and transiently transfected with 50 ng of the transfection control pRL-TK plasmid (*Renilla* luciferase containing thymidine kinase promoter plasmid) and 200 ng of the luciferase reporter plasmid (*Firefly* luciferase containing plasmid) of *IFNβ* and *ISRE* promoters. After 18 h cells were treated with PM_{10} at the different dosage as explained in the PM_{10} dose standardization method section (PM(I), PM(II) and PM(III)) and blank as a control for 24 h. Then after cells were infected with NDV (MOI 2) for 24 h. The cells were lysed at 24 h after final infection, and finally the luciferase activity in total cell lysates was measured with Glomax (Promega, Madison, WI, USA).

2.8. Protein estimation of cytokines by enzyme-linked immunosorbent assay (ELISA)

A549 cells were treated with PM_{10} at the different dosage as explained in the PM_{10} dose standardization method section (PM(I), PM(II) and PM(III) and blank as a control after 24 h of seeding. The culture media were harvested at 36 h after PM_{10} treatment and were analysed by specific ELISA kits (Becton Dickinson) according to the manufacturer's instructions to determine the amounts of *IL6* that were secreted by the cells.

2.9. Cell counting by trypan blue assay to estimate dead cells

A549 cells were seeded and after 24 h treated with PM_{10} and blank for 24 h before NDV infection. Cell supernatant were collected after 36 h of infection, mixed with trypan blue dye (Sigma) in the ratio 1:1. The mixture then used for counting the dead cells under the microscope.

2.10. Viral quantification of GFP signals by microscopy

A549 cells were seeded along with cover slips in low confluency and next day treated with PM_{10} at a dosage of 1:1 [PM: DMEM] for 24 h prior to virus infection. Cells were then infected with NDV-GFP (3 MOI − multiplicity of infection 3) in serum free media for 1 h. After infection cells were again supplemented with complete media and treated with PM_{10} at a dosage of 1:1(PM_{10}: DMEM) for 24 h at 37 °C, 5% CO_2. Cells were then washed twice with PBS for 5 min and fixed in 4% PFA (paraformaldehyde) for 20 min again washed in PBS and incubated with DAPI (20 mg/ml) for 30 min at room temperature and finally washed thrice with PBS. Cover slips then containing cells were carefully mounted on to the glass slides using Fluoroshield (Sigma) as mounting media. Slide was then kept for few hours for drying before imaging. Images were visualised at 40X with Apotome − AXIO fluorescence microscope by Zeiss. Image analysis (intensity of GFP and GPF positive cells) was done by using Image J software (Fiji).

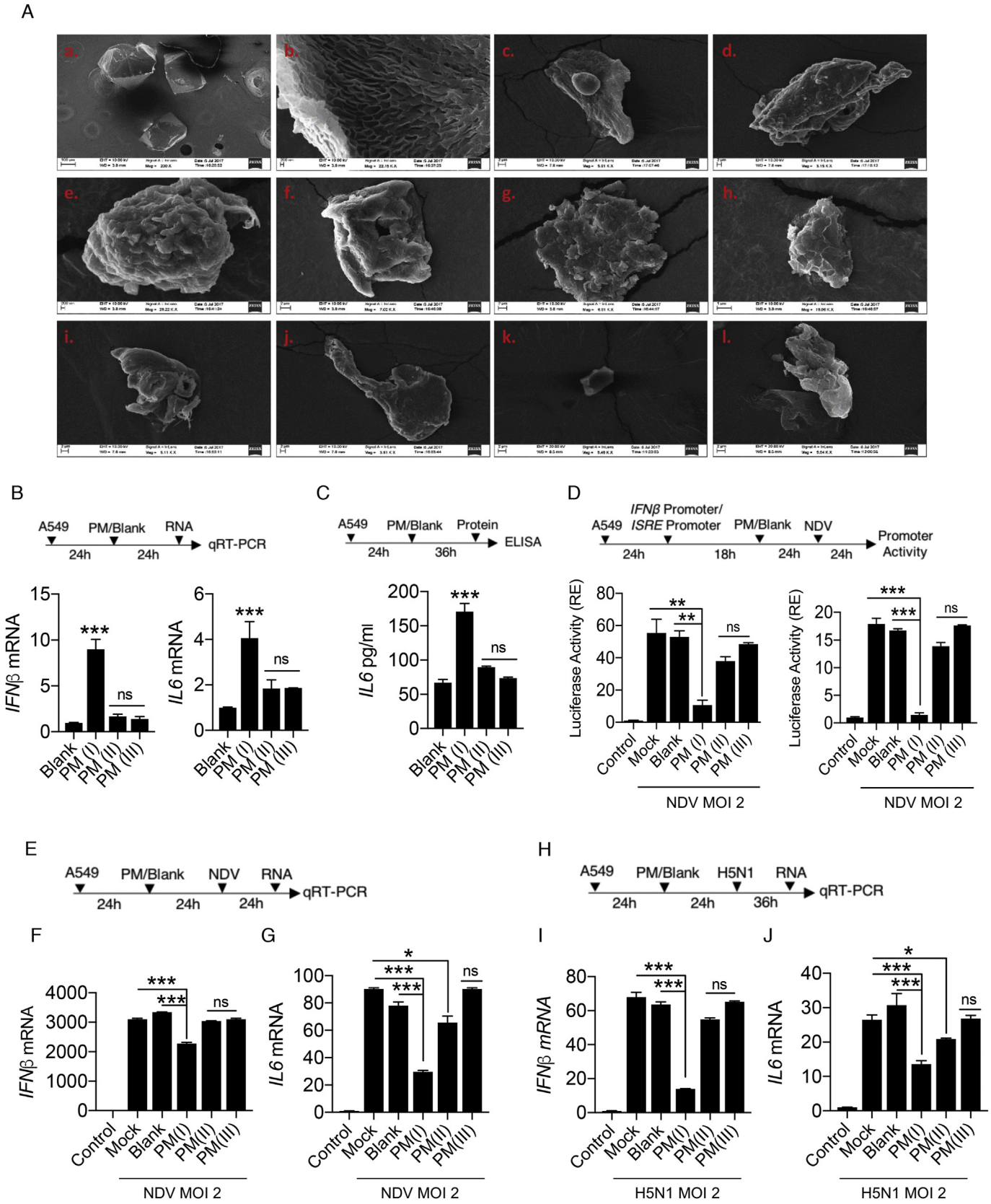


Fig. 1. PM_{10} regulates the innate immune response upon RNA virus infection – (A) Scanning electron images of coarse airborne particulate matter PM_{10}. (a) Image of blank (control) solution with no PM_{10} dissolved in it. (b–l) Images of different shapes with varied structures representing the different characteristic morphological features of PM_{10} in the samples. Quantification of innate immune response. A549 cells were treated with PM_{10} (at different dosage: PM(I), PM(II), and PM(III) – details are mentioned in the methods section) and control mentioned as blank for (B) 24 h then harvested in Trizol to quantify the mRNA expression of *IFNβ* and *IL6* by qRT-PCR. (C) 36 h then cell supernatant was collected to measure the protein level of *IL6* by ELISA. (D) Schematic representation of workflow for quantification of *IFNβ* and *ISRE* promoter activities by luciferase assay as

2.11. Next generation sequencing (NGS) analysis for transcriptomic profiling

Total RNA was extracted using TRIzol reagent (Ambion/Invitrogen) and assessed for quality. The RNA-Seq paired end libraries were prepared from the QC passed RNA samples using Illumina Trueseq stranded mRNA sample prep kit. Libraries were sequenced using NextSeq500 with a read length (2 × 75 bp), by Eurofins Genomic India Private Limited, India. The Raw reads were assessed for quality using FastQC (Andrews et al., 2010). The filtering of reads and the removal of adapters were performed using the tool Trimmomatic (Bolger et al., 2014). Approximately 18 million base pair reads were mapped to the human transcriptome (hg38), using Kallisto (Bray et al., 2016) and the abundance of the assembled coding transcriptome were projected as transcripts per million (TPM). The transcripts level abundance counts were converted into gene-level abundance counts using the R package, Tximport (Soneson et al., 2015). Differential expression analysis was performed using Limma package (Ritchie et al., 2015) implemented in Biojupies tool (Torre et al., 2018). The genes which were differentially expressed ($-1.5 < \text{Log FC} < 1.5$) were selected and the gene ontology analysis were performed using DAVID tool (Huang et al., 2007). Bubble plots, circle plot, chord plots were generated from the gene ontology and pathway enrichment results generated by DAVID tool, using the R package GOplot (Walter et al., 2015).

Data availability: The NGS (RNA-Sequencing) data for expression profiling reported in this paper have been deposited in the GenBank database (accession no. GSE147227).

2.12. Statistical analysis

All experiments were carried out along with the appropriate controls, indicated as untreated/uninfected cells (Control) or treated/infected with the appropriate reagent/virus infection alone (Mock). Experiments were performed in duplicates or triplicates for at least two or three times independently. GraphPad Prism 8.0 (GraphPad Software, La Jolla, CA, USA) was used for statistical analysis. The differences between two groups were compared by using an unpaired two-tailed Student's t-test. While the differences between three groups or more were compared by using analysis of variance (ANOVA) with Tukey test. Differences were considered to be statistically significant when $p < 0.05$. Statistical significance in the figures is indicated as follows: $***p < 0.001$, $**p < 0.01$, $*p < 0.05$; *ns*, not significant.

3. Results

3.1. Exposure of PM_{10} reduces innate immunity upon RNA virus infection

As reported previously, PM or similar substances like smog, diesel exhaust,cigarette smoke extract causes activation of the inflammatory responses when comes in contact with host's airways and lungs (Oeder et al., 2012; Proud et al., 2012). To understand the effect of PM_{10} on host cells, we initially characterized the coarse size particulate matter (PM_{10}), that were collected and used in the study. We performed SEM-EDS (scanning electron microscopy – energy dispersive X-ray spectrometer) analysis of PM_{10} collected from Bengaluru city, India and concluded that various shapes were embedded in the PM_{10}. We found different characteristic morphological features within the PM_{10} (Fig. 1A), consists of biologically active shapes like air ash, spherical, irregular, well-defined, aggregates and rounded. We additionally, performed energy dispersive spectroscopy (EDS) analysis and got different peaks in the spectrum obtained upon analysing the sample at different points with the pulse of electrons (Fig. S1A). The peaks in the spectra correspond to the presence of different elements including both metals and non-metals (% by weight) in the PM_{10} (Fig. S1B) which are hazardous and have potency to cause lung damage. Therefore, characterization of PM_{10} prompted us to examine whether PM_{10} can induce any innate immune responses in human lung epithelial carcinoma cells, A549. Interestingly, we have found that when cells were exposed to PM (I), where (I) is the dose, which corresponds to equal volume of PM_{10} and DMEM media (50% PM10 in DMEM media) composition; the type I interferon, *IFNβ* (Fig. 1B) and inflammatory cytokine, interleukin 6 - *IL6* (Fig. 1C) were induced moderately as compared to the other dosage forms such as PM(II) and PM(III), where (II) represents 25% of PM_{10} composition in DMEM media and (III) represents 10% of PM_{10} composition in DMEM media respectively. Furthermore, we performed *IFNβ* and *ISRE* promoter assay after infection with NDV in presence of PM_{10} and found that there was significant reduction in the promoter activities at the dosage of PM_{10} (I) compared to other dosage of PM_{10}; PM(II) and PM(III) respectively (Fig. 1D), suggesting that though PM_{10} induces innate immune responses alone but in presence of virus infection that induction compromised due to combined effect of PM_{10} exposure and NDV infection. Additionally, we concluded that in different set of experiments dual treatment of PM_{10} and virus infection (NDV) to A549 cells as shown in schematic representation (Fig. 1E) reduces the mRNA transcript levels of interferon *IFNβ* and cytokine *IL6* (Fig. 1F and G). These findings further prompted us to investigate whether the currently characterized PM_{10} is associated with any respiratory diseases because majority of infectious respiratory diseases are mainly caused by RNA viruses.

Previously, it has been shown that cigarette smoke extract (CSE) affects various regulatory pathways during Rhinovirus (RV) infection using human bronchial cell lines by microarray analysis (Proud et al., 2012). We re-analyse the microarray-based gene expression omnibus (GEO) dataset: GSE27973 in context to our prospective and found that there are several important cellular machineries associated genes (Fig. S2A) were modulated due to CSE exposure and RV infection. We next analysed the regulation of important genes involved in diseases particularly Influenza (flu) virus infection and key immune signalling pathways (Figs. S2B–D). Gene profile analysis concluded that various antiviral genes were prominently downregulated upon CSE exposure and RV infection. Here, in current study we used Influenza virus infection along with PM_{10} treatment in the A549 cells, because Influenza virus infection is severely fatal compared to any other virus that causes respiratory damage and Influenza virus is regularly active upon the evolutionary scale and regarded as one of the hazardous threats according to WHO to humans. Therefore, to get insights about PM_{10} exposure and Highly Pathogenic Avian Influenza infection (HPAI), we treated the A549 cells with PM_{10} and infected them HPAI H5N1 (MOI 2 – multiplicity of infection 2) as shown in schematic

indicated in A549 cells. NDV represents New-Castle Disease Virus infection at MOI = 2. (E) Schematic work flow of PM_{10} exposure (at different dosage: PM(I), PM(II), and PM(III) – details are mentioned in the methods section) and NDV infection. (F–G) Quantification of *IFNβ* and *IL6* mRNA transcripts in uninfected (control), mock infected, blank treated and PM_{10} exposed cells by qRT-PCR. (H) Schematic work flow of PM_{10} (at different dosage: PM(I), PM(II), and PM(III) – details are mentioned in the methods section) exposure and H5N1 Influenza infection. (I–J) Quantification of *IFNβ* and *IL6* mRNA transcripts in uninfected (control), mock infected, blank treated and PM_{10} exposed cells by qRT-PCR. Data are mean ± SEM of triplicate samples from single experiment and are representative of three independent experiments. $***p < 0.001$, $**p < 0.01$ and $*p < 0.05$ by one-way ANOVA Tukey test and unpaired *t*-test.

representation (Fig. 1H). We observed that the dose PM(I) of PM_{10} significantly reduces the mRNA expression levels of both *IFNβ* and *IL6* in presence of H5N1 infection (Fig. 1I and J), compared to other dosage of PM_{10} (PM(II) and PM(II)) and control groups, indicating that during pathogenic infection by RNA viruses, particularly Influenza virus, PM_{10} reduces the innate immune response in the cells.

3.2. PM_{10} enhances viral replication upon RNA virus infection

Curtailed immune responses upon PM_{10} treatment and virus infections: both in case of NDV and H5N1 Influenza virus infections, prompted us to measure the viral load in presence of PM_{10}. We thus demonstrated the experiment of PM_{10} exposure and virus infection like NDV, H1N1 (PR8) and H5N1 in A459 cells respectively. Using virus-specific primer, it was observed that PM_{10} significantly enhances the viral replication of all the RNA viruses ubiquitously in its optimal concentration − PM(I) compared to other concentration (PM(II) and PM(II)) and control groups. PM_{10} enhances the virus replication of NDV (Fig. 2A), H5N1 (Fig. 2B) and H1N1 (Fig. 2C). Additionally, microscopy analysis demonstrates similar results in which GFP (green fluorescent protein) tagged NDV was used to infect the PM_{10} pre-exposed cells (Fig. 2D). Increased NDV infection was quantified by measuring the intensity of GFP signal and number of GFP positive cells (Fig. 2E and F). Furthermore, presence of PM_{10} along with NDV infection induces the cell death as an additional detrimental effect on cells, quantified by the trypan blue assay (Fig. 2G). Altogether, our results conclude that PM_{10} enhances the viral replication pertaining to lower immune responses.

3.3. RNA-seq analysis of H5N1 infected cells in presence of PM_{10}

PM_{10} enhances the viral replication and suppress the immune responses. To further understand the global outcome of immune responses within the human cell and to dissect the mechanism about the current physiological effect, we performed RNA sequencing to profile the overall changes in the host genes and cellular pathways upon PM_{10} treatment and HPAI H5N1 infection. Schematic workflow of the experiment and transcriptomic sequencing shown in Fig. 3A. Differential expression of host genes analysis was performed between PM_{10}-treated_H5N1-infected and subsequently mock-treated_H5N1-infected samples. After aligning raw RNA-sequence reads to the human transcriptome using Kallisto algorithm, the read counts of each genes were estimated and differential expression was performed using Limma package by using Biojupies tool as shown in the methods. The differential expression of genes analysis were visualised through the volcano plot (Fig. 3B), which is in general a scatter plot, plotted between the statistical significance of genes and the magnitude of change in gene expression. In the volcano plot, the genes which were upregulated are scattered towards the right of the x-axis and the genes which were downregulated are scattered towards the left of the x-axis. The genes which were statistically significant and altered more than 1.5 fold change were plotted in red colour and the genes which were altered more than 1.5 fold change between the two experimental groups (PM_{10}-treated_H5N1-infected and mock-treated_H5N1-infected) were plotted in blue colour. And the remaining genes which were altered less than 1.5 fold were plotted in black colour. In principal, this could be noted that the enhanced viral replication, in presence of PM_{10} might be the result of down-regulation of innate immune genes and simultaneous upregulation of other cellular factors, particularly metabolic pathways-related factors, namely, *VIPR1, CYP1A1, AlDH1A3* and *PPP1R14A*. Moreover, gene ontology analysis performed by using the DAVID tool revealed the enriched biological processes (BP), cellular components (CC) and molecular functions (MF) for the genes which were altered more than 1.5 fold change between the experimental groups. The results were visualised through the bubble plot (Fig. 3C(i)). The Z-score obtained from the **d**atabase for **a**nnotation, **v**isualization and **i**ntegrated **d**iscovery (DAVID) tool output was assigned to x-axis and the negative logarithm of the *p-value* was assigned at the y-axis in the bubble plot. The area of the circles in the bubble plot corresponds to the number of genes assigned to the particular ontology term and the colour corresponds to the gene ontology categories-green, pink and blue for biological process (BP), cellular components (CC) and molecular function (MF) respectively. The threshold of labelling was set to 3, based on the negative logarithm of the *p-value*. Inflammatory response (GO:0006954), integral component of plasma membrane (GO:0005887) and cytokine activity (GO:0005125) were the top enriched terms in the gene ontology categories; BP, CC and MF respectively. Inflammatory response and cytokine activity were known to be associated with Influenza infection as well as PM_{10} treatment in A549 cells. Circle plot was used to visualize the top 10 enriched ontology terms in which the outer ring shows the scatter plotting of log FC values of each genes in the ontology term, in which red circles signifies the upregulated genes and the blue circles signifies the down-regulated genes within each terms. The inner ring shows the bar plot where the size of the bar represents the significance of terms and the colour corresponds to the Z-score of the associated term (Fig. 3C(ii)). Additionally, the chord plot represents the connection of common significant differentially expressed genes with the significant enriched ontology terms (Fig. S3A). Pathway analysis was performed using the DAVID tool and top enriched pathways of differentially expressed genes with $-1.5 < \log FC < 1.5$ were represented by the chord plot (Fig. 3D(i)) depicting the network between significant differentially expressed genes and their enriched pathways. Additionally, circle plot depicts the connection of top enriched pathways with the status of the genes contributing to the pathway represented by their log FC and Z-score (Fig. 3D(ii)).The Pathway enrichment analysis revealed neuroactive ligand-receptor interaction (hsa04080), cytokine-cytokine receptor interaction (hsa04060), chemical carcinogenesis (hsa05204) as the top enriched pathways between the experimental groups.Gene ontology analysis and pathway enrichment analysis revealed that significantly down-regulated genes during H5N1 infection in presence of PM_{10} were involved majorly in various immune signalling pathways and innate immune responses, in accordance with our experimentally validated results. On contrary, comprehensive analysis revealed that significantly up-regulated genes were majorly involved in various metabolic pathways. Furthermore, representative of up-regulated genes from significantly regulated metabolic pathways were validated by qRT-PCR analysis and found the enhanced mRNA expression levels of *VIPR1* (Vasoactive Intestinal Polypeptide Receptor 1), *CYP1A1* (Cytochrome P450 Family 1 Subfamily A Member 1), *AlDH1A3* (Aldehyde Dehydrogenase Family Member A3) and *PPP1R14A* (Protein Phosphatase 1 Regulatory Inhibitor Subunit 14A) genes upon H5N1-infection in A549 cells in presence of PM_{10} (Fig. 3E–H). Similar results were obtained in NDV-infected A549 cells in presence of PM_{10} (Figs. S3B–E). Related results were obtained by re-analysing the GEO dataset GSE27973 of Rhinovirus infection and CSE exposure in human bronchial epithelial cell lines (Figs. S4A–B). Additionally, theses metabolic pathways-related genes were found to be associated with many pathological states (Fig. S4C). Overall our data concludes that upon PM_{10} treatment during RNA virus infection, particularly, Influenza virus infection, PM_{10} significantly enhances the virus infection by down-regulating innate immune responses and upregulating different metabolic processes, that might potentiate air pollutant to enhance virus infectivity within the cells and

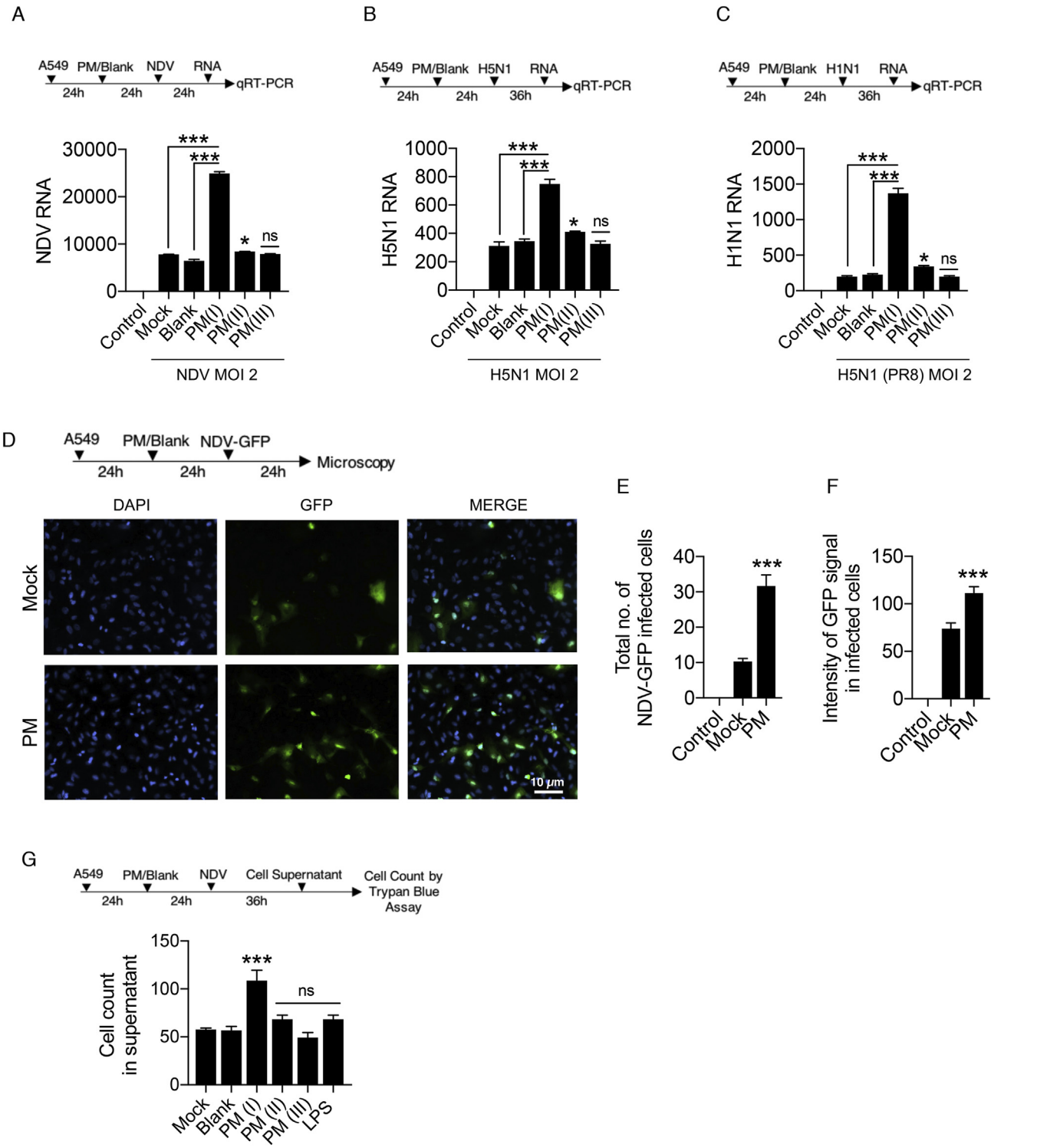
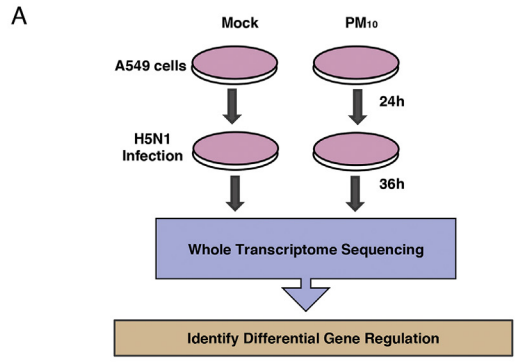
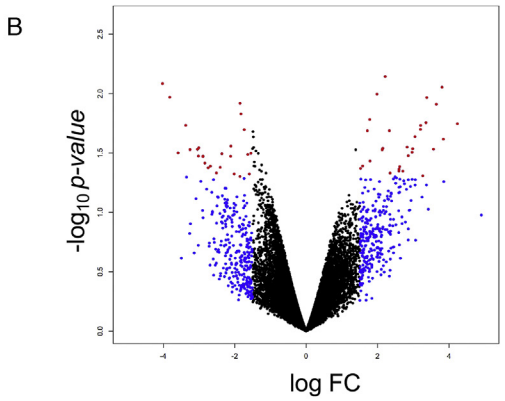
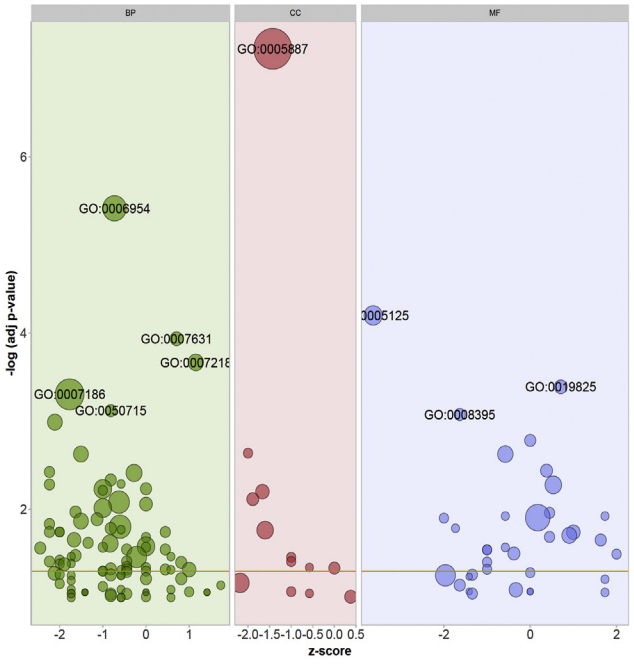


Fig. 2. PM_{10} elevates the RNA virus infection – (A–F) Estimation of viral replication in A549 cells exposed with PM_{10} for 24 h before virus infection at MOI = 2. (A) Schematic work flow of the experiment, PM_{10} exposure (at different dosage: PM(I), PM(II), and PM(III) – details are mentioned in the methods section; PM_{10} dose standardization) enhances the NDV abundance (viral transcripts) in the cells compared to the control groups (uninfected control, mock infected, and blank treated cells respectively). (B–C) Schematic work flow of the experiment, PM_{10} exposure (at different dosage: PM(I), PM(II), and PM(III) – details are mentioned in the methods section; PM_{10} dose standardization) enhances the H5N1 and H1N1 abundance (viral transcripts) in the cells compared to the control groups (uninfected control, mock infected, and blank treated cells respectively). (D) Schematic work flow for microscopy: A549 cells were exposed with PM_{10} (labelled as PM; corresponds to PM(I) dosage form - details are mentioned in the methods section; PM_{10} dose standardization) then after infected with GFP – labelled NDV for 24 h, cells on the cover slips were then fixed (as per the protocol described in methods section) and estimated for the GFP positive signals, quantified as (E) total number of NDV-GFP infected cells and (F) intensity of GFP signals in the infected cells. (G) Schematic work flow to estimate the cell death in cell supernatant after PM_{10} exposure (at different dosage: PM(I), PM(II), and PM(III) – details are mentioned in the methods section; PM_{10} dose standardization) and NDV infection in A549 cells. Cells (dead) were counted by the trypan blue counting assay. Data are mean ± SEM of triplicate samples from single experiment and are representative of three independent experiments. ***$p < 0.001$ by one-way ANOVA Tukey test and unpaired *t*-test. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

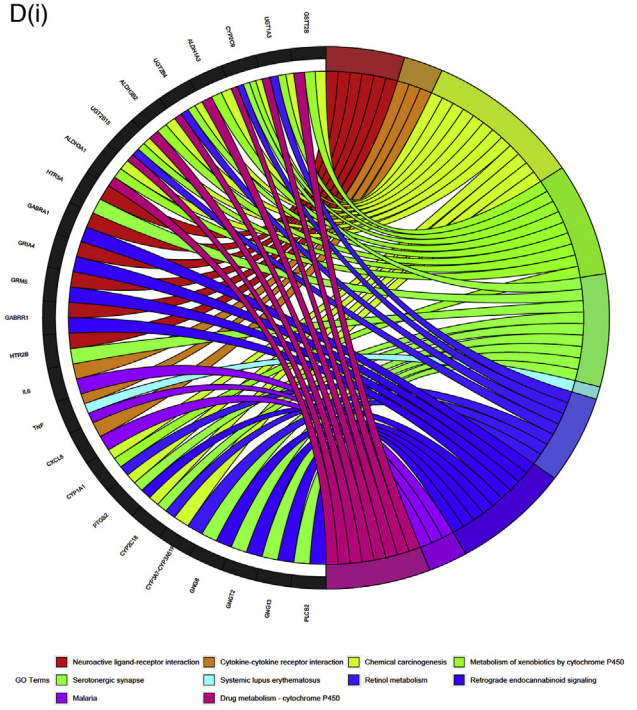




C(i)



D(i)



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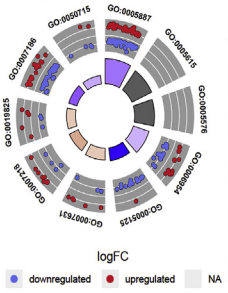
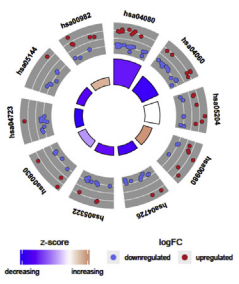
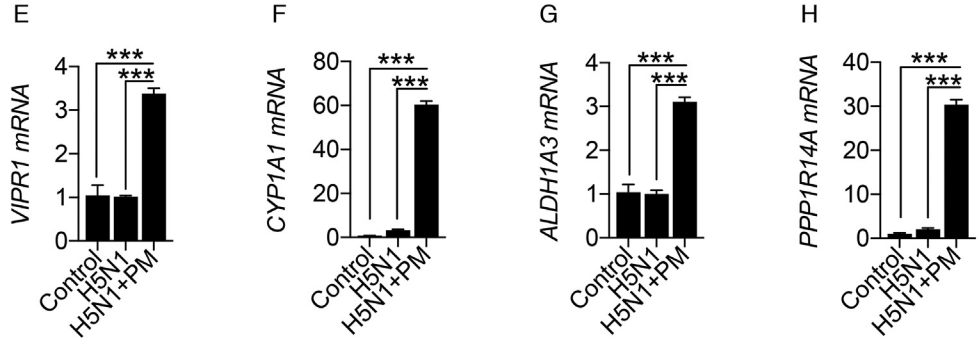


ID	Description
GO:0005887	integral component of plasma membrane
GO:0005615	extracellular space
GO:0005576	extracellular region
GO:0006954	inflammatory response
GO:0005125	cytokine activity
GO:0007631	feeding behavior
GO:0007218	neuropeptide signaling pathway
GO:0019825	oxygen binding
GO:0007186	G-protein coupled receptor signaling pathway
GO:0050715	positive regulation of cytokine secretion

D(ii)



ID	Description
hsa04080	Neuroactive ligand-receptor interaction
hsa04060	Cytokine-cytokine receptor interaction
hsa05204	Chemical carcinogenesis
hsa00980	Metabolism of xenobiotics by cytochrome P450
hsa04726	Serotonergic synapse
hsa05322	Systemic lupus erythematosus
hsa00830	Retinol metabolism
hsa04723	Retrograde endocannabinoid signaling
hsa05144	Malaria
hsa00982	Drug metabolism - cytochrome P450



causes the respiratory damage.

3.4. Knockdown of metabolism-associated genes involved in virus replication

To investigate the correlation between the upregulated metabolic pathways-related genes and their influence on virus infection upon PM_{10} treatment, we selected *CYP1A1*, *VIPR1* and *PPP1R14A* genes because these genes were significantly upregulated in our RNA sequencing analysis and their roles are poorly understood. The *CYP1A1* involved in xenobiotic metabolic pathways, which is one of the metabolic pathways in aiding virus infections, *VIPR1* is associated with G-protein coupled receptor pathway and *PPP1R14A* involved in vascular smooth muscle contraction and oxytocin pathway which were directly or indirectly related to virus infectivity within the host cell. To this end, we performed knockdown study of *CYP1A1*, *VIPR1* and *PPP1R14A* in A549 cells. We used two different short hairpin (*sh)*-clones for each gene to knockdown the expression of *CYP1A1*, *VIPR1* and *PPP1R14A* genes respectively as shown in the schematic workflow (Fig. 4A–C). Particularly, knock down of these genes in presence of NDV infection in A549 cells, leads to significant suppression the virus infection and upregulation of innate immune response-related or antiviral genes, *IL6* (interleukin 6) and *IFIT1* (interferon induced protein with tetratricopeptide repeats 1), notably, the knockdown substantially reduced the gene expression (Fig. 4A–C), suggesting that upregulated metabolic pathways-related genes in presence of ambient PM_{10} (Fig. 3E–H) support the virus infections and supress the innate immune responses-related genes, that further contribute to the severity of respiratory related diseases or highly pathogenic respiratory virus infections, like Influenza. Since transcriptomic analysis clearly reveals the involvement of these metabolic-pathway-related cellular genes in H5N1 infection and reduction of antiviral cytokines (Fig. 3). And these cellular factors were found to be involved in the pathogenesis of other RNA virus infection. Therefore, performing the knockdown studies of these genes in presence of an experimental RNA virus, NDV will further add to the value of these results and mechanism associated with dual effect of PM_{10} exposure and virus infection in enhancing the severity of respiratory illness, especially flu infection. In addition, reanalysis of GEO dataset GSE34607, reveals that the expression of these metabolic genes in BEAS-2B bronchial epithelial cells were significantly modulated in presence of PM_{10} (Fig. S5A) along with other cellular inflammatory genes (Fig. S5B). Moreover, *VIPR1*, *CYP1A1* and *PPP1R14A* are reported to be strongly associated with the modulation of inflammatory and/or innate immune responses as their downregulation results in the elevation of immune responses (Abad et al., 2016; Blackham et al., 2010; Delgado et al., 2001; Fang et al., 2016; Paton and Renton, 1998; Yin et al., 2017; Zhang et al., 2018) during microbial infections, autoimmunity and dendritic cell differentiation respectively. Altogether, our result concludes that these metabolic pathways-related genes or cellular factors contributes in elevation of RNA virus infection and causing lung disease severity in presence of airborne ambient PM. A schematic representation of the overall study is shown in Fig. 4D.

4. Discussion

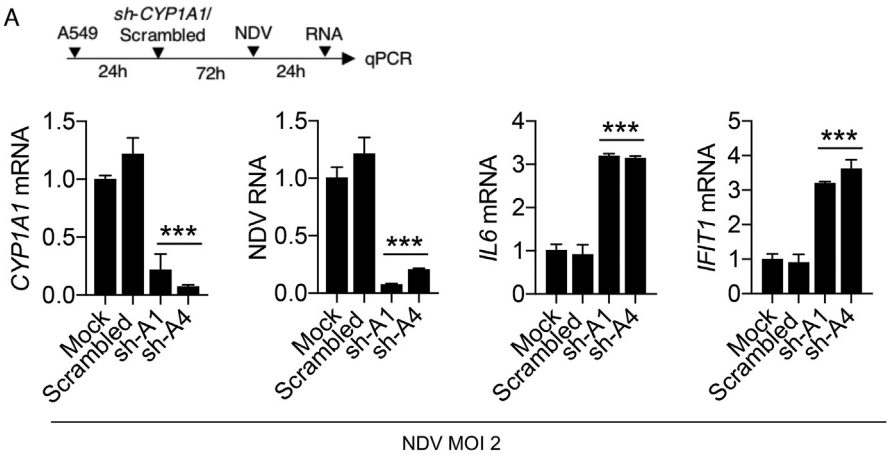
Airborne PM were considered as one of the abiotic hazardous determinants for the development diseases such as cancer (Quezada-Maldonado et al., 2018), respiratory (Miller and Peden, 2014), cardiovascular (Ulrich et al., 2002) and neurological disorders (Bandyopadhyay, 2016; Crinnion, 2017; Genc et al., 2012; Loane et al., 2013; Xu et al., 2016). Presently, emergence of novel infectious microbial pathogens along with air pollution is one of severe threats to the human society globally. Infection through respiratory route has been included as the top threat to global health in the 13th General Programme of work, by WHO which aims to cover more than three billion people against the health emergencies from 2019 to 2023 (WHO, 2018).

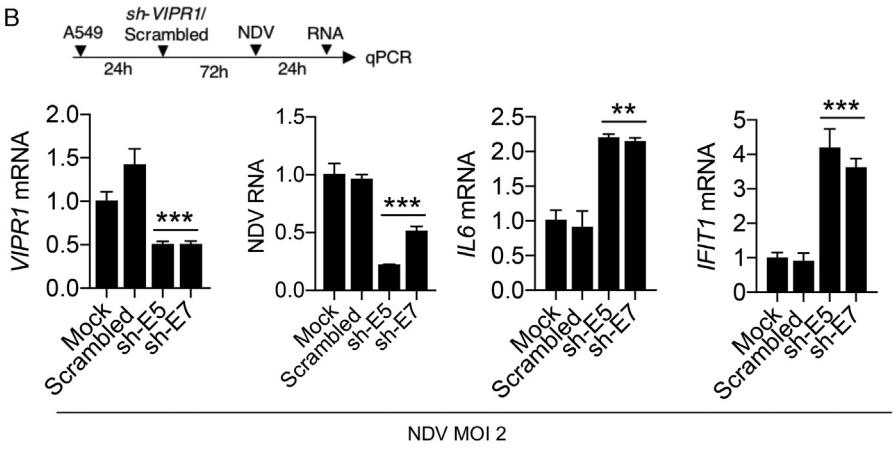
Air pollution is also one of the key risk factor for metabolism-associated diseases along with respiratory route infections, and its adverse effects including comorbidity or even death of such coinfected patients throughout the world (Landrigan, 2017). Among the different air pollutants, PM contributes to the majority of lethal effects, which differs according to the geographical area, climatic conditions and sources of occurrence or release into the environment, particularly in developing country like India, where air pollution is predominant factor in major cities like, New Delhi, Pune, Bengaluru, Kolkata, and Chennai.

PM exists in different sizes and directly interacts with various cells particularly epithelial cells of respiratory tract and lungs through complex physicochemical and biochemical interactions. They are classified into three sizes; $PM_{0.1}$, $PM_{2.5}$ and PM_{10}. $PM_{2.5}$ were considered to be the most dangerous and hazardous airborne particles as they have the tendency to penetrate deeper into the lung tissues and cause permanent damage (de Barros Mendes Lopes et al., 2018; Horne et al., 2018). However in recent past, severe lung damage and occupational respiratory tract-related disorders has been reported to be caused by the PM_{10}, the coarse size PM, mainly found in road dust, agricultural dust, construction and demolition work site, mining operations, desert dust and dust storm apart from industrial and vehicle exhaust (Celis et al., 2004; Chirino et al., 2015; Falcon-Rodriguez et al., 2016; Goudarzi et al., 2019; Hutchison et al., 2005; Kliengchuay et al., 2018; Naimabadi et al., 2016; Song et al., 2016; Van Den Heuvel et al., 2016). PM_{10} have been extensively reported to cause toxic effects due to its diverse shape, size and composition in different parts of the world and a study additionally suggests that PM_{10} risk coefficient is several times higher than the usual risk caused by $PM_{2.5}$ (Gerlofs-Nijland et al., 2007; Janssen et al., 2013; Mallone et al., 2011; Perez et al., 2008; Pope et al., 1992; Sandstrom et al., 2005).

To date, there were few studies which links PM with respiratory viral infections (Khilnani and Tiwari, 2018; MacNee and Donaldson,

Fig. 3. Transcriptomic analysis shows PM_{10} enhances abundance of metabolic pathways-related transcripts (genes) during H5N1 infection – (A) Schematic outline of PM_{10} exposure and H5N1 infection (MOI 2) in A549 cells at indicated time. Cells were subjected to whole transcriptome sequencing and differential gene expression analysis. (B) Volcano plot represents differential expression of genes between two groups of samples (mock H5N1 infected and PM_{10} exposed plus H5N1 infected) during H5N1 infection in A549 cells. For each gene: *p-value* is plotted against fold change (mock vs PM_{10}). Significantly differentially expressed genes are marked in red colour while genes which are altered (>1.5-fold) are marked in blue colour. (C) Gene Ontology analysis performed as per the protocol mentioned in methods section represents the top differentially expressed genes in ontology terms: BP (biological processes), CC (cellular components) and MF (molecular functions) respectively depicted by bubble plot and circle plot generated through R package GOPlot. (D) Pathway enrichment analysis performed as per the protocol mentioned in methods section. Chord plot represents the differentially expressed genes and their connection with the top enriched pathways. Circle plot represents the top enriched pathways and status of the genes contributing to the pathways by their log FC and Z-score. (E–H) Quantification (measured by qRT-PCR) and validation of the fold changes in the abundances of significantly expressed metabolic pathways related transcripts: *VIPR1*, *CYP1A1*, *ALDH1A3* and *PPP1R14A* in the samples of A549 cells; untreated (control), mock H5N1 infected (H5N1) and PM_{10} exposed (labelled as PM; corresponds to PM(I) dosage form - details are mentioned in the methods section) plus H5N1 infected (H5N1+PM), analysed by RNA- Sequencing. For figure (E–H): Data are mean ± SEM of triplicate samples from single experiment and are representative of two independent experiments. ***$p < 0.001$ and **$p < 0.01$ by one-way ANOVA Tukey test and unpaired *t*-test. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)


A
sh-CYP1A1/ Scrambled
A549
NDV
RNA
qPCR
24h
72h
24h
CYP1A1 mRNA
NDV RNA
IL6 mRNA
IFIT1 mRNA
***
Mock
Scrambled
sh-A1
sh-A4
NDV MOI 2


B
sh-VIPR1/ Scrambled
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NDV
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24h
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IFIT1 mRNA
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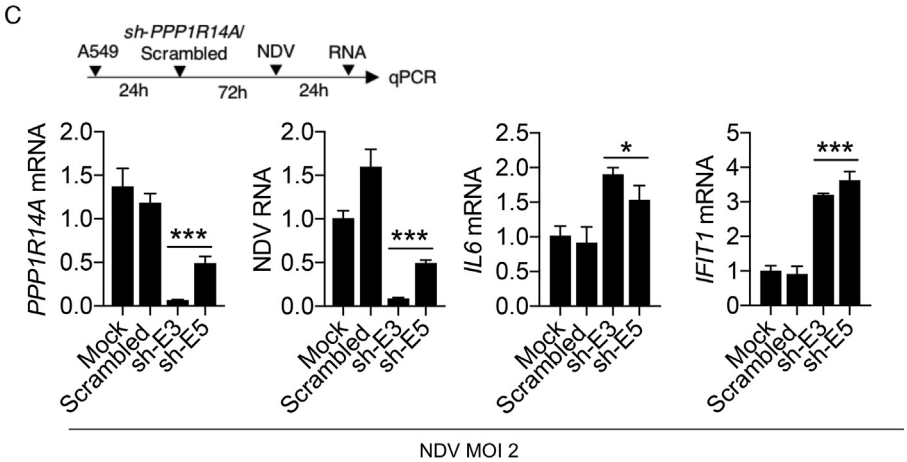
Mock
Scrambled
sh-E5
sh-E7
NDV MOI 2


C
sh-PPP1R14A/ Scrambled
A549
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RNA
qPCR
24h
72h
24h
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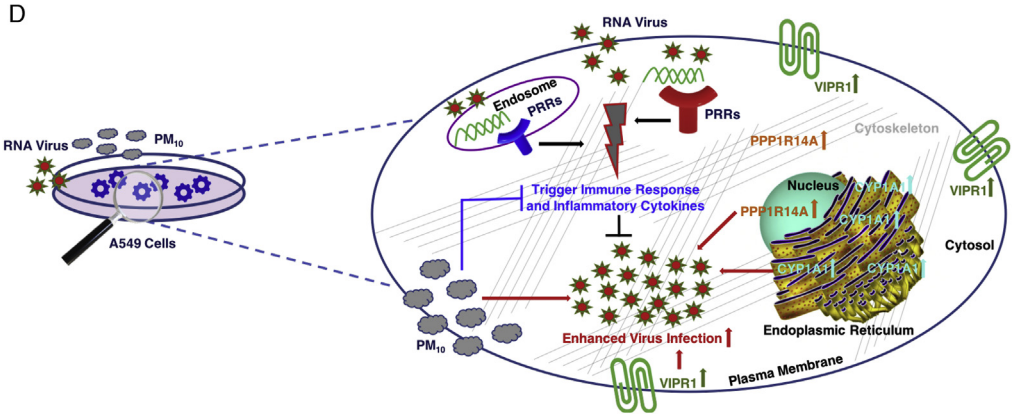
NDV RNA
IL6 mRNA
IFIT1 mRNA
*
***
Mock
Scrambled
sh-E3
sh-E5
NDV MOI 2


D
RNA Virus
PM10
A549 Cells
Endosome
PRRs
Trigger Immune Response and Inflammatory Cytokines
VIPR1
Cytoskeleton
PPP1R14A
Nucleus
CYP1A1
Cytosol
Endoplasmic Reticulum
Enhanced Virus Infection
Plasma Membrane

2003). Though these pollutants modulate the host defense and enhance the susceptibility and severity during virus infection, the underline molecular mechanisms are poorly understood (Becker and Soukup, 1999). Although, top pandemics and global threats were majorly caused by viral outbreaks, particularly RNA viruses, PM could be one of the key determinants in the spread of these viruses. Viruses are broadly, categorized as DNA and RNA based on the type of genetic material. It is evident that RNA viruses are critically harmful to the mankind and cause epidemics or pandemic for example, Influenza (flu virus), Severe Acute Respiratory Syndrome (SARS) CoV, SARS CoV-2, Middle East Respiratory Syndrome (MERS) CoV (coronaviruses), Dengue virus and HIV (human immunodeficiency virus). Recently, before coronavirus outbreak, Influenza is also included among the topmost threats by WHO and suggested to have pandemic potential. As among all the RNA viruses, its genetic complexities allow to evolve at the rapid rate and infect the host in its completely new format with high rate of fatality. Influenza infection peaks during the winter season and cause frequent seasonal epidemics, as well as sudden unforeseen pandemics. It spreads readily, and there is no proper vaccination available, although drug (Tamiflu) for Influenza is available, drug-resistant Influenza viruses are also emerged and are severe threat to the mankind. Therefore, it has been a major health as well as an economic burden throughout the world. Among various controlling factors, environmental factors, especially air pollution play an essential role in emergence of Influenza virus with pandemic potential. Combined effect of both air pollutant and virus infection is the current interest of research community to underpin the mechanism that contributes towards the severity of the respiratory damage and spread of lung infection. Few studies explained the direct causative effects of ambient pollutants and other similar causative agents like cigarette smoke extracts, diesel exhaust on various viral lung infections especially on the severity of common cold caused by Rhinovirus (MacNee and Donaldson, 2003; Paulin and Hansel, 2016; Proud et al., 2012). Different studies provide the range of impacts by PM to the lung infections from different geographical origins (Feng et al., 2016; Huang et al., 2016; Liu et al., 2019b). In a developing country like India, the levels of airborne ambient PM, especially PM_{10}, increased in the past decade due to heavy industrialization. PM_{10} isolation from Indian subcontinent and its deleterious effects on human health in terms of innate immunity during RNA virus infections are not being reported yet.

In this study, we sought to understand whether PM_{10} exposure leads to significant modification of innate immune responses and viral infectivity in human lung epithelial cell lines, A549. Additionally, we focused to explore the overall molecular changes occurred when cells were exposed to PM_{10} and virus together. We also aimed to underpin the mechanism behind the enhanced viral replication of Influenza (H5N1) virus and other RNA virus infections like NDV in presence of airborne (PM_{10}). In continuation, the cellular transcriptomic changes in presence of PM_{10} during viral infection were analysed by RNA sequencing data analysis. We used PM_{10} in our study obtained from the Bengaluru city. Bengaluru is one of the heavily industrialized, populated and urbanized cities in India. Therefore, studying about the ambient PM_{10} and its impact on respiratory health holds great importance. Initially, we characterized the PM_{10} by performing SEM-EDS analysis, and reported the topological features and chemical composition of the PM_{10} as revealed by imaging analysis (Fig. 1A and S1). Characterization of PM_{10} is of significant importance as it classifies the air pollutant into active and hazardous as well as non-active and unharmful particles. Many studies support the notion of PM characterization through SEM-EDS analysis and has reported different morphological shapes and chemical composition of PM, that cause severe lung diseases and illness (Anake et al., 2016; Bahadar Zeb et al., 2018; Galvez et al., 2013; Genga et al., 2013; Grassi et al., 2004; Labrada-Delgado et al., 2012; Ličbinský et al., 2010; Ramirez-Leal et al., 2009; Ramirez-Leal et al., 2014; Tuvjargal et al., 2019). Then, we demonstrated the effectiveness of PM_{10} exposure on A549 cells, by testing its inflammatory property within the cells, as it was previously reported that PM causes dysregulated inflammation in lungs to cause severe damage and toxicity (Cevallos et al., 2017; de Barros Mendes Lopes et al., 2018; Horne et al., 2018).

PM_{10} and its impact on lung environment and airway was investigated by exposing the lung epithelial cells with PM_{10} and infecting them with different RNA viruses like NDV and H5N1 flu virus. Our results demonstrate the consequences of both air pollutant and virus infection. Interestingly, we observed that PM_{10} suppress innate immunity and significantly elevated the viral replication. We have performed several experiments with different dosage of PM_{10} to demonstrate the phenomena of reduced immune responses (Fig. 1B–J) and enhanced viral load (Fig. 2). We also demonstrate by using luciferase assay, that PM_{10} exposed cells in presence of NDV infection reduced the promoter activity of the key antiviral genes of innate immunity, *IFNβ* and *ISRE* (Fig. 1D). The transcripts of antiviral innate immune genes were quantified to be suppressed during NDV and H5N1 infection estimated by qRT-PCR (Fig. 1E–J). Similarly, by quantifying the viral transcripts and viral GFP signals through qRT-PCR and microscopy respectively, it was concluded that PM_{10} at its optimal dosage significantly enhance the virus replication (Fig. 2A–F) and contributes to the severity of infection. Additionally, optimal dose of PM_{10} exposer along with NDV infection to the cells showed highest cell death, as estimated by the trypan blue assay (Fig. 3F). This demonstrates *ex-vivo* toxicity and severity of air pollutant and virus infection on cells. Previously, it has been shown that antiviral response was supressed upon CSE exposure during Rhinovirus infection in human bronchial epithelial cell lines (Proud et al., 2012). Reports also revealed that geogenic PM_{10} modulates Influenza infection (Clifford et al., 2015). This prompted us to test the effect of PM_{10} on the enhanced infectivity of highly pathogenic avian H5N1 Influenza virus infection and decipher the molecular mechanism. Next, to support the notion in connection to these studies related to the respiratory disease severity and its dual effect of PM exposure and virus infection, we extensively reviewed previous studies along with recent published work and preprints research related to unprecedented COVID-19, caused by SARS CoV-2 through respiratory route leading to pandemic and its correlation with disease severity under air pollution, particularly in presence of PM.

PM concentration has been shown to correlate with increased mortality and morbidities in many viral diseases. $PM_{2.5}$ and PM_{10} showed a positive correlation with RSV (respiratory syncytial virus) infection rates (Ye et al., 2016). Increased spread of measles virus is strongly correlated with the $PM_{2.5}$ concentration in China (Chen et al., 2017a; Peng et al., 2020). A global mortality impact modelling study showed PM_{10} concentration among one of the factors, associated with increased mortality during Influenza (H1N1) 2009 pandemic (Morales et al., 2017). An ecological study showed that

Fig. 4. Knockdown of validated genes reduces RNA virus infection by enhancing innate immune response – A549 cells were transiently transfected with 1.5 μg of two of the respective *sh*-clones of each indicated genes or scrambled control for 72 h then infected with NDV (MOI 2) for 24 h and subjected to the quantification of the respective indicated transcripts or genes; (A) *CYP1A1*, (B) *VIPR1* and (C) *PPP1R14A*; NDV viral RNA transcripts and antiviral cytokines; *IL6* and *IFIT1* transcripts. (D) Schematic representation of the overall conclusion of the study. Data are mean ± SEM of triplicate samples from single experiment and are representative of three independent experiments. ***$p < 0.001$ and **$p < 0.01$ by one-way ANOVA Tukey test and unpaired *t*-test.

exposure to air pollutants such as PM_{10} has been associated with increased mortality during the previous SARS-CoV outbreak in China in 2003. The study showed that the regions having moderated Air Pollution Index (API) has increased risk of death than those areas having less API. It is important to note that PM_{10} was the major pollutant among the five air pollution indices considered in this study (Cui et al., 2003). Similar to previous coronavirus outbreak (SARS-CoV), PM is also shown to be strongly associated with increased death during the ongoing coronavirus pandemic, COVID-19 (Conticini et al., 2020; Dutheil et al., 2020b; Peng et al., 2020; Sciomer et al., 2020; Setti et al., 2020a; Zhu et al., 2020). According to a United States nationwide cross-sectional study, 1 μg/m^3 increase in the $PM_{2.5}$ concentration has been linked with 15% increase in death rate due to COVID-19 (Wu et al., 2020). Additionally, in a recent study it is shown that the regions with higher Air Quality Index (AQI) also reported higher mortality rate due to COVID-19 (Conticini et al., 2020; Fattorini and Regoli, 2020; Piazzalunga-Expert, 2020). As discussed earlier, PM strongly results in high mortality when associated with any respiratory diseases. In this connection, another study revealed strong correlation of air quality data in 71 provinces of Italy with the number of deaths occurred in those provinces due to COVID-19 (Fattorini and Regoli, 2020). Air pollutants including PM, thus, promotes the viral spread. Moreover, a recent study showed the first evidence of the presence of SARS-CoV2 RNA from the PM_{10} collected from Bergamo region of Italy (Dutheil et al., 2020a; Setti et al., 2020b). These studies strongly support the positive association of PM including PM_{10}, with the increased mortality during virus outbreaks. Although, there may be certain uncontrollable confound factors which might be biased towards the observations, future studies which includes more variables must be considered along with the PM to find the important co-factors and/or host's cellular factors, cellular machineries and signalling pathways as suggested by the Italian aerosol study (Italian-Aerosol-Society, 2020). As one such report suggests the role nitrogen dioxide (NO_2), another class of air pollutant, might severely contributes towards the COVID-19 fatality (Ogen, 2020).

Although, few studies reported the global transcriptomic changes, in presence PM_{10} by microarray analysis. Our study for the first time, used high throughput RNA sequencing to study the overall changes in the gene expression upon PM_{10} exposure during the viral infection of highly pathogenic avian Influenza (HPAI) H5N1 virus in the lung epithelial carcinoma cells, A549. RNA sequencing analysis identified that majority of genes are significantly downregulated were involved in immune-related pathways, cytokine signalling, and few other inflammatory pathways (Fig. 3). In addition to this, we observed a significant increase in the expression of genes involved in various metabolic pathways, which were previously remain uncategorized, particularly in case of airborne ambient PM exposure during flu infection. We validated RNA sequencing results for four of the top upregulated genes, namely *VIPR1* (vasoactive intestinal peptide 1), *CYP1A1* (cytochrome P450, family 1, subfamily A memeber1 also known as aryl hydrocarbon hydroxylase), *ALDH1A3* (aldehyde dehydrogenase 1, family member 3A) and *PPP1R14A* (protein phosphatase 1 regulatory inhibitor subunit 14A) using quantitative qRT-PCR analysis. These selected genes - *VIPR1*, mainly located on plasma membrane and *PPP1R14A* majorly located on nucleus and cytoskeleton were moderately found to be involved in virus infections like HIV-1 and Influenza as reported by an *in-vitro* study and an *in-silico* phosphoproteomics study in human macrophages respectively (Bokaei et al., 2006; Soderholm et al., 2016; Temerozo et al., 2018). *CYP1A1* was recently reported to be involved in many virus infections especially hepatitis B and hepatitis C virus (Fattahi et al., 2018; Ohashi et al., 2018; Stavropoulou et al., 2018). One such report superficially uncovers the induction of *CYP1A1* in presences of PM_{10} (Kim et al., 2018). Additionally, induction of *CYP1A1* in presence of diesel exhaust particles were extensively reported in human bronchial cells (Totlandsdal et al., 2010). *ALDH1A3*, apart from being involved in studies related to different types of cancers (Croker et al., 2017; Flahaut et al., 2016), was also previously reported in connection with virus infections like human papilloma virus and respiratory syncytial virus (Diamond et al., 2012; Puttini et al., 2018; Tulake et al., 2018). Altogether, differentially expressed genes identified in our study related to different metabolic modifications were reasonably linked to virus infections. Therefore, we selected these genes to validate further, in context to RNA virus infectivity. We demonstrated by short hairpin *(sh)*-RNA mediated transient silencing, that these genes significantly reduced the viral replication and moderately elevated the expression of antiviral genes (Fig. 4), suggesting the importance of these metabolic pathway-related genes in regulation of pathogenic burden during viral infection.

Overall, this study highlights the effect of PM_{10} exposure upon virus (flu) infection that affects the lung airways to cause severe respiratory damage. The high throughput RNA sequencing was performed in our study for the first time, in context to Indian subcontinent distribution of PM_{10}. PM_{10} which is collected and isolated in this study demonstrate the transcriptomic changes upon its exposure during Influenza virus infection in A549 cell lines. There were very few studies that reported the link between PM_{10} exposure and enhanced viral infections (Hirota et al., 2015; Wang et al., 2015). However, the implication attached to our study model is that, we have identified and validated the host's cellular metabolic-pathway related genes and these genes contribute in enhancing the severity of infection (flu infection) in presence of ambient air pollutant, PM_{10}. Since we have incorporated both air pollutant and H5N1 strain of virus from the Indian origin and obtained the conclusions related to the mechanism by which PM_{10} enhances the virus replication within the lung epithelial cells, therefore, it could be proposed that possibly adoption of our study model applies to other strains of Influenza virus and additionally to the other class of RNA viruses, for example coronavirus in connection with different types and standards of PM. One such strong hypothesis could be built through the transcriptomic analysis performed in the study, and opens up the interesting question to test in future to decipher the mechanism how air pollutant could affect the virus infection or its severity. *ACE2* is the receptor for SARS-CoV-2 (Hoffmann et al., 2020) and there are reports that *ACE2* expression increases upon exposure to $PM_{2.5}$ (Lin et al., 2018) and PM_{10} (Miyashita et al., 2020). This frames an important question whether the prolonged exposure to PM increases the susceptibility to infection by SARS-CoV-2 due to the enhanced expression of *ACE2*. Also, increased *ACE2* is crucial for the regulation of lung tissue homeostasis and prevention of lung injury due to excessive production of pro-inflammatory cytokines (Imai et al., 2005). *ACE2* expression is downregulated not only after the SARS-CoV (Glowacka et al., 2010) and SARS-CoV-2 infection (Verdecchia et al., 2020), but also after the H5N1 (Zou et al., 2014), H1N1 (Liu et al., 2014) infection. From the extended RNA-seq results in our study (Fig. S4), *ACE2* expression was downregulated in PM10 treated and subsequently H5N1 infected samples, compared to the mock treated H5N1infected samples. Downregulation of *ACE2* has been associated with severe lung injury and increased lethality of H5N1 virus (Zou et al., 2014) as well as increased viral replication of human coronavirus (NL-63) (Dijkman et al., 2012). Meanwhile, *Ace2*-knockout mice develop severe lung injury after the exposure to $PM_{2.5}$ (Lin et al., 2018). We can speculate that PM exposure leads to the upregulation of *ACE2*, which increases the susceptibility to SARS-CoV-2 and virus mediated downregulation of *ACE2* post

infection, which in turn contributes to the severe lung injury and increased lethality. This observation supports the "double-hit" hypothesis, in a recent study (Frontera et al., 2020), which explores the involvement of $PM_{2.5}$ in SARS-CoV-2 leading to severe complications that might be applicable in case of PM_{10} association with RNA viruses, particularly H5N1 and/or SARS-CoV2.

The limitation of our study adheres to fact that, we could have included more *in-vitro* lung origin cell lines/tissues (de Barros Mendes Lopes et al., 2018; Graff et al., 2012; Huang et al., 2009; O'Beirne et al., 2018) and an *in-vivo* mouse model in order to explore the effect of pollutant under physiological conditions after PM_{10} exposure which will fully elucidate the outcomes of the study. Additionally, we could have included more variety of different ambient PM related to both seasonal variations and different geographical origins (instil with different chemical composition, shape of the particle or both) of Indian subcontinent, as that would add to the strength and scientific depth of the study in providing better insights about the effects of PM_{10} over various lung infections including Influenza and other RNA virus infection and also add to formulate the policies related to control the air pollution. However, we understand the gravity of this research area and looking forward to answer the underexplored questions, by using *in-vivo* models, associated herewith in coming future studies.

5. Conclusion

In conclusion, PM_{10} enhances RNA virus replication by suppressing the innate immunity along with the upregulation of several metabolic pathways-related genes, as evident from the transcriptomic profiling results. Knocking down these metabolism-related upregulated genes, decreases the viral replication and subsequently increases the innate immune response. As several epidemiological studies suggest that PM_{10} exposure is associated with severity of lung infections, our results also support these studies. In this study, we used lung epithelial cells in which we demonstrated that Influenza (flu) virus replication were elevated in presence of PM_{10} through suppression of antiviral innate immune cytokines. The outcome of this study may encourage future studies to uncover the mode of action of PM_{10} over different RNA viruses which have the potential to cause future outbreaks and guide the policy makers to regulate the level of PM for public healthcare purposes.

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CRediT authorship contribution statement

Richa Mishra: Conceptualization, Investigation, Validation, Formal analysis, Writing - original draft, Writing - review & editing, Project administration. **Pandikannan Krishnamoorthy:** Data curation, Writing - review & editing. **S. Gangamma:** Conceptualization, Investigation, Funding acquisition, Resources. **Ashwin Ashok Raut:** Resources. **Himanshu Kumar:** Conceptualization, Formal analysis, Writing - original draft, Writing - review & editing, Project administration, Funding acquisition, Resources, Supervision.

Declaration of competing interest

The authors declare no conflict of interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2020.115148.

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