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RESEARCH ARTICLE

Airborne particulate matter from goat farm increases acute allergic airway responses in mice

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ABSTRACT

Background: Inhalation exposure to biological particulate matter (BioPM) from livestock farms may provoke exacerbations in subjects suffering from allergy and asthma. The aim of this study was to use a murine model of allergic asthma to determine the effect of BioPM derived from goat farm on airway allergic responses.

Methods: Fine (<2.5 μm) BioPM was collected from an indoor goat stable. Female BALB/c mice were ovalbumin (OVA) sensitized and challenged with OVA or saline as control. The OVA and saline groups were divided in sub-groups and exposed intranasally to different concentrations (0, 0.9, 3, or 9 μg) of goat farm BioPM. Bronchoalveolar lavage fluid (BALF), blood and lung tissues were collected.

Results: In saline-challenged mice, goat farm BioPM induced 1) a dose-dependent increase in neutrophils in BALF and 2) production of macrophage inflammatory protein-3a. In OVA-challenged mice, BioPM induced 1) inflammatory cells in BALF, 2) OVA-specific Immunoglobulin (Ig)G1, 3) airway mucus secretion-specific gene expression. RNAseq analysis of lungs indicates that neutrophil chemotaxis and oxidation-reduction processes were the representative genomic pathways in saline and OVA-challenged mice, respectively.

Conclusions: A single exposure to goat farm BioPM enhanced airway inflammation in both saline and OVA-challenged allergic mice, with neutrophilic response as Th17 disorder and eosinophilic response as Th2 disorder indicative of the severity of allergic responses. Identification of the mode of action by which farm PM interacts with airway allergic pathways will be useful to design potential therapeutic approaches.

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BioPM; goat farm; allergic airway disease; Th2; Th17; genomic pathways; murine; ovalbumin

Introduction

Asthma has a major impact on the global burden of respiratory disease, with approximately 320 million people affected worldwide. It is characterized by recurrent airflow obstruction, hyperresponsiveness and inflammation. Allergic asthma is characterized by type 2 helper T cells (Th)2 immune responses, which promotes eosinophilic inflammation, increased Immunoglobulin (Ig)E/IgG, mucus hypersecretion, and airway remodeling (De Monchy et al. 1985; Bentley et al. 1992). Moreover, asthmatic patients suffer from exacerbations with acute or subacute phases, from exposures to environmental triggers such as airborne particulate matter (PM) that originates from wood smoke or traffic exhaust (Bui et al. 2013).

The prevalence of asthma has increased in the past 20 years, and PM could be one of the most important environmental factors that can be accounted for this increase (Koren 1997; Bach 2002; Pope III and Dockery 2006). Epidemiological studies demonstrate that asthmatics are more sensitive to airways responses from PM exposure when compared to healthy individuals (Lipsett et al. 1997). The effect of allergens from indoor air is more strongly linked to asthma prevalence and severity than outdoor allergens (Johnston 2000).

Livestock density in the Netherlands reached 3.8 livestock units per hectare of agricultural area in 2016, which is almost 5 times greater than that of Europe in that year. Furthermore, the Netherlands has also reported the highest increase rate of livestock farms among the European countries(https://ec.europa.eu/eurostat/statistics-explained/index.php/Agri-environmental-_indicator_livestock_patterns). The number of dairy goats increased rapidly from 2000 to 2019 in the Netherlands(https://www.statista.com/statistics/1097651/number-of-dairy-goat-farms-in-the-netherlands-by-number-of-goats/). Animal farming operations produce a complicated mixture of airborne PM, that is able to elicit pro-inflammatory respiratory responses (de Rooij et al. 2016). We define BioPM as a complex biological mixture of

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airborne, biogenic PM (mammalian, bacterial, fungal). These livestock BioPM contain microorganisms which are suggested to induce respiratory inflammatory responses in both farms and residents living in close proximity (Cambra-López et al. 2010). Q fever outbreaks, a zoonotic disease resulting from a respiratory infection with *Coxiella burnetti*, occurred in the Netherlands between 2007 and 2020 (van Asseldonk et al. 2013). The association between ambient *C. burnetti* DNA and residential areas near goat farms has been found in an epidemiological study (de Rooij et al. 2016). Yet, to our best knowledge, no information is available on the potential for BioPM emitted by goat farms to promote or modulate airway inflammatory diseases in experimental animal allergic studies. The mechanism by which BioPM mediates these effect is not entirely clear but appears to be associated with the ability of livestock BioPM to shift T-cell polarization in mice and humans (Robbe et al. 2014). RNA sequencing technology was used to generate pulmonary gene expression data, which was analyzed using pathway enrichment approaches, to provide possible leads on the immunological events related to BioPM induced airway inflammation (Fabregat et al. 2018).

In the present study, we hypothesized that goat farm BioPM could modulate airway allergic responses in an acute allergic murine model. For this hypothesis, BALB/c mice were OVA sensitized and then challenged with saline + BioPM (controls) or OVA + BioPM to assess several parameters of pulmonary inflammation and airway epithelial remodeling (i.e. mucus cell metaplasia).

Materials and methods

Airborne BioPM sampling period, sites and procedure

Indoor ambient fine [<2.5 µm, Mass Medium Aerodynamic Diameter (MMAD)] BioPM was collected at goat farm located in the central region of the Netherlands during February 2017 to April 2017. Around 900 Dutch White Goats, average 5 years old, were raised at goat farm during sampling period. Indoor air sampling was carried out for 2–6 days and for 6 hours per day (between 09:00 and 16:00 h). Daily collected BioPM was pooled to collect sufficient material to carry out the current study. All fine BioPM was collected in demineralized water using the Versatile Aerosol Concentration Enrichment System (VACES) to avoid BioPM damage. The collecting procedure has been previous described (Kim et al. 2001). Detailed description of the sampling dates, procedures during the sampling campaign and BioPM microbial composition are described elsewhere (Liu et al. 2019). No relevant contributions from other sources such as combustion (traffic) is anticipated as BioPM is sampled immediately from stables.

Experimental protocol

Female BALB/c mice (female mice are more susceptible than male mice to develop allergic airway inflammation; Melgert et al. 2005; Takeda et al. 2013), 6–8 weeks old, were obtained from Charles River Laboratory (Portage, MI) and randomly assigned to treatment groups of 6. Husbandry conditions were maintained at the Michigan State University (MSU) animal housing facilities at room temperature of 21–24 °C and relative humidity of 45–70%, with a 12 h light/dark cycle starting at 7:30 A.M. All animal procedures and experimental protocols were approved by the MSU Institutional Animal Care and Use Committee, MSU is an AAALAC accredited institution.

Based on the results of previous pilot studies that measured acute airway inflammation in non-allergic mice, doses of 0, 0.9, 3 and 9 µg BioPM were chosen in our allergy protocols. In order to check the airway allergic responses murine model sensitized and challenged with OVA with previous experiments, we have used diesel soot (0, 0.9 and 3 µg) and standard air PM samples (SRM1648a; 0, 0.9, 3 and 9 µg) collected by the NIST (Gaithersburg, MD, USA) as our benchmark. All subsequent experiments were conducted as follows: On Day 0, all mice (n = 6 animals/group) were OVA sensitized by intraperitoneally injection of 0.25 ml saline containing 20 µg OVA (Sigma-Aldrich) with 1 mg alum (aluminum potassium sulfate, Sigma-Aldrich; allergen sensitization). On Day 10, all mice were boosted with an intraperitoneal injection with 20 µg OVA in 0.25 ml saline and an intranasal instillation of 30 µl 0.5% OVA in saline. For the challenge, on Days 17 and 18, OVA-sensitized mice were intranasally instilled with/without 30 µl 0.5% OVA in saline or treated with saline alone (vehicle control) to produce either non-allergic (Groups A–D) or allergic airways (Groups E–H) (Table 1). Two days later, mice were intranasally instilled with 30 µl 0, 0.9, 3, or 9 µg BioPM in saline, 24 h prior to being sacrificed on Day 21 after which effects of BioPM exposure was measured (Table 1).

Mice were anesthetized, followed by performing a midline laparotomy. For plasma separation, about 0.5 mL of blood was drawn from the vena cava and collected in heparinized tubes (BD Microtainer, Franklin Lakes, NJ). Animals were exsanguinated via the abdominal aorta. The plasma was stored at −80 °C for later biochemical analysis (OVA-specific IgE and IgG1). Immediately after death, a cannula was placed in the trachea and the heart and lungs were excised en bloc.

Table 1. Experimental groups.

Group	Sensitization Day 0,10	Challenge Day17,18	BioPM Day20	Necropsy Day21
A	OVA	Saline	Saline	
B	OVA	Saline	0.9 µg	
C	OVA	Saline	3 µg	
D	OVA	Saline	9 µg	
E	OVA	OVA	Saline	
F	OVA	OVA	0.9 µg	
G	OVA	OVA	3 µg	
H	OVA	OVA	9 µg	

Mice were intraperitoneally sensitized with 20 µg OVA with alum on day 0, followed by intraperitoneal administration of 20 µg OVA and intranasal administration of 0.5% OVA on day 10. On days 17 and 18, OVA-sensitized mice were challenged intranasally with 0.5% OVA or saline. BioPM derived from goat farm was administered with intranasal doses of 0, 0.9, 3, or 9 µg on day 20. All animals were sacrificed 24 hours after the last intranasal challenge.

Bronchial alveolar lavage fluid (BALF) was recovered using a volume of 0.8 ml sterile saline, a second intratracheal lavage was performed and combined with the first. After BALF collection, the right lobes were clipped and placed in RNAlater (Qiagen, CA). Samples were kept at −80 °C until for RNA isolation.

Lung histopathology

The left lung lobe was intratracheally fixed with 10% neutral buffered formalin at a constant pressure (30 cm H_2O) for 2 h and then stored in the same fixative to preserve pulmonary architecture until further tissue processing. Twenty-four hours later, two sections were excised at the level of the 5th and 11th airway generation along the main axial airway (G5 and G11), to sample proximal and distal bronchiolar airways, respectively (Harkema and Hotchkiss 1992). The detailed protocols are described in Supplementary (Lung Morphometry). Briefly, tissue blocks were then embedded in paraffin and 5- to 6-µm-thick sections were cut from the anterior surface. Lung sections were stained with hematoxylin and eosin (H&E) for light microscopic examination and with Alcian Blue (pH 2.5)/Periodic Acid–Schiff (AB/PAS) for identification and quantification of intracellular mucus (acid and neutral mucosubstances) in pulmonary bronchiolar epithelium. Other lung tissue sections were immunohistochemically stained using a polyclonal rabbit antibody directed against murine eosinophil-specific major basic protein (MBP; 1:500; Mayo Clinic, AZ) for histologic identification of MBP-laden eosinophils.

BALF cytometry and analyses for inflammatory cytokines

The total number of intact cells in BALF was counted by using a hemocytometer. Cytological slides were prepared and centrifuged at 400 g at RT for 10 min using a Shandon cytospin 3 (Shandon Scientific, PA) and stained with Diff-Quick (Dade Behring, DE). Differential cell counts for macrophages, neutrophils, eosinophils and lymphocytes were assessed from a total of at least 200 cells.

The BALF was centrifuged at 2400 *g* at 4 °C for 15 min, the supernatant collected and stored at −80 °C for cytokine analysis. Cell-free BALF was assayed for the inflammatory cytokines interleukin (IL)-17E/IL-25, GM-CSF, interferon gamma (IFN-γ), macrophage inflammatory protein (MIP)-3a, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-21, IL-22, IL-28B, IL-10, IL-23, IL-12p70, IL-27, IL-13, IL-15, IL-17A, IL-17F, IL-33, IL-31, tumor necrosis factor (TNF)-β, TNF-α and CD40L. All cytokines were measured using Luminex (Millipore, Billerica, MA) which were performed according to the manufacturer's instructions. Cytokine/chemokine data were considered as detectable using the following criteria: at least three detectable (>10 pg/ml) individuals out of six, which is the animal number per group.

Immunoglobulins (Ig) in blood plasma

Plasma was separated from blood and analyzed for OVA-specific IgE and IgG1 using an ELISA kit (Cayman, Chemicals, Sanbio, Uden, the Netherlands) according to the manufacturer's instructions. Pre-coated ELISA plates were incubated with diluted plasma samples and standards for 2 h. After washing steps, antibodies were detected using biotin-conjugated anti-mouse IgE or IgG1 antibody. Finally, streptavidin-horseradish peroxidase was added and followed by incubation with reaction substrate. Optical density was read at 650 or 450 nm wave length. For OVA-specific IgE, the plasma from all mice was diluted 1:20. For OVA-specific IgG1, the plasma from the saline-challenged mice was diluted 1:1000, and plasma from the OVA-challenged mice was diluted 1:16,000.

RNA isolation from mouse lungs

Total RNA was extracted from the caudal lobes (right lungs) using phenol extraction in combination with the miRNeasy RNA isolation kit (Qiagen, Hilden, Germany). 3 µg BioPM were chosen as representative dose to study gene expression in lungs of OVA/Sal and OVA/OVA mice. In short, murine lung tissue was homogenized by using the bead homogenization (MP Biomedicals, Illkirch, France) in 600 µl Trizol (Thermofisher, Landsmeer, The Netherlands). RNA isolation was carried out according to the manufacturer's instructions. The RNA concentration was assessed using a NanoDrop 2000 spectrophotometer (Isogen Life Science, Netherlands). The RNA quality and integrity were determined using Lab-on-Chip analysis on an Agilent 2100 Bioanalyzer (Agilent Technologies) using the RNA 6000 Nano kit. The RNA integrity numbers (RIN) of all RNA samples had values above 8.4.

Transcriptome profiling

Gene expression profiles in the mouse lungs were obtained by RNAseq. Therefore, libraries for the Illumina NextSeq were generated using the TruSeq Stranded mRNA Library Prep Kit using 400 ng of total RNA as input according to the manufacturer's instructions. Briefly, the mRNA fraction was purified from total RNA by polyA capture, fragmented and subjected to first-strand cDNA synthesis with random hexamers in the presence of Actinomycin D. The second-strand synthesis was performed incorporating dUTP instead of dTTP. Barcoded DNA adapters were ligated to both ends of the double-stranded cDNA and subjected to PCR amplification. Libraries were subsequently validated for fragment size on a QiaXcel (Qiagen) and quantified using RT-qPCR with a Kapa-kit (Roche Sequencing Store). In each sequencing run, 24 libraries were pooled at equimolar concentrations and sequenced using the Illumina NextSeq 500/550 High Output Kit v2.5 (single-end, 75 Cycles). Basecalling and demultiplexing was performed using bcl2fastq2 Conversion Software v2.20, and demultiplexed FASTQ files were generated based on sample-specific barcodes (>16

million reads/sample). RNAseq reads were aligned to the Mus musculus reference genome (UCSC mm10) and most recent transcript annotations using STAR (2.6.1a). A count table was generated and DESeq2 (v1.1) (Love et al. 2014) was used for statistical analysis and identification of differentially expressed genes (DEG). Genes were considered significantly different with a false discovery rate (FDR) <0.1.

Pathway enrichment analysis

The pathway enrichment analysis was performed using a web-based application, Database for Annotation, Visualization and Integrated Discovery (DAVID: https://david.ncifcrf.gov/) (Huang et al. 2009). DAVID uses annotations classified as metabolic pathways Kyoto Encyclopedia of Genes and Genomes (KEGG), Gene Ontology (GO), Keywords and others to provide a comprehensive set of functional pathways. The significance of the pathways is determined by the following criteria: (1) a ratio of the total number of DEGs that were list to website divided by the number of genes that are related to the certain pathways; (2) Fisher's exact test p values were calculated to determine the possibility of the association between the DEGs in the data set and the significant pathways, then these p values were changed to Benjamini-Hochberg p values for multiple correction, and only the ones with FDR <0.05 were selected.

Statistics

Each study group consisted of 6 mice and all values are expressed as mean ± standard deviation of the mean (SEM). Outlier test (Grubbs) was performed and recognized outliers were removed from the analysis. For the continuous normal datasets, a one-way ANOVA analyses was performed, followed by a Student Neuman–Kuels post-hoc multiple comparisons test comparing groups exposed to saline/OVA with BioPM to groups exposed to saline/OVA alone. For non-normal distributed datasets, a non-parametric Kruskal Wallis or Mann–Whitney tests were performed to check for differences between groups exposed to saline/OVA with BioPM and groups exposed to saline/OVA alone. All analyses were conducted using GraphPad Prism (version 8.0.2). Significance was assigned to p-values less than or equal to 0.05.

If the medians of animal groups indicated a tendency of increase or decrease, a linear trend analysis was performed between these groups to investigate whether effects could be related to dose of BioPM. A large class of dose–response models was fitted for describing the change in any continuous or quantal endpoint as a function of BioPM dose and saline/OVA treatment by using software PROAST (https://www.rivm.nl/en/proast). A member from this family was selected using a likelihood-ratio test for depicting the best model fit (Slob 2002).

Results

Pulmonary histopathology

Control mice that received only intranasal instillations of saline (0 µg BioPM, Figure 1(A)) had no treatment-related pulmonary histopathology. BioPM-induced pulmonary histopathology in mice intranasally challenged with saline (no OVA) consisted of a minimal bronchiolitis and alveolitis that was primarily located in the hilar region of the lung lobe (proximal transverse section of the lung lobe at the level of axial airway generation 5) with lesser involvement of the more distal lung lobe section (at the level of axial airway generation 11) (Figure 1(B)).

The BioPM-induced inflammatory response in the airway wall and peri-bronchiolar interstitial tissue (bronchiolitis) consisted of a mixed inflammatory cell influx of polymorphonuclear leukocytes (neutrophils) and lesser numbers of mononuclear leukocytes (mainly lymphocytes) and eosinophils (type 1 immunity/inflammation characteristic of a nonallergic toxic response). Large and small diameter bronchioles were affected as well as centriacinar regions including the terminal bronchioles and proximal alveolar ducts and associated alveoli. Associated with the airway inflammation in the large-diameter, but not small-diameter, bronchioles, was an occasional mucous cell metaplasia (secretory cells with AB/PAS-stained mucosubstances not normally found in bronchioles of mice) in the luminal airway epithelium. However, this epithelial change was of minimal severity.

In the affected alveolar regions of these BioPM-instilled mice, there were small widely scattered focal accumulations of neutrophils and alveolar macrophages/monocytes. In general, the severity of the bronchiolitis/alveolitis was greater in mice receiving the higher amounts of BioPM (dose response), which was most apparent in mice receiving 9 µg BioPM (Figure 1(A,B)).

In contrast, mice intranasally challenged with OVA exhibited a very different inflammatory cell response (type 2 immunity/inflammation characteristic of an allergic response) consisting of a mixed cellular infiltrate of large numbers of eosinophils, lymphocytes, plasma cells and lesser number of neutrophils. This allergic inflammatory response was of much greater severity (moderate to marked; Figure 1(C)) than the minimal type 1 immunity/inflammation in mice that received intranasal BioPM but no OVA challenge (Figure 1(A)). The most severe type 2 immune/inflammatory cell responses in the lung were in OVA challenged mice that were intranasally instilled with 9 µg BioPM (Figure 1(D)).

Morphometric determinations (unbiased quantitative image analysis) of the pulmonary density of AB/PAS-stained mucosubstances in bronchiolar epithelium confirmed that there were significantly greater amounts of mucosubstances (marker of mucus cell metaplasia) after OVA challenge (Figure 2(A,C)), but no significant changes with subsequent BioPM instillations in OVA-challenged mice (Figure 2(C,D)).

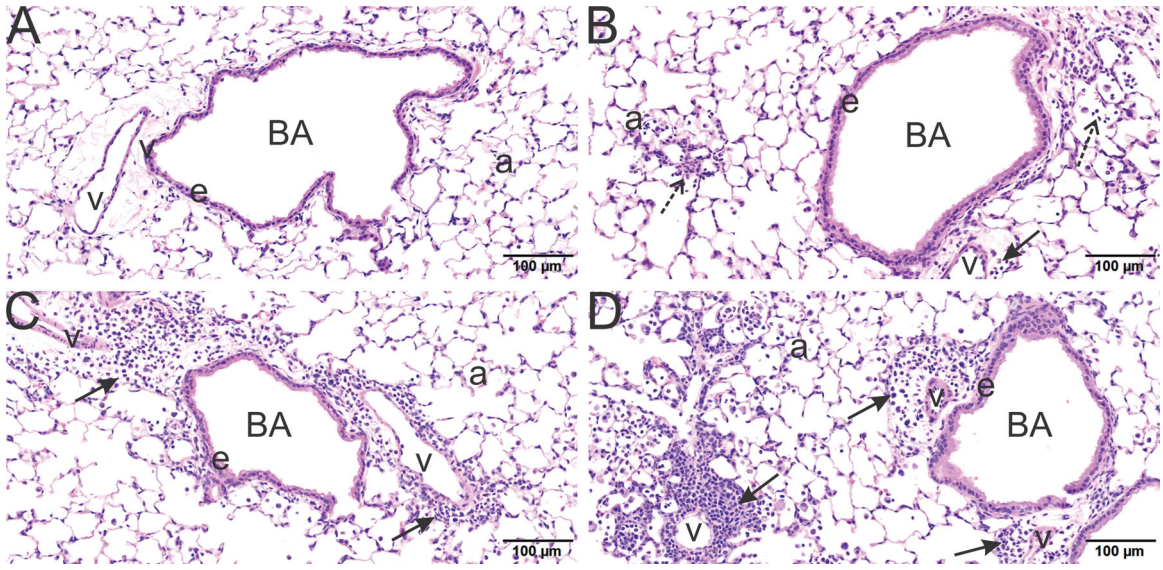

Figure 1. Light photomicrographs of lung tissue sections from mice intranasally challenged with (A) saline (vehicle control; 0 µg BioPM), (B) saline and 9 µg of goat farm BioPM, (C) OVA (0 µg BioPM), or (D) OVA and 9 µg of goat farm BioPM. In (B), stippled arrows identify a mixed inflammatory cell influx in the alveolar parenchyma (a; alveolitis) composed mainly of neutrophils, macrophages/monocytes and lesser numbers of lymphocytes and eosinophils (T1 immune/inflammatory response). Sold arrow in (B) identifies a mild perivascular inflammatory influx composed of a similar mixture of inflammatory cells. In (C) and (D), solid arrows identify a moderate to marked perivascular and peribronchiolar inflammatory cell influx composed predominantly of lymphocytes, plasma cells, and eosinophils, and lesser numbers of neutrophils (T2 immune/inflammatory response). In (D), there is also an alveolar infiltrate of an inflammatory cell mixture composed of eosinophils, neutrophils, macrophages/monocytes and lymphocytes. v: blood vessel; BA: bronchiolar airway; e: bronchiolar epithelium.

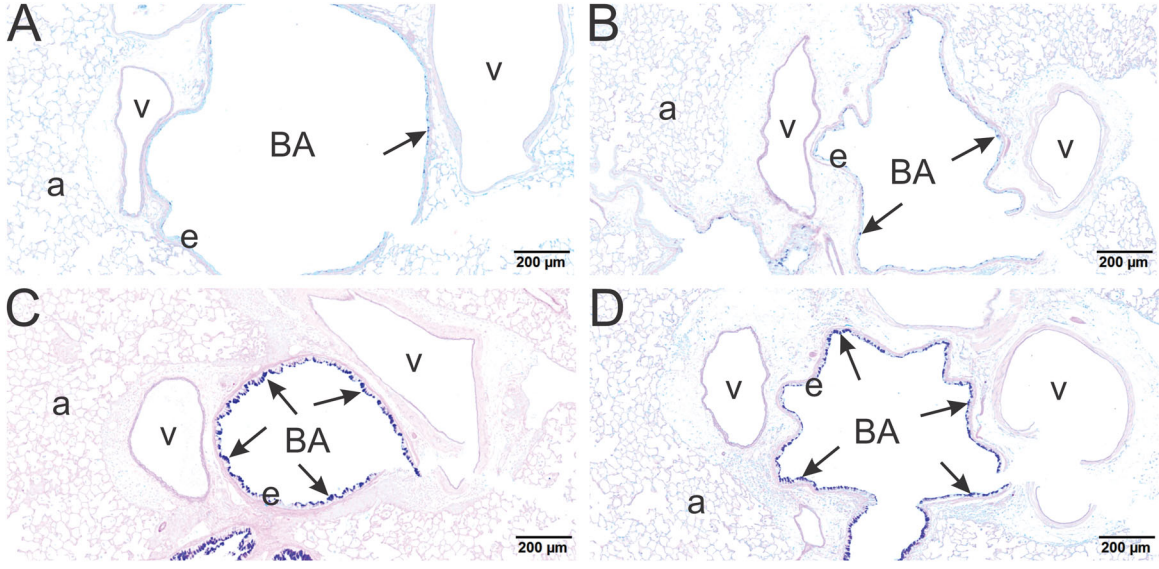

Figure 2. Light photomicrographs of large-diameter bronchiolar airway (BA; proximal axial airway in left lung lobe) from mice intranasally challenged with (A) saline (vehicle control; 0 µg BioPM), (B) saline and 9 µg of goat farm BioPM, (C) OVA (0 µg BioPM), or (D) OVA and 9 µg of goat farm BioPM. Tissues were histochemically stained with Alcian Blue (pH 2.4)/Periodic Acid Schiff (AB/PAS) for identification of mucosubstances (arrows; magenta chromagen) in airway epithelium (e). Scant amounts of AB/PAS-stained intraepithelial mucosubstances in saline-challenged mice (A and B). In contrast, large amounts of AB/PAS-stained intraepithelial mucosubstances in OVA challenged mice (C and D). v: blood vessel; BA: bronchiolar airway; a: alveolar parenchyma.

Allergen-induced airway epithelial remodeling (mucus cell metaplasia)

To confirm the effectiveness of OVA sensitization and challenge in inducing allergic asthma, proximal and distal bronchiolar airways of the left lung lobe were sampled and intraepithelial mucosubstances and eosinophils were quantified.

Morphometrically, the volume density of AB/PAS-stained mucosubstances storage in epithelia was significantly greater in OVA/OVA mice when compared to OVA/Sal mice. BioPM slightly enhanced the mucus production in the OVA/OVA mice, but this effect was not significant (Figure 3(A,B)). Lung tissues of OVA-challenged mice had a significant increase in expression of the mucin glycoprotein

5 AC (*Muc5ac*, 11.6-fold), mucin 5 B (*Muc5b*, 4.3-fold), mucin 4 (*Muc4*, 1.8-fold) and *Clca3a*1 (1.2-fold) genes compared to OVA/Sal mice (Table 2). Gene expression analysis of *Muc5ac*, *Muc4* and *Clca3a1* was 1.9, 1.3 and 1.4-fold overexpressed, respectively, in 3 µg BioPM/saline as compared to control mice (Table 2). All these genes are associated with airway mucus production and secretion (Nakanishi et al. 2001; Bonser and Erle 2017). Interestingly, however, no significant changes in expression of these genes were observed after BioPM treatment in OVA/OVA mice (Table 2).

No eosinophilic inflammation was observed in the lung tissue of OVA/Sal mice with or without BioPM (data not shown). OVA/OVA mice, however, had a marked eosinophilic and lymphocytic inflammatory infiltrate in the interstitium surrounding bronchiolar airways and arterial and venous blood vessels. Exposure of BioPM did not modify this specific OVA-driven pulmonary histopathology (Supplementary Figure 1).

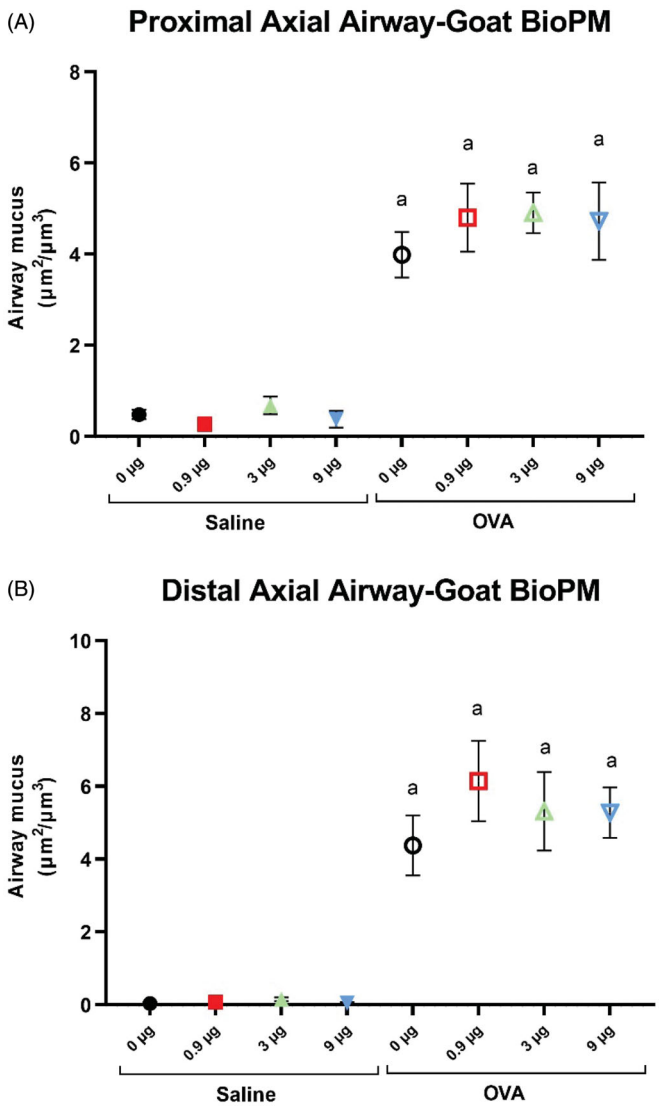

Figure 3. Airway epithelial mucus storage in Proximal (A), and in Distal (B) axial airway from mice intranasally challenged with saline + goat farm BioPM or OVA + goat farm BioPM. Increase in AB/PAS stained epithelium to indicate intraepithelial mucosubstances as a feature of allergic airway disease, was analyzed in proximal and distal bronchial airways of mice challenged with Saline or OVA, as indicated. Graph represents mean (SEM). 'a': significantly different from respective saline group ($p < 0.05$).

Airway inflammatory cell influx in BALF

To investigate whether BioPM could enhance the allergic airway inflammation, inflammatory cell numbers in the BALF were counted and differentiated.

As graphically illustrated in Figure 4, the number of neutrophils in the saline-challenged animals depended on the BioPM dose. However, BioPM exposure did not modify the numbers of macrophages, eosinophils or lymphocytes in saline-challenged mice except for a small increase in eosinophils in the mice exposed to 3 µg BioPM (Figure 4).

In the OVA-challenged animals, the enhancement of macrophages was significant at a dose of 3 or 9 µg BioPM. Although all doses of BioPM caused a 2-fold increase in eosinophils and lymphocytes, statistically significant

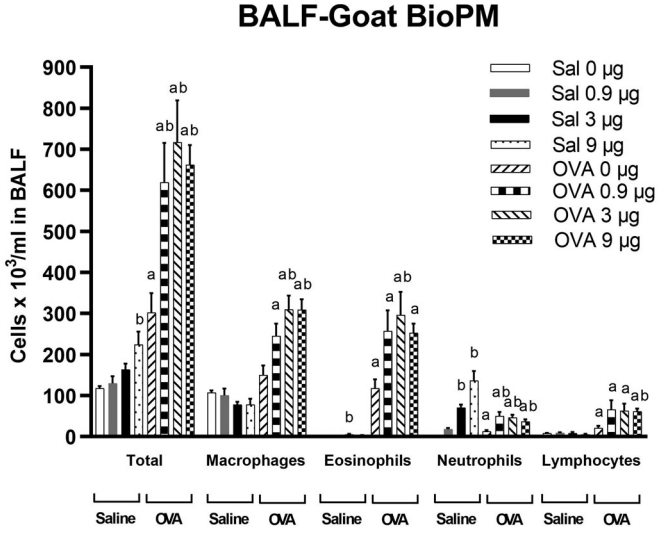

Figure 4. Differential macrophages, eosinophils, neutrophils and lymphocytes cell counts in BALF from mice intranasally challenged with saline + goat farm BioPM or OVA + goat farm BioPM. 'a': significantly different from respective saline group ($p < 0.05$); 'b': significantly different from respective 0 µg group ($p < 0.05$).

Table 2. Gene expression analysis of mucus related genes.

	OVA/OVA vs OVA/Sal			OVA/Sal 3 vs 0 µg			OVA/OVA 3 vs 0 µg		
Gene	log2 fold change	*p* Value	FDR	log2 fold change	*p* Value	FDR	log2 fold change	*p* Value	FDR
Muc5ac	3.53*	1.58E − 28	6.34E − 26	0.89*	2.20E − 05	8.33E − 04	−0.21	0.31	0.52
Muc5b	2.11*	1.25E − 17	1.06E − 15	0.38	0.07	0.18	−0.30	0.14	0.32
Muc4	0.86*	6.23E − 07	8.58E − 06	0.40*	4.70E − 04	6.63E − 03	−0.10	0.60	0.76
Clca3a1	1.15*	8.45E − 06	8.69E − 05	0.47*	0.024	0.09	−0.04	0.87	–

*Significantly changed ($p < 0.05$) compared to non-BioPM exposed mice; FDR, false discovery rate, '–': data is not available.
Note: Positive values represent up-regulation and negative values represent down-regulation.

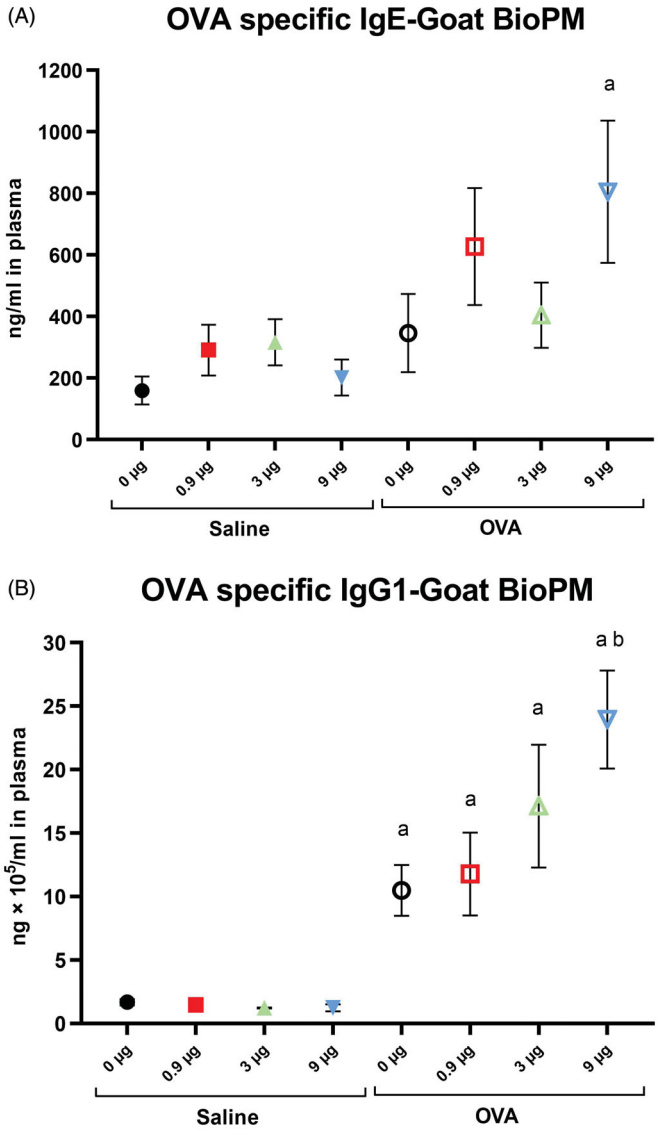

Figure 5. OVA specific IgE (A) and IgG1 (B) production from mice intranasally challenged with saline + goat farm BioPM or OVA + goat farm BioPM. The levels were analyzed by an ELISA. 'a': significantly different from respective saline group ($p < 0.05$); 'b': significantly different from respective 0 µg group ($p < 0.05$).

increases were only found with 3 µg BioPM for eosinophils and with 9 µg BioPM for lymphocytes (Figure 4). The number of neutrophils was significantly increased after BioPM exposure in the OVA group, this increase was less pronounced compared to the saline group (Figure 4).

To confirm the OVA-specific antibody responses, IgE and IgG1 levels specific for OVA were assayed in plasma.

OVA-specific IgE and IgG1 levels were markedly increased by OVA treatment. In OVA/Sal mice, BioPM slightly (not significant) enhanced the OVA-specific IgE levels and no increase in plasma levels of OVA-specific IgG1. In the OVA/OVA mice, a tendency for a BioPM dose-dependent response was observed for both OVA-specific IgE and IgG1 levels (Figure 5).

Soluble mediators in BALF were assayed to detect T helper (Th) 1 (IL-2, IL-28B and IL-27), Th2 (IL-17E/IL-25, IL-5, IL-13 and IL-31) and Th17 cytokines (IL-23, MIP-3a and IL-17A), IL-10. BioPM treatment had no effects on any Th1 cytokine or for IL-10 in saline and OVA-challenged mice groups (Table 3). There were also no effects on Th2 related cytokines or gene expression (Table 3), including IL-4, IL-5 and IL-13, detected in lungs of mice exposed to BioPM in both saline- and OVA-challenged.

BioPM induced a dose dependent increase in the production of the Th17 cytokine, MIP-3a, in saline-challenged mice. No effect on MIP-3a was seen in OVA-challenged mice. In OVA-challenged animals, BioPM increased IL-23, another Th17 cytokine (Figure 6).

Lung gene expression and pathway enrichment analysis

To investigate BioPM-mediated mechanisms/pathways on the development/worsening of allergic airway disease, gene expression analysis was performed in lung tissue. The complete list of DEGs altered by goat farm BioPM is reported in Supplementary Table 1. Mice treated with OVA without PM had significantly altered gene expression (2576 upregulated and 2397 downregulated genes) compared to the control mice. Specifically, 1662 (Saline), 1318 (OVA) genes were upregulated and 2277 (Saline), 1795 (OVA) genes were downregulated in lung tissues of mice that were treated with goat farm BioPM (Supplementary Table 2). The upregulated and the downregulated DEGs were uploaded in DAVID separately to look for enrichment of biological processes, resulting in 204 (Saline), 147 (OVA) and 316 (Saline), 303 (OVA) annotation clusters in upregulated and downregulated groups, respectively. OVA sensitization and challenge also caused 344 upregulated and 276 downregulated clusters (Supplementary Table 2). The top 20 annotation clusters and their associated genes are listed in Supplementary Table 3.

The upregulated genes related to 79 pathways of which 73 were significant (based on the Benjamini–Hochberg multiple testing correction) in saline-challenged mice, 64 pathways of which 52 were significant in OVA-challenged mice in top 10 annotation clusters. Representative pathways of interest were selected and are listed in Table 4. Based on the relationship with inflammatory or allergic responses, 'neutrophil chemotaxis' is the most apparent pathway induced by BioPM in saline-challenged mice, while 'oxidation-reduction process' is the most apparent pathway induced in OVA-challenged mice after BioPM treatment. The detailed gene expression of neutrophil and eosinophil related genes are presented in Tables 5 and 6. The downregulated genes identified 65 pathways of which 62 were significant in saline-challenged mice, compared to 75 pathways of which 71 were significant in OVA-challenged mice in top 10 annotation clusters. However, no downregulated pathways were found to be related to an inflammatory or allergic response (Supplementary Table 4).

Overall, the type of responses was not significantly different from those induced by the benchmark particles, i.e. diesel soot, only somewhat less strong per microgram PM compared to farm BioPM (data not shown).

Table 3. Gene expression in lungs and cytokine levels in BALF related to Th1, Th 2, and Th 17.

		Gene expression OVA/OVA vs OVA/Sal				Gene expression OVA/Sal				Gene expression OVA/OVA			
						3 vs 0 μg			Cytokine OVA/Sal	3 vs 0 μg			Cytokine OVA/OVA
Gene/protein	Function	log2 fold change	*p* Value	FDR	Cytokine OVA/Sal	log2 fold change	*p* Value	FDR	3 vs 0 μg	log2 fold change	*p* Value	FDR	3 vs 0 μg
IL-2	Th1 cytokine	0.14	0.66	NA	+	−0.03	0.84	NA	+	0.02	0.90	NA	+
IFNL3/IL-28B	Th1 cytokine	0.08	0.48	NA	+	NA	NA	NA	+	4.86E−03	0.93	NA	+
IL-12A/IL-12p70	Th1 cytokine	−0.1	0.53	0.69	ND	0.19	0.21	0.39	ND	−0.05	0.81	0.90	ND
IFN-γ	Th1 cytokine	0.89	0	NA	ND	0.42	0.05	NA	ND	−0.12	0.59	NA	ND
IL-27	Th1 cytokine	0.09	0.78	NA	+	−0.12	0.54	NA	+	−0.21	0.26	NA	+
IL-15	Th1 cytokine	−0.2	0.18	0.34	ND	0.15	0.30	0.48	ND	0.26	0.15	0.32	ND
IL-25	Th2 cytokine	−0.08	0.5	NA	ND	−0.02	0.74	NA	ND	NA	NA	NA	ND
IL-5	Th2 cytokine	1.16	0	NA	+	0.59	2.00E−03	NA	+	0.15	0.46	NA	+
IL-13	Th2 cytokine	2.1	2.05E−10	5.48E−09	ND	0.40	8.11E−03	NA	ND	0.11	0.61	0.77	ND
IL-31	Th2 cytokine	NA	NA	NA	+	NA	NA	NA	+	NA	NA	NA	+
IL-4	Th2 cytokine	1.06	0	NA	ND	0.41	0.05	NA	ND	0.61	5.82E−03	NA	ND
IL-33	Upstream Th2 instruction	0.71	5.50E−07	7.67E−06	ND	0.52*	3.31E−08	5.03E−06	ND	0.25	0.10	0.26	ND
CCL20/MIP-3	Th17 chemokine	1.15	1.50E−05	1.50E−04	↑	0.46*	0.02	0.09	↑	0.28	0.19	0.38	+
IL-17A	Th17 cytokine	2.06	2.54E−10	6.70E−09	+	0.14	0.21	NA	+	−0.11	0.60	NA	+
IL-17F	Th17 cytokine	0.75	6.00E−03	NA	ND	0.16	0.07	NA	ND	0.08	0.61	NA	ND
IL-23a/IL-23	Th17 cytokine	0	0.99	NA	+	0.13	0.37	NA	+	0.13	0.35	NA	+
IL-21	Th17 cytokine	2.18	1.41E−11	NA	ND	8.39E−03	0.94	NA	ND	−0.05	0.80	NA	ND
IL-22	Th17 cytokine	NA	NA	NA	ND	0.02	0.62	NA	ND	NA	NA	NA	ND
IL-6	Acute phase response cytokine	0.74	0.02	NA	ND	0.67	1.41E−06	NA	ND	0.05	0.83	NA	ND
IL-1β	Acute phase response cytokine	0.4	0.05	0.14	ND	0.63*	2.76E−05	9.60E−04	ND	0.43*	0.02	0.09	ND
Tnfrsf1a/TNF-α	Acute phase response cytokine	−0.31	0.98	0.99	ND	−0.31*	5.24 E−04	7.13E−03	ND	−0.33*	7.73E−07	1.58E−04	ND
Lta/TNF-β	Acute phase response cytokine	1.6	8.02E−11	2.30E−09	ND	0.30	0.14	0.30	ND	0.09	0.65	0.80	ND
CD40LG/CD40L	Acute phase response cytokine	0.5	0.03	0.09	ND	0.28	0.14	0.30	ND	−0.07	0.74	NA	ND
IL-10	T-reg cytokine	2.76	2.51E−19	2.83E−17	ND	0.20	0.18	NA	ND	0.04	0.86	NA	ND
Csf2/GM-CSF	Growth factor	0.46	0.02	0.05	ND	0.70*	1.58E−05	6.54 E−04	ND	0.17	0.37	0.57	ND

*Significantly changed ($p < 0.05$) compared to non-BioPM exposed mice; FDR: false discovery rate; ND: not detectable; NA: data is not available; +: detectable; ↑: enhancement after BioPM exposure.
Note: Positive values represent up-regulation and negative values represent down-regulation.

Discussion

The results of this study indicate that airway exposure to BioPM from a goat farm potentiated several airway inflammatory parameters in an experimental allergic mouse model, including significant increases of inflammatory cells, OVA-specific IgG1 levels, Th17 cytokine (MIP-3a and IL-23) production and airway mucus secretion-specific gene expression. RNAseq analysis indicates that neutrophil chemotaxis and oxidation-reduction process were the representative pathways in saline and OVA-challenged mice after BioPM treatment, respectively. These findings in animals suggest that exposure to BioPM has the potential to enhance the airway inflammatory responses.

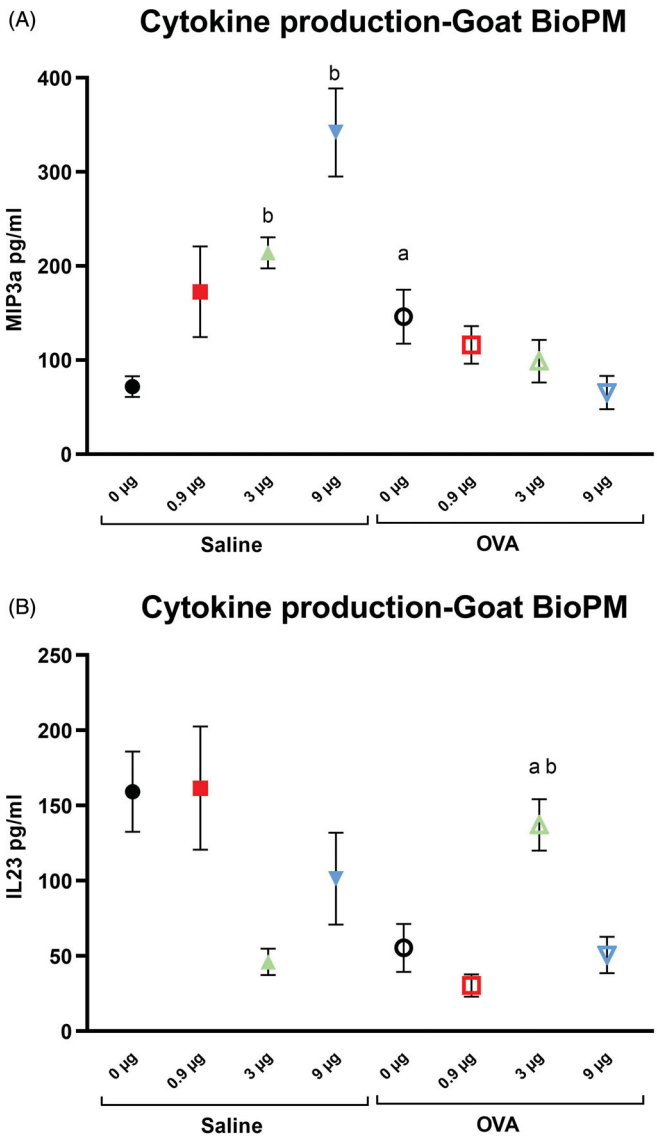

Figure 6. Production of BALF cytokines and chemokines from mice intranasally challenged with saline + goat farm BioPM or OVA + goat farm BioPM. MIP3a (A) and IL23 (B) were analyzed by a Luminex. 'a': significantly different from respective saline group ($p < 0.05$); 'b': significantly different from respective 0 μg group ($p < 0.05$).

In this study, the enhancement of OVA-specific IgE/IgG1 and eosinophil recruitment was observed in plasma and BALF, respectively, following farm BioPM challenge in allergic (OVA-challenged) animals. The presence of IgE and IgG1 antibodies has been shown to prime the vessel wall for eosinophil extravasation, driven by IL-4 (Corry et al. 1996). IL-13 was found to be necessary to initiate mucus thickening (Wills-Karp et al. 1998). Ambient fine PM has been associated with an acute increase of airway inflammatory cells, Th2 cytokines (IL-4, IL-13) production and IgE expression in an OVA mouse model (Liu et al. 2017). Nevertheless, in our study, neither *IL-4* nor *IL-13* gene expression was changed by goat farm BioPM. The enhancement of IgE and IgG1 might be due to the instillation of OVA, which is still present in the airways after 2 days and may penetrate through the protective epithelial barrier, which was damaged by high concentrations of BioPM.

It was found that gene expression of MIP-2b (CXCL3), eotaxin1/2 (CCL11/CCL24), monocyte chemotactic protein 5 (MCP-5, CCL12), human macrophage-derived chemokine (CCL22) and IL-33 were increased in mice treated with OVA + BioPM. These chemokines/cytokines can recruit and prime eosinophils and Th2 allergic response (Aghasafari et al. 2019). Despite our observation of eosinophil increases in BALF of mice treated with OVA + BioPM, no eosinophil related genes (except *MIP-2b*) were affected by BioPM, not even upstream Th2 steering genes, such as *IL-33*, *IL-25*, thymic stromal lymphopoietin (*TSLP*) and high mobility group box (*HMGB*) (Ullah et al. 2014). Moreover, the expression of TNF-α, a cytokine that contributes to the enhancement of allergic inflammation (Nakae et al. 2007), was lower after OVA + BioPM treatment. Repeated exposure to pig farm dust extract potentiated the production of eotaxin1 (CCL11), MCP-5 (CCL12) and TNF-α protein levels in mice when asthmatic symptoms were present, although the contents of this extract dust are not well

Table 4. Pathway enrichment analysis for all DEGs.

Pathways (upregulated)	OVA/Sal 3 vs 0 μg	OVA/OVA 3 vs 0 μg	Number of Genes	Enrichment Score	Adjusted *p* Value
Innate immune response	+		84	26.5	1.9E−14
Endoplasmic reticulum	+		183	13.1	2.0E−13
Protein folding	+		34	10.3	1.6 E−07
C-type lectin	+		34	9.7	6.5E−08
Carbohydrate binding	+		49	9.7	4.6E−08
Neutrophil chemotaxis	+		27	7.4	1.2E−09
Eosinophil chemotaxis	+		7	4.9	0.004
mitochondrion		+	272	40.3	5.9E−50
ribosome		+	58	20.0	4.2E−23
oxidation-reduction process		+	97	9.0	1.6E−12

Adjusted *p* value, corrected by Benjamini-Hochberg multiple testing correction; '+' means the terms were identified in the PM treatment group.

Table 5. Neutrophil related gene expression in lung tissue of OVA/Sal and OVA/OVA mice treated with BioPM.

	OVA/Sal 3 vs 0 μg			OVA/OVA 3 vs 0 μg		
Gene	log2 fold change	*p* Value	FDR	log2 fold change	*p* Value	FDR
CXCL1	1.57*	1.26E−18	1.80E−15	−2.11E−03	0.99	1.00
CCL3	1.42*	1.92E−13	1.52E−10	0.38	0.06	0.18
CCL2	1.30*	8.55E−10	2.49E−07	0.09	0.69	0.82
CXCL3	3.06*	1.03E−54	1.47E−50	0.60*	9.30E−04	0.01
CXCL2	1.45*	5.01E−12	2.88E−09	0.30	0.17	0.37
CCL9	1.27*	7.91E−14	6.65E−11	0.51*	0.02	0.08
CCL8	1.04*	8.52E−07	7.03E−05	0.25	0.21	0.41
CXCR2	0.49*	9.36 E−04	0.01	0.23	0.22	0.41
ITGB2	0.41*	0.01	0.04	0.26	0.16	0.34
CCL4	1.17*	9.61E−09	1.85E−06	5.11E−04	0.98	0.99
CCL7	1.51*	7.66E−13	5.47E−10	0.08	0.71	0.83
CCL6	0.61*	0.0035582	0.02	0.34	0.12	0.29
CCL24	0.69*	3.08 E−04	4.96E−03	0.31	0.16	0.34
CCL22	0.90*	7.86E−07	6.65E−05	0.15	0.41	0.61
CCL20	0.46*	0.02	0.09	0.28	0.19	0.38
CKLF	0.39*	1.95 E−03	0.02	0.42	0.02	0.08
IL1B	0.63*	2.76E−05	9.60E−04	0.43	0.02	0.09
FCER1G	0.56*	9.16E−04	0.01	0.34	0.08	0.22
AMICA1	0.24*	0.02	0.07	0.16	0.12	0.29
LGALS3	0.43*	0.01	0.05	0.51*	0.02	0.08
NCKAP1L	0.34*	3.74E−03	0.03	0.23	0.10	0.25
VAV1	0.31*	2.90 E−03	0.02	0.07	0.54	0.71
CCL17	1.19*	2.68E−10	9.82E−08	0.36	0.10	0.26
CCL11	1.01*	3.07E−07	3.13E−05	0.11	0.62	0.78
CCL12	1.28*	8.22E−12	4.20E−09	0.21	0.30	0.50
CXCL15	0.40*	1.60E−05	6.58E−04	0.18	0.17	0.36
TREM1	0.47*	6.37E−03	0.04	−0.06	0.79	0.88

*Significantly changed ($p < 0.05$) compared to non-BioPM exposed mice; FDR: false discovery rate.
Note: Positive values represent up-regulation and negative values represent down-regulation.

Table 6. Eosinophil related gene expression in lung tissue of OVA/Sal and OVA/OVA mice treated with BioPM.

	OVA/Sal 3 vs 0 μg			OVA/OVA 3 vs 0 μg		
Gene	log2 fold change	*p* Value	FDR	log2 fold change	*p* Value	FDR
CCL24	0.69*	3.08E−04	4.96E−03	0.31	0.16	0.34
CCL11	1.01*	3.07E−07	3.13E−05	0.11	0.62	0.78
CCL3	3.06*	1.03E−54	1.47E−50	0.60*	9.30E−04	0.01
CCL2	1.45*	5.01E−12	2.88E−09	0.30	0.18	0.37
LGALS3	0.43*	0.01	0.05	0.51*	0.02	0.08
CCR3	0.52*	4.27E−03	0.03	0.37	0.07	0.20
CCL7	1.51*	7.66E−13	5.47E−10	0.08	0.71	0.83

*Significantly changed ($p < 0.05$) compared to non-BioPM exposed mice; FDR: false discovery rate.
Note: Positive values represent up-regulation and negative values represent down-regulation.

characterized (Warren et al. 2019). A possible explanation for the effect of BioPM on only a part of the allergy parameters is that the OVA protocol produced such a robust response that BioPM co-treatment was unable to elicit additional inflammation. A sensitization and challenge protocol that induces with a less robust phenotype that is sensitive to BioPM-induced exacerbation can be considered in future studies.

Histopathologically, significant accumulations of lymphocytes were clearly recognized in OVA-challenged animals after BioPM exposure. In addition to Th2 cells that participate in allergic responses, group 2 innate lymphoid cells (ILC2) can also produce Th2 related cytokines and contribute to lung eosinophilic inflammation and mucus production in allergic responses (Moro et al. 2010). It has been shown that ILC2 cells represent more than half of the cells producing Th2 cytokines in the lung of OVA- and HDM-induced allergic mice (Wolterink et al. 2012). The role of ILCs in the effects of air pollutants on classic innate immune cells and lung ILCs are recognized (Estrella et al. 2019). In a study that examined the effect of ambient PM on lung ILC2 in an OVA-induced mouse model, *IL-5*, *IL-13* and *GATA3*, a transcription factors of ILC2 (Lund et al. 2013), were significantly increased (Lu et al. 2018). However, in our study, *GATA3* expression was significantly decreased by goat farm BioPM in allergic mice. Future studies may consider immunostaining *GATA3* to indicate ILC2 by using specific antibodies in lung tissues or collect lung tissues for RNAseq analysis at a series of time points after BioPM treatment.

Significant lung neutrophilic infiltration was noted in both non-allergic mice (saline-BioPM) mice and allergic mice (OVA + BioPM), while non-allergic mice responded with a twofold greater BALF neutrophil count than allergic mice. A recent study found that PM exposure during the sensitization phase in a house dust mite (HDM) allergy mouse model resulted in Th17-associated neutrophilic inflammation (Zhang et al. 2018). Neutrophil chemotaxis is also one of the most representative pathways identified by pathway enrichment analysis in the current study. In mice that were instilled with saline during challenge, the enhancement of neutrophil influx and the MIP-3a (CCL20) increase in BALF, both direct to a Th17 response by BioPM exposure, although the level of other Th17 cytokines (IL-17A and IL-17F) remained unchanged. Regarding the gene expression of Th17 cytokines (Jin and Dong 2013; Manni et al. 2014), the expression of *CXCL1*, MIP-2a (*CXCL2*), MCP-1 (*CCL2*) and MCP-3 (*CCL7*) increased, while the expression of *IL-*

17A, *IL-17F*, *IL-21* and *IL-22* remained unchanged. IL-17 also mediates the immune response via matrix metalloproteinase (*MMPs*) (Bullens et al. 2006), which may explain the elevated level of *MMP-3* we observed with BioPM. In sputum and BALF from severe asthmatic patients, high levels of IL-17 were detected (Molet et al. 2001) and IL-17 has been proposed to be involved in neutrophilic inflammation induction and. airway remodeling in severe asthma (Al-Ramli et al. 2009). Asthmatic patients with more severe forms have been reported to have neutrophilic inflammation and a mixed Th1 and Th17 cytokine profiles (Shaw et al. 2007; McKinley et al. 2008). Nonetheless, the neutrophilic response and Th17 cells were increased when Th2 cytokines were suppressed (Choy et al. 2015), indicating Th17 cells may be acting like Th1 to counter Th2 cells. The role of Th17 cells in allergic asthma still remains to be explored.

We established an enhanced neutrophilic Th17 and eosinophilic Th2 response after BioPM exposure in OVA/Sal and OVA/OVA mice, respectively. We determined other key pathways by using pathway enrichment analysis. Several pathways, such as the innate immune response and the C-type lectin pathway, were identified in non-allergic mice after BioPM treatment. Dendritic cells (DCs), playing a central role in linking the innate to the adaptive immune response, can recognize and present allergens to T cells and activate various phenotypes of asthma (Lambrecht and Hammad 2015). In the past decade, much progress has been made in understanding the role of DCs in the process of BioPM-induced allergic response. Ambient PM induced cytokine production by DCs required for Th2 skewing and a mixed Th1/Th2 response of cytokine production in an *in vivo* study (Williams et al. 2007). We have recently shown that the BioPM from goat farm activated Toll-like receptors and monocytic cells, indicating the involvement of innate immune receptors and cells (Liu et al. 2019). The interactions between these pathways could also be responsible for the observations made in the present study. For instance, allergens could trigger C-type lectin receptors such as dectin-2 on DCs, which could contribute to a mixed Th2 and Th17 response (Norimoto et al. 2014; Hadebe et al. 2018).

In this study, the oxidation reduction process and the mitochondrion pathway were identified in OVA + BioPM mice. It has been reported that antioxidants are effective in inhibiting the effect of diesel exhaust particles in an OVA-induced animal asthma model, supporting PM can be linked to reactive oxygen species generation (Whitekus et al. 2002). In our previous study (Groot, Liu et al. submitted for publication), peripheral blood mononuclear cells (PBMCs) that had been isolated from asthmatic patients and healthy people were exposed to BioPM from various animal farms. Upon exposure to pig farm BioPM, a small (but significant) increased level of oxidative stress, assessed by malondialdehyde, appeared in PBMCs that were isolated from asthmatic patients compared to healthy people. This enhancement was, however, not seen after exposure to goat farm BioPM. Both pig and goat farms BioPM-induced inflammatory events were abrogated by pretreatment with antioxidant in PBMCs from both healthy and asthmatic patients, suggestive of a mechanism (partly) related to intracellular oxidative stress. In future studies, Nrf2 deficient mice and their wild-type controls can be used to explore the role of oxidative stress in exacerbating allergic responses by BioPM.

Lung function measurements are used to evaluate effects on asthma (symptoms). A methacholine challenge test, which includes a Forced Expiratory Volume (FEV1) measurement to assess lung function, is a typical test used to help diagnose (the severity of) airway hypersensitivity, a hallmark of asthma (Leroyer et al. 1998). Lung function reduction has been attributed to effects on airway remodeling. Inflammation, increased IgE/IgG1 level, and mucus production are strongly associated with the development of airway hypersensitivity (Chesné et al. 2015; Sibilano et al. 2016). In our study, we did not evaluate the lung function of the animals. However, we did measure markers that are known to be strongly correlated with lung function (i.e. Th2 and Th17 related cytokines, eosinophil counts and pro-inflammatory cytokines such as TNF-α and IL-6) (Hamelmann et al. 1999).

The effects of BioPM derived from indoor chicken and pig farms modified allergic airway responses in a mouse model very similar to the model used in the present study (Liu et al. 2020). Variation in the responses between farms was observed and this may be linked to the different micro-organism compositions of the various farms. Contrasting the agricultural sources of BioPM that may affect the airway allergic responses may result in a better understanding of the relationship between farm type and health effects. Overall, our finding that acute exposure to goat farm BioPM showed a combined neutrophilic and eosinophilic inflammatory response in an OVA sensitization and challenge experimental animal model, supports the notion that animal industry BioPM exposure could be a potential factor to worsen allergic symptoms in occupational farmers and residents living nearby.

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Ethics approval

All animal procedures and experimental protocols were approved by the MSU Institutional Animal Care and Use Committee, MSU is an AAALAC accredited institution. Human participates and human data are not applicable.

Disclosure statement

The authors report no conflict of interest.

Availability of data

Data and materials are available upon request of the correspondence author (Rob J. Vandebriel, rob.vandebriel@rivm.nl).

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