



Livestock farm particulate matter enhances airway inflammation in mice with or without allergic airway disease

Dingyu Liu^{a,b}, James G. Wagner^c, Jack R. Harkema^c, Miriam E. Gerlofs-Nijland^a, Elena Pinelli^a, Gert Folkerts^d, Rob J. Vandebriel^a and Flemming R. Cassee^{a,b*}

ABSTRACT

Effects of airborne biological particulate matter (BioPM; from livestock farms) on the pulmonary airways are not well studied. The aim of the present study was to investigate whether fine (<2.5 μm) BioPM derived from indoor animal stables (two chicken and two pig farms) could modify airway allergic responses by using a mouse model of allergic airway disease (allergic asthma). After intraperitoneal ovalbumin (OVA) sensitization mice were either intranasally challenged with OVA (allergic mice) or saline (non-allergic controls). Mice were also intranasally treated with farm-derived BioPM. Bronchoalveolar lavage fluid (BALF), blood and lung tissues were collected one day after intranasal exposure. BioPM from all the farms caused an acute neutrophilic inflammatory response in non-allergic mice. In allergic mice, BioPM derived from pig farm 2 induced a larger cellular inflammatory response than other farm-derived BioPM. All farm BioPM elicited Th17 cytokine (Interleukin (IL)-23) production except chicken farm 2, whereas Th2 cytokine (IL-5) increase was only induced by BioPM collected from chicken farm 2. These results indicate the exposure of BioPM from chicken and pig farms may cause the enhancement of airway allergic response in mice following exposure to OVA. More variation in the responses between farms was observed in allergic than non-allergic mice. Understanding the source and doses of BioPM that may affect the airway allergic response could help susceptible individuals to avoid worsening their respiratory diseases.

Keywords: BioPM, Livestock, Allergic airway disease, Murine, Ovalbumin

INTRODUCTION

Air pollution is a major environmental health problem throughout the world. In particular,

inhalation of particulate matter (PM) has been associated with common respiratory diseases, such as asthma and allergic rhinitis.^{1,2} Livestock farming is an important source of emissions of PM, here referred to as BioPM, and comprised of a complex mixture of airborne biogenic particulates of mammalian, bacterial, fungal origins.^{3,4} BioPM that originates from livestock farm operations can absorb gases, odorous compounds, and microorganisms and components thereof. There is growing evidence that BioPM that is produced during agricultural activities is a possible factor for worsening airway function in farmers⁵ and

^aNational Institute for Public Health and the Environment, Bilthoven, 3720 BA, the Netherlands

*Corresponding author. National Institute for Public Health and the Environment, Postbus 1, Bilthoven, 3720 BA, Utrecht, the Netherlands. E-mail: flemming.cassee@rivm.nl

Full list of author information is available at the end of the article

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people living in an area with a high livestock density.^{3,6}

Asthma, characterized by bronchial hyper-responsiveness, inflammation, and airflow obstruction, is a heterogeneous disease with multiple phenotypes. Allergic asthma, which is related to type 2 helper T cells (Th2)-dependent airway inflammation and immunity, is considered one of the most common asthma phenotypes. Pathologically, allergic airway disease is characterized by a mixed inflammatory cell influx, consisting of mainly eosinophils, lymphocytes, and plasma cells and a lesser number of neutrophils, and concomitant with conspicuous amounts of Th2 cytokines (such as Interleukin [IL]-4, IL-5, IL-13). This is the principal pathway for asthma progression or worsening.^{7,8} Severe asthmatic patients with high Th2 activity can be controlled by Th2 targeted therapies.⁹ Ambient fine PM exposure is associated with an acute increase of airway inflammatory cells, increased Th2 cytokines production, and upregulation of Immunoglobulin (Ig)E, which together indicates that naïve T cells are shifted toward a Th2 phenotype by ambient PM exposure.¹⁰ Besides the Th2 pathway, the Th17 pathway, involving activation of Th17 cells and production of Th17 cytokines (IL-17, IL-23), has recently received attention in asthma pathology. Contributions from both Th1 and Th17 cells mediate airway inflammatory responses after exposure of mice to BioPM derived from cattle and pig farms.^{11,12} The polarization of T cells and prevalence of asthma are variable, which may be caused by microorganisms that originate from different farm microenvironments. A large variety of Gram positive and negative bacteria and fungi is present in livestock farms,¹³ but little is known about the mechanism by which livestock BioPM might affect the allergic response in case of pre-existing asthma. Thus, it is important for the development of improved preventative strategies to determine the effects of animal farming on allergic respiratory disease.

In the present study, indoor airborne BioPM was collected from four livestock farms (two chicken and two pig farms) located in the Netherlands. Mice sensitized with ovalbumin (OVA) were intranasally treated with various doses of BioPM in the absence (non-allergic mice) and presence of intranasal OVA exposure (allergic mice), a

common experimental murine model of asthma-like disease. We hypothesized that BioPM derived from farms exacerbates allergic airway response. In addition, we hypothesized that the type of livestock exposure significantly affects the allergic response.

MATERIALS AND METHODS

BioPM sampling period, sites and procedure

Indoor ambient fine (<2.5 µm, Mass Medium Aerodynamic Diameter (MMAD)) BioPM were collected at four farms during July 2016 to July 2017, two chicken farms (Chicken 1 [breeding farm], Chicken 2 [egg-laying farm]) and two pig farms (Fig 1 [commercial farm], Fig 2 [university test farm]), all located in the central region of the Netherlands. Per site, sampling was carried out for 2–6 days and for 6 h per day (between 09:00–16:00 h). Daily collection of BioPM were pooled to ensure sufficient material was available to carry out the *in vivo* studies. All BioPM were collected in demi water using the Versatile Aerosol Concentration Enrichment System (VACES) that has been previously described.¹⁴ The BioPM is put immediately in water to avoid artifacts known for sampling on filters.¹⁵ The BioPM concentration of the collected samples ranged from 0.15 mg/ml (Fig 2) to 3.78 mg/ml (Chicken 2). To enable testing equal BioPM mass concentrations, BioPM samples were diluted to 30, 100 and 300 µg/ml in sterile water before administration. Characteristic features of the collected livestock BioPM for each site and detailed description of the sampling dates and procedures during the sampling campaign are described elsewhere.¹³

Experimental protocol

Female BALB/c mice, 6–8 weeks old, were obtained from Charles River Laboratories (Portage, MI) and randomly assigned to a treatment group of 6. Husbandry conditions were maintained at the Michigan State University (MSU) animal housing facilities at room temperature of 21°C–24 °C and relative humidity of 45–70%, with a 12 h light/dark cycle starting at 7:30 a.m.

Pilot studies were conducted to assess how varying doses of BioPM affected changes in airway inflammation in non-allergic mice. Based on the results of these pilot studies (data not shown),

doses of 0.9, 3 and 9 μg as appropriate dose range of BioPM. All subsequent experiments were conducted as follows: On Day 0, all mice were intraperitoneally injected with 0.25 ml saline containing 20 μg OVA (Sigma-Aldrich) with 1 mg alum (aluminum potassium sulfate, Sigma-Aldrich) ($n = 6$ animals/group). On Day 10, all mice were boosted with an intraperitoneal injection with 20 μg OVA in 0.25 ml saline and intranasally instilled with 30 μl 0.5% OVA in saline. On Days 17 and 18, OVA-sensitized mice were challenged intranasally with/without 30 μl of 0.5% OVA in saline or saline alone (vehicle control). Two days later (Day 20), mice were intranasally treated with 0, 0.9, 3, or 9 μg (if available) BioPM derived from various farms 24 h prior to being sacrificed on Day 21 to assess the effects of BioPM exposure (Fig. 1).

Intranasal instillation, necropsy, lavage collection, and tissue preparation

Mice were euthanized with an intraperitoneal injection of sodium pentobarbital, the abdomen and thorax were opened, and blood was drawn from the aorta and collected in heparinized tubes (BD Microtainer, Franklin Lakes, NJ) for isolation of plasma, and then mice were euthanized by exsanguination. 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.

Bronchoalveolar lavage fluid (BALF) was recovered using 2×0.8 ml sterile saline. Only BALF with high recovery rates were included, which occurred in all but 1 out of 144 animals. In the remaining 1 case, accidental damage to the lung before or during flushing occurred.

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.¹⁶ The details are described in the supporting information (Lung Morphometry). Briefly, tissue blocks were 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 the pulmonary bronchiolar epithelium. Other lung tissue sections were evaluated immunohistochemically using a polyclonal rabbit antibody directed against murine eosinophil-specific major basic protein (MBP; 1:500; Mayo Clinic, AZ) for histologic detection of MBP-laden eosinophils. All lung tissue sections were examined by a board-certified veterinary pathologist for exposure-related histopathology. The incidence and severity of pulmonary lesions were semi-quantitatively scored for pulmonary inflammation (bronchiolitis/alveolitis) and airway epithelial remodeling (mucous cell metaplasia). Severity scores for the lung histopathology were based on the following criteria: (0) no significant findings; (1) minimal, less than 10% of the lung section affected; (2) mild, 10% to less than 25% lung affected; (3) moderate, greater than 25%

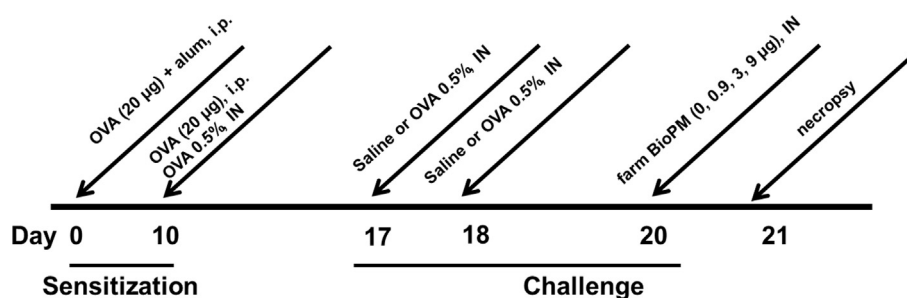


Fig. 1 Experimental protocol. Study design. Mice were intraperitoneally sensitized with 20 μg OVA with alum on day 0, followed by intraperitoneal administration of 20 μg OVA and intranasal administration of 0.5% OVA on day 10. On days 17 and 18, OVA-sensitized mice were challenged intranasally with 0.5% OVA or saline alone. BioPM derived from various farms were administered intranasally with doses of 0, 0.9, 3, or 9 μg on day 20. All animals were sacrificed 24 h after the last intranasal challenge

but less than 50% of lung affected; (4) marked, greater than 50% but less than 75% of the lung affected; (5) severe, greater than 75% of the lung affected.

BALF cytometry and Luminex analyses for inflammatory cytokines

The total number of cells in BALF was estimated 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 neutrophils, eosinophils, macrophages/monocytes, 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 and the supernatant fraction was collected and stored at -80 °C for cytokine analysis. Cell-free BALF was assayed for the inflammatory cytokines IL-17E/IL-25, GM-CSF, IFN- γ , macrophage inflammatory protein-3 (MIP-3), IL-1 β , 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 used when the following criteria were met: at least 3 out of 6 animals per group showed cytokine levels >10 pg/ml.

ELISA OVA- IgE/IgG1

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, details can be found in Vandebriel et al.¹⁷ 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 (HRP) was added and followed by incubation with reaction substrate. Optical density was read at 650 and 450 nm wave length. For OVA-specific IgE, plasma from all mice was diluted 1:20. For OVA-specific IgG1, plasma from the saline-challenged mice was diluted 1:1000, and plasma

from the OVA-challenged mice was diluted 1:16000.

Statistics

The statistical analysis was performed using R statistical software (version 3.6.0). Descriptive statistics (mean, standard deviation) were computed using GraphPad Prism (version 8.0.2). Outlier test (Grubbs) was performed and recognized outliers were removed from the analysis. For the continuous normal datasets, a one-way ANOVA analysis 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 was 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.

A large class of dose-response models was used for describing the change in any continuous or quantal endpoint as a function of BioPM dose and saline/OVA treatment. A member from this family was selected using a likelihood-ratio test for depicting the best model fit.¹⁸ Data from the 2 chicken farms were pooled to increase sample size; the same was done for the 2 pig farms. The resulting exponential or log-logistic models were further used to compute the benchmark dose, stratified per treatment level.

The benchmark response (BMR) was based on expert judgement by choosing a predetermined change in response compared to non-BioPM treated groups for each endpoint. The counts of BALF inflammatory cells (macrophages, eosinophils, neutrophils, lymphocytes) were analyzed as quantal data as only a limited number of cells (200 cells) was counted.¹⁹ No defaults are available for this type of response; therefore, based on expert judgment, the BMR was chosen to 20%.¹⁹ For the continuous endpoints (OVA specific IgE/IgG1 and BALF cytokine), a 100% change was chosen.²⁰ A 50% change in response was chosen for mucous secretion and tissue eosinophils in airway G5/G11.

RESULTS

Pulmonary histopathology

BioPM-induced pulmonary histopathology in mice intranasally challenged with saline (no OVA) were similar in character whether they were intranasally instilled with BioPM from either chicken or pig farms. These lung lesions 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 [G5]) with lesser involvement of the more distal lung lobe tissue section (at the level of axial airway generation 11 [G11]). Control mice that received only intranasal instillations of saline (0 μg BioPM) (Fig. 2 A) had no significant pulmonary histopathology.

This 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 non-allergic response). Large and small diameter bronchioles were affected as well as some 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 there was some mucous cell metaplasia (secretory cells with AB/PAS-stained mucosubstances not normally found in bronchioles of

mice) in the luminal airway epithelium, but of minimal severity.

In the affected alveolar regions of these BioPM-instilled mice, there were small widely scattered focal accumulations of predominantly neutrophils and alveolar macrophages/monocytes. In general, the severity of the bronchiolitis/alveolitis was slightly greater in mice receiving the higher amounts of BioPM. This was most apparent in mice receiving 3 μg (figure not shown) or 9 μg (Fig. 2 B) BioPM from Chicken 1, and for 9 μg from Chicken 2 (figure not shown) and 9 μg from Pig 1 (Fig. 2 C).

In contrast to the mice that received saline, mice 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 (Fig. 2 D). This allergic inflammatory response was of a much greater severity (moderate to marked) than the minimal type 1 inflammation found in the mice that received saline.

In general, the pulmonary histopathology of OVA challenged mice that were also intranasally instilled with BioPM, from either a pig or chicken farm, was not of greater severity than that of OVA challenged mice that received only intranasal saline (controls). Mice, however, that were instilled with 9 μg of BioPM from Pig 1 (Fig. 2 F), did exhibit slightly more lung histopathology (based on semi-quantitative scores [S Fig. 1 C]) than the OVA

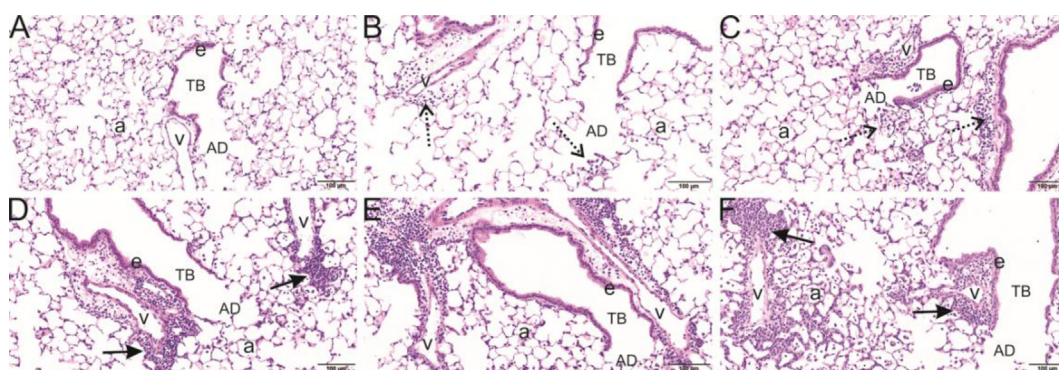


Fig. 2 Pulmonary histopathology. Light photomicrographs of lung tissue sections from mice intranasally challenged with (A) saline (vehicle control; 0 μg BioPM), (B) saline and 9 μg of Chicken 1 BioPM, (C) saline and 9 μg of Pig 1 BioPM, (D) OVA (0 μg BioPM), (E) OVA and 9 μg of Chicken 1 BioPM, or (F) OVA and 9 μg of Pig 1 BioPM. Notes: Stippled arrows, minimal to mild mixed inflammatory cell influx composed mainly of neutrophils, monocytes and lesser numbers of lymphocytes and eosinophils (T1 immune response); Solid arrows, moderate to marked inflammatory cell influx, mainly perivascular and peribronchiolar, composed predominantly of lymphocytes, plasma cells, and eosinophils, and lesser numbers of neutrophils (T2 immune response); v, blood vessel; a, alveolar parenchyma; TB, terminal bronchiole, AD, alveolar duct; e, bronchiolar epithelium.

challenged, saline-instilled control mice (0 µg BioPM) (Fig. 2 D). Morphometric determination of the pulmonary densities of AB/PAS-stained mucosubstances in bronchiolar epithelium (S Fig. 2A-D) or major basic protein-laden eosinophils (S Fig. 3A and B) did not show significant difference among the OVA-challenged mice except an increase in the perivascular/peribronchial area elicited by Pig 2 BioPM (S Fig. 3 B).

Inflammatory cells in BALF

Compared to saline treated control animals, mice that were challenged only with OVA and no BioPM had a significant increase in BALF total cells ($1.1 \times 10^5 \pm 2.2 \times 10^4$ versus $3.1 \times 10^5 \pm 1.5 \times 10^5$ in BALF of non-allergic and allergic mice, respectively). This increase in total cells was due to the dramatic increase in eosinophils and neutrophils.

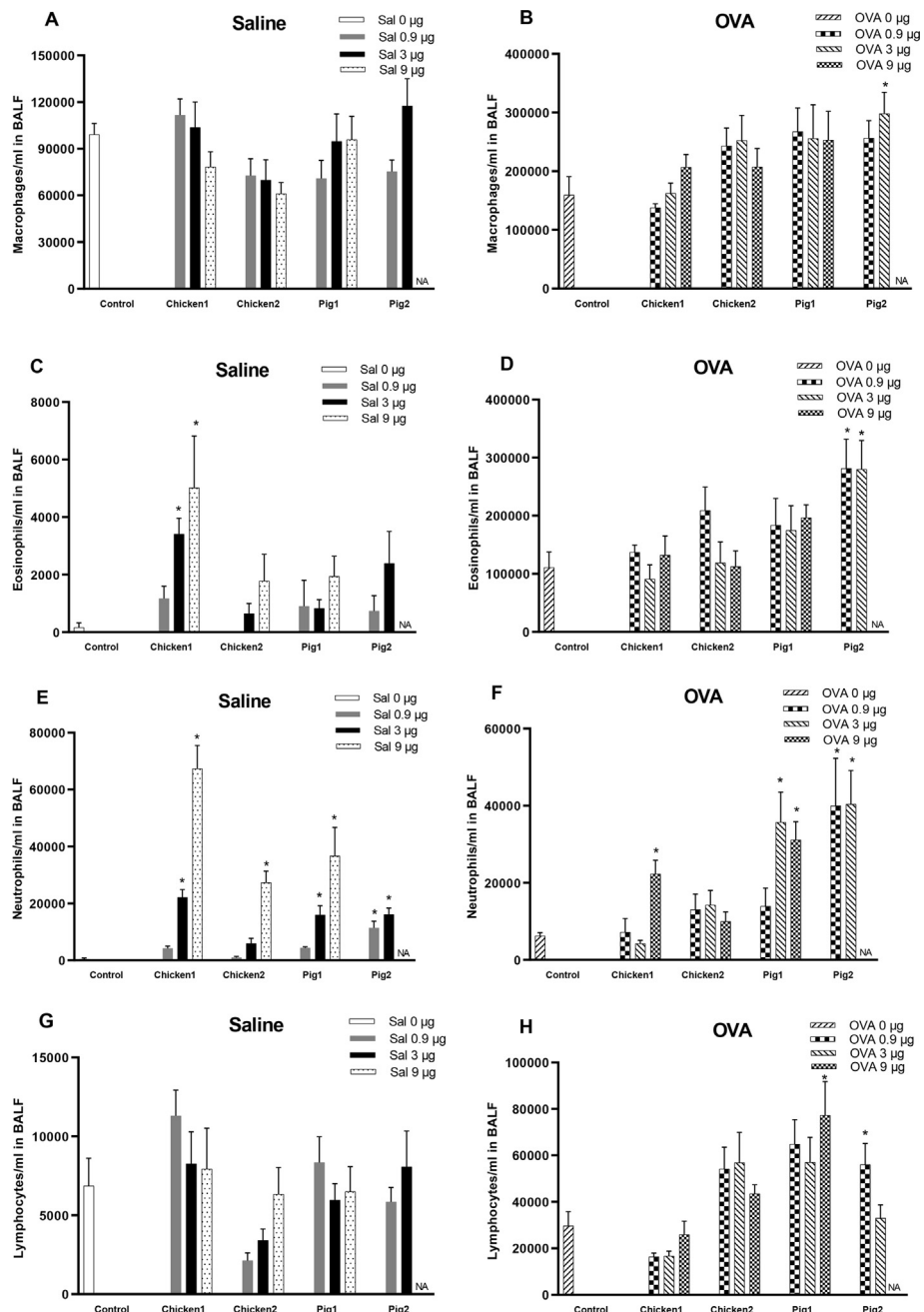


Fig. 3 Inflammatory cells in BALF. Macrophages (Sal (A), OVA (B)), eosinophils (Sal (C), OVA (D)), neutrophils (Sal (E), OVA (F)) and lymphocytes (Sal (G), OVA (H)) cell counts in BALF. Note: Samples of 9 µg BioPM from Pig 2 were absent (NA: data not available) due to low BioPM concentration. Graphs represent mean (SEM). * significantly different from respective 0 µg group (p < 0.05)

In saline-challenged non-allergic mice, all BioPM elicited a significant dose-dependent increase in BALF neutrophils (Fig. 3 E), which is suggestive of an acute non-allergic T1 inflammatory response. Dose-response analysis using the benchmark dose (BMD) suggests that the percentage neutrophils is a very sensitive parameter that is influenced by exposure of livestock farm BioPM (S Fig. 5H and I). A tendency of increase in eosinophils, though minimal compared to the neutrophil response, was also observed after administration of BioPM collected from all farms, but was greatest in BioPM from Chicken 1 (Fig. 3 C). Exposure to BioPM did not alter other BALF macrophages (Fig. 3 A) or lymphocytes (Fig. 3 G).

In OVA-challenged allergic animals, Pig 2 BioPM elicited a relatively larger increase in BALF eosinophils compared to that elicited by the other pig farm BioPM, which induced changes similar to that induced by BioPM from both chicken farms (Fig. 3 D). In addition, BioPM from Pig 2 induced a significant increase in macrophages (Fig. 3 B), neutrophils (Fig. 3 F) and lymphocytes (Fig. 3 H). Treatment of OVA-challenged mice with BioPM from either of the chicken farms failed to alter the BALF inflammatory cell numbers with the exception of neutrophils (Fig. 3 F).

OVA-specific immunoglobulin levels in blood plasma

In saline-challenged non-allergic mice, BioPM from Chicken 1 resulted in a dose-dependent increase in OVA-specific IgE production (Fig. 4 A), a characteristic feature of allergic immune responses to antigens. A tendency of OVA-specific IgG1 enhancement, though not statistically significant, was observed in saline-challenged non-allergic

mice that were exposed to Chicken 1 BioPM (S Fig. 4 A).

In the OVA-challenged mice, serum OVA-specific IgE levels were significantly elevated in 0.9 μ g BioPM from Chicken 1 compared to non-PM exposure mice (Fig. 4 B). No OVA-specific IgG1 changes were induced by BioPM from all farms (S Fig. 4 B).

Cytokine and chemokine expression in BALF

In saline-challenged non-allergic mice, Th1 (IL-2, IL-28B and IL-27), Th2 (IL-17E/IL-25, IL-5, IL-13 and IL-31) and Th17 associated cytokines/chemokines (IL-23, MIP-3a and IL-17A) were detectable, while IL-10 was also detected (S Tab 1 A).

No BioPM treatment related effects were found for Th1 cytokines and IL-10 (S Tab 1 A). BioPM from Chicken 1 and Pig 1 elicited a dose-dependent increase in Th17 cytokine (MIP-3a) production (Fig. 5 A) in saline-challenged mice. BioPM from Pig 2 increased IL-23 production (Fig. 5 E). No other significant changes by BioPM were observed.

Treatment of OVA-challenged mice resulted in the same profile as measured for the saline-administered group, since no BioPM treatment related effects were found for Th1 cytokines and IL-10 (S Tab 1 B).

IL-23 is increased by BioPM from Chicken 1 and Pig 2, whereas no significant changes were observed by BioPM from the other chicken or pig farm (Fig. 5 F). The Chicken 2 BioPM induced IL-5 increase was found (Fig. 5 D), which indicates a Th2 response could be involved in BioPM-induced airway allergic response. In contrast to the enhancement of MIP-3a in saline-challenged

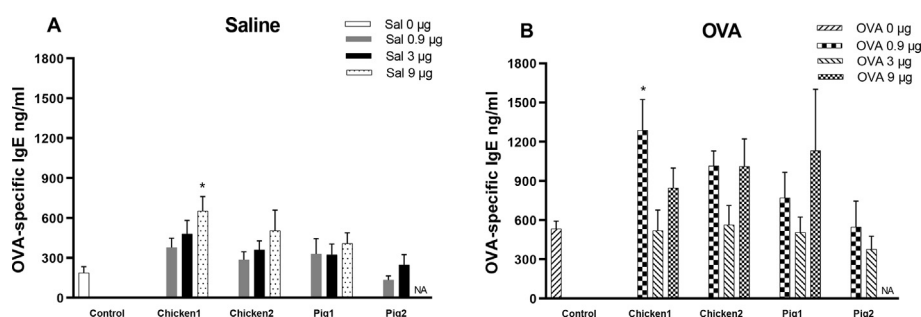


Fig. 4 OVA-specific Immunoglobulin levels in blood plasma. OVA specific IgE production (Sal (A), OVA (B)) in plasma. Note: Samples of 9 μ g BioPM from Pig 2 were absent (NA: data not available) due to low BioPM concentration. Graph represents mean (SEM). * significantly different from respective saline group ($p < 0.05$)

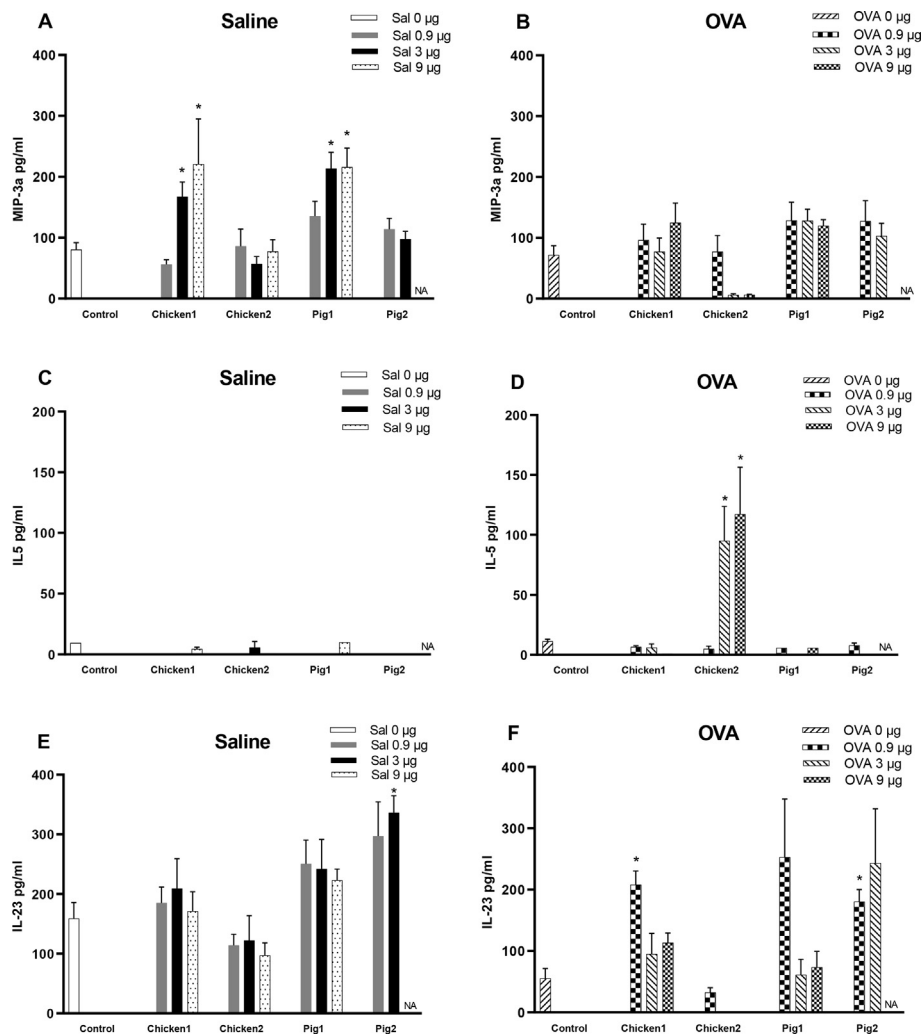


Fig. 5 Cytokine and chemokine expression in BALF. Production of cytokines and chemokines in BALF. MIP-3a (Sal (A), OVA (B)), IL-5 (Sal (C), OVA (D)) and IL-23 (Sal (E), OVA (F)) were analyzed by a Luminex. Note: Samples of 9 µg BioPM from Pig 2 were absent (NA: data not available) due to low BioPM concentration. Graphs represent mean (SEM). * significantly different from respective saline group ($p < 0.05$)

mice, BioPM did not alter the production of this chemokine in OVA mice.

DISCUSSION

Exposure to livestock farm BioPM has been shown to have a range of effects to promote or inhibit different facets of allergic respiratory diseases.^{21,22} The results of this animal study indicate that exposure to livestock BioPM elicited airway inflammatory and epithelial cell changes in both non-allergic and allergic airways that differed by the type of immune response (Th2 vs Th17). Furthermore, we observed more variation in the responses between farms with regard to inflammatory cells and cytokine/chemokine production in allergic airways, compared to saline challenged, non-allergic airways.

In non-allergic mice treated with BioPM, without OVA challenge, a mild inflammatory response was induced as indicated by peribronchiolar mixed inflammatory cells, a dose-dependent neutrophil recruitment, eosinophil influx, increased MIP-3a production and enhancement of OVA-specific IgE. A similar finding, dust collected from a pig confinement facility inducing airway inflammation dominated by neutrophils, was made by Mc Govern et al.²³ These authors showed that pig BioPM driven airway hyperresponsiveness may be dependent on oxidative stress and be mediated by neutrophils, as was shown by treating the mice with antioxidant and neutrophil-depleting antibody, respectively. Healthy volunteers exposed to pig farm BioPM showed pulmonary inflammation that was characterized by an increase in several inflammatory cell populations,

with neutrophils being dramatically increased in BALF or nasal lavage fluid.^{24,25} It is widely accepted that neutrophil influx, which may result in an acute lung inflammation, is largely mediated by Toll like receptor 4 (TLR4) signaling.^{26,27} Lipopolysaccharide (LPS), a ligand for TLR4, has been reported to induce neutrophil recruitment in a similar animal model as used in the present study.²⁸ Airborne LPS commonly found in agricultural aerosols and PM is associated with increased airway inflammation in occupational lung disease.²⁹ BioPM from the four farms used in this study contain mainly ligands for TLR4 and the activation was blocked by using a TLR4 antagonist in monocytic cells.¹³ The average concentration of LPS from the two pig farms was about 24 times higher than that in chicken farms (S Tab 2) at the same BioPM concentration. Nevertheless, our *in vivo* results show that the effect of pig farms BioPM is slightly higher than chicken farms BioPM in neutrophil influx expressed per unit mass, which indicates that in the present study LPS is not (or only partially) responsible for the neutrophil increase induced by livestock BioPM in saline-challenged mice. This notion is supported by data from a study in which the effect of heated (inactivating biological material) PM and non-heated PM collected from ambient air was studied in healthy volunteers. Both heated and non-heated PM induced airway neutrophil influx regardless of whether LPS is active in PM.³⁰ It may also be that a relative small amount of LPS is sufficient to induce neutrophil influx and a 24-fold higher dose only induces a small increase in the cellular response.

Many rodent PM studies describe airway neutrophilic inflammation that is accompanied by pro-inflammatory cytokine release in BALF. The abundant MIP-3a amount induced by all farm BioPM used in the present study may play a role in neutrophil influx as MIP-3a is known to attract neutrophils.³¹ In non-allergic mice exposed to BioPM, the dose-dependent response in neutrophils is consistent with the tendency of an increase in MIP-3a, a Th17 chemokine, after exposure to pig farms BioPM. Chemotaxis of Th17 cells was largely suppressed by anti-MIP-3a antibody in the supernatants from activated human neutrophil, indicating a potential crosstalk

between Th17 cells and neutrophils recruitment and activation.³²

Our OVA sensitization and challenge protocol resulted in conspicuous allergic airway symptoms. The effectiveness of allergy induction in the present model was histologically confirmed by the extensive AB/PAS-stained mucosubstances in the bronchiolar epithelium and MBP-stained eosinophils counts in the lung tissues. Exposure to pig farm BioPM two days after the OVA challenge enhanced airway inflammation as indicated by histopathology and inflammatory cell and cytokine accumulation in BALF. Similar findings of enhancement of lung inflammation by inhalation of dust extracts collected from a pig farm have been reported in an OVA allergic mouse model.³³ However, dust collected from dairy/goat stables³⁴ and certain bacteria isolated from animal farms^{35,36} have shown contradictory results with protective effects in the experimental allergic model. This protective effect is supportive of the hygiene hypothesis, which is that early life farming exposure may reduce the development of allergic asthma in children.³⁷ These contradictory results can be explained by distinct microbial compositions of BioPM that are released from different farms and farming practices. For example, despite the genetic similarity, the prevalence of asthma was low in Amish children compared to Hutterites probably due to their microbial variety.³⁸ The protective effects of dust extracts from Amish farms was abrogated in MyD88 and Trif knock-out mice, which indicates innate immune signaling is critical. The differences in microbial composition that were observed in dust samples from their homes might provide a possible explanation. As we mentioned previously, the allergic inflammatory response and underlying immune mechanisms maybe altered following specific microorganism exposure or the interaction between multiple microorganisms.

We suggest that Th17 (IL-23) and Th2 (IL-5) responses could be involved in Chicken 1 and Chicken 2 BioPM-induced exacerbation of the allergic response respectively. We have recently described the application of a 16S amplicon sequencing to characterize the bacterial components in these livestock farms, which demonstrated that the bacterial profiles differ between the two

chicken farms.¹³ IL-5 could drive eosinophilia in lung tissue, which supports the development of eosinophils in bone marrow.³⁹ However, the eosinophils density (both in parenchyma and perivascular) was not altered by BioPM from Chicken 2 in allergic mice. When comparing the two pig farms, a significant eosinophil increase and a mixed Th2 (IL-31)/Th17 (IL-23) response were elicited by BioPM from Pig farm 2 only. This may be in line with the observation that eosinophils contribute not only as inflammatory cells, but also by producing IL-31, driving Th2 polarization, leading to the symptoms of allergic asthma.^{40,41} The different responses between the two pig farms could be associated with a high variation in their fungal communities (mycobiome profiles) in the two pig farms, as was shown in our previous study using Internal Transcribed Spacer (ITS) sequencing.¹³ Further studies could consider isolating specific microorganism to identify which components in ambient air of farms would contribute to allergic responses.

The results of our study indicate that livestock farm-derived BioPM contain substances that are capable of priming the development and exacerbation of allergic response in an experimental murine allergy model. Variations in responses/mechanisms observed in PM from different livestock microenvironments are possibly based on the microbial or fungal diversity. Identifying the relevant microorganisms in farm BioPM might be considered in future studies that aim to quantify the health risks for people living close to or working on specific farms. In the current study, we have applied only a single and relatively high dose of BioPM to study the acute response, whereas the people living nearby farms are probably exposed to lower concentrations but over a larger period (resulting in a similar or comparable accumulative dose). Therefore, further studies are needed to investigate the potential relevance to human exposure.

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Potential competing interests

The authors report no competing interests.

Ethics statement

All animal procedures and experimental protocols were approved by the MSU Institutional Animal Care and Use Committee, MSU is an AAALAC accredited institution.

Author contributions

All authors contributed to design this study, read and approved the final version of manuscript. DL contributed to carried out partial experiments, analyzed data, prepared figures, prepared the manuscript and edited the manuscript. JGW carried out the conduction of animal experiments and edited the manuscript. JRH contributed to the lung histopathology, captured the images and prepared the manuscript. EP, GF, RJV and FRC contributed to edit the manuscript.

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Appendix A. Supplementary data

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Author details

^aNational Institute for Public Health and the Environment, Bilthoven, 3720 BA, the Netherlands. ^bInstitute for Risk Assessment Sciences, Utrecht University, Utrecht, 3508 TC, the Netherlands. ^cPathobiology and Diagnostic Investigation, College of Veterinary Medicine, Michigan State University, East Lansing, MI 48824, USA. ^dDepartment of Pharmacology and Pathophysiology, Utrecht Institute for Pharmaceutical Sciences, Utrecht University, Utrecht, 3508 TC, the Netherlands.

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