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Microorganisms and their components present in livestock ambient air in relation to respiratory symptoms

De aanwezigheid van micro-organismen en hun componenten in de omgevingslucht van vee in relatie tot luchtwegklachten

(met een samenvatting in het Nederlands)

Proefschrift

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Chapter 1

General Introduction

Background

Exposure to airborne particulate matter (PM) has been suggested to have various adverse health impacts. It ranks fourth among all risks accounting for about 4.2 million deaths worldwide [1]. The reported effects of PM exposure are heterogeneous, involving allergic as well as non-allergic respiratory symptoms [2-4]. PM sources may affect the properties of PM [5]. Although the chemical composition of PM has been thoroughly studied [6], little is known about the presence of biological products that can influence respiratory disease, such as asthma. For example, animal farming contributes to air pollution by emitting PM [7], containing living microorganisms or parts released thereof, which comprises of bacterial cells and cellular fragments, fungal spores, viruses, and by-products of microbial metabolism [8, 9]. This complex and heterogeneous mixture is known as BioPM. Exposure to such diverse components in the farming environment is likely to trigger the immunological responses associated with various respiratory diseases.

Asthma is a global and increasingly prevalent disease, with at least 383,100 attributed deaths in 2016 [10], and it is estimated to increase in the next two decennia (28% more persons with asthma), resulting in the Netherlands in expenditures up to 900 million euro per year. There is considerable evidence that asthma prevalence is associated with air pollution including dust from livestock and other environmental aerosols.

1. BioPM in farming environment

1.1. BioPM exposure

The Netherlands has one of the highest animal farm densities worldwide. Livestock farms are known to diminish the air quality in surrounding areas by emitting BioPM. Major sources of BioPM are animals, animal wastes, feed and bedding material [11]. PM can stay suspended in the air for long periods and reach long distances from their source [12]. Therefore, BioPM exposure may result in health problems to farmers and nearby communities.

There is no uniform international definition of regulations for BioPM emission limits. Pig and poultry farms within the European Union are required to use appropriate operational practices to manage environmental and health impacts known as the Best Available Techniques (BAT) [13]. Implementation of the BAT requirements aim at meeting dust emission limits that will help to reduce BioPM emissions. However, the specific regulatory limits vary between countries. For example, the Netherlands considered

producing a risk assessment framework for intensive farms and recommended an exposure limit for the general population of 30 endotoxin units per cubic meter (EU m^{-3}), based on applying a safety factor of three to the occupational limit of 90 EU m^{-3} [14]. BioPM composition varies considerably from region-to-region, farm-to-farm, and even room-to-room [15, 16]. BioPM consists of complex mixtures and available toxicological and epidemiological studies provide insufficient information to define health related exposure limits as well as to set health based regulations for distance between farms and residential areas.

1.2. Respiratory health effect of BioPM

A study in the Netherlands has suggested that areas with high farm density contribute to the PM concentrations [17]. Air is an important transfer medium for microorganisms that could have potentially major consequences for the health of people surrounding animal farms. Several occupational studies have reported a negative impact of BioPM-exposure on respiratory health outcomes such as increase in impaired lung function, respiratory symptoms and inflammatory biomarkers in Bronchoalveolar lavage fluid (BALF), sputum and blood among farm workers [18-21]. Interestingly, Eduard *et al.*[22] reported an association between endotoxin, fungal spores and increased risk of non-atopic asthma, and an inverse relationship with atopic asthma, which suggests possible inflammatory adaptation or tolerance responses in the latter. The “hygiene hypothesis” states that living in a farm confers protection against development of asthma and allergy in early life, with airborne bacteria suggested as playing a role [23-25]. It has been investigated that children growing up in farms are less likely to develop allergic diseases compared to children living in the nearby areas [26, 27]. A few studies suggest that exposure to microbes is protecting for both the atopic and non-atopic phenotype not only during early life, but also sustained in adult life [28-31]. The contrasting findings could be due to timing and dose of BioPM exposure in the different studies. These contrasting findings can be explained by the potential underlying mechanisms [9, 22]. For example, it is not entirely understood which microbial exposures are responsible for the health effect. Furthermore, complex BioPM-induced responses may have a potential synergist effect on health outcomes compared to the effect of exposure to single agent [32].

Concerns have been raised about public health risks in people living at short distances from livestock farms that are potentially exposed to BioPM, but not all results are consistent. Most of the studies show no association between distance to nearest pig or poultry farm and asthma [33], and some even reported significant inverse correlations [34]. Hoopmann *et al.* [35] reported a positive association between endotoxin, used

as BioPM exposure measure, and self-reported asthma rates in children living near an intensive animal farming area. In this study, the children were from atopic parents only. Most experimental studies were conducted with pig farms and there was insufficient evidence to determine whether the type of farm had any health effect due to BioPM exposure. Collectively, BioPM pollution is suggested to potentially play a role in respiratory health effects of residents near intensive farms, although which specific components in BioPM are primarily responsible for the association with respiratory disorders and the underlying mechanisms are still unknown.

2. Ambient air microbiome

There are several methods for the detection of airborne microorganisms. To study viable microorganisms, classical cultivation in selective culture media has been used traditionally. However, cultivable microorganisms comprise only a minor fraction of the total airborne microbial composition [36]. Molecular typing using qPCR allow the detection of uncultivable microorganisms in the air, but is restricted to single species. Most pathogenic airborne bacteria have been identified by this technique as described in this chapter. To characterize the microbiome in an unbiased way, next generation sequencing (NGS) technologies can be used. NGS provides the opportunity to profile entire microbial communities from complex air samples, in a cost-effective and reproducible way [37, 38]. Bezhad *et al.* [36] have reviewed the current state-of-the art with respect to airborne metagenomics using NGS, and summarized the challenges such as the extreme low density of airborne microorganisms, the variety of collection devices that are used, and lack of standardized protocols and methodologies for sequencing and data analysis. Nevertheless, in the next paragraphs we aim to summarize the current knowledge regarding the various bacteria and fungi associated with these BioPM in the context of intensive farming.

2.1. 16S profiling

16S amplicon sequencing is widely used for the profiling of bacterial communities due to it cost-effectiveness in comparison to shotgun metagenomics [39]. Bacterial 16S rRNA gene profiling relies on the high degree of conservation of the 16S rRNA gene across the domains of bacteria. Universal primers have been designed for the conserved region of this gene in order to PCR amplify the hypervariable regions (earthmicrobiome.org) [40, 41]. The sequence of the variable region contains sufficient sequence diversity to phylogenetic classification of bacteria to the genus level.

In the context of pig farms, Arfken *et al.* reported that 16S sequencing revealed that *Actinobacteria*, *Bacteroidetes*, *Firmicutes*, and *Proteobacteria* are the dominant airborne bacterial inside the farms and on the corresponding spray fields. Analysis of the sequencing data at the genus and species level also demonstrated the presence of opportunistic pathogenic bacteria [42]. This approach has also been applied on size resolved PM from five different French dairy farms, with emphasis on fine dust particles ranging from 3 - 7.5 µm [43]. Although the bacterial composition differed between the farms, dominant phyla detected at all location comprise again *Actinobacteria*, *Bacteroidetes*, *Firmicutes* and *Proteobacteria*. Although not directly measured in air, 16S sequencing of mattress dust from farming households and controls showed several bacterial genera positively associated with farming, including *Clostridium*, *Facklamia*, and *Ruminococcaceae* [44], suggestive for airborne dispersal of these bacteria.

We have shown that microbial composition in farms is not affected by size fraction (Mariman *et al*, manuscript in preparation). Multivariate analysis of microbiome profiles obtained by 16S and Internal Transcribed Spacer (ITS) profiling revealed distinct clusters of farms with laying hens, broiler chicken and pigs, indicating that type of animals and farming type is driving the microbial composition. Similar findings were obtained when comparing the airborne microbiome in chicken, goat and pig farms [45]. Furthermore, in this study we not only showed full bacterial and fungal profiles based on DNA sequencing, but also confirmed presence of microbial ligands for toll-like receptor (TLR) 2, 4 and 5 using cell lines expressing individual TLRs.

Similar studies in dairy farms also revealed that the airborne microbe in farms spans all size fractions of the inhalable region, and are predominantly derived from animal feces. Here, they also found immune activating and potential pathogenic bacteria such as *Staphylococcus*, *Pseudomonas*, and *Streptococcus* [46].

In contrast, airborne bacteria in the proximity of intensive farming might also have positive health effects, as it seems to protect against the development of allergies [47]. Airborne bacteria were more abundant and diverse in pig farms than in suburban homes, including bacterial species previously suggested to contribute to protect against allergies [48].

2.2. Infectious Airborne bacteria

2.2.1. Molecular methods

In contrast to unbiased microbiome profiling using shotgun or amplicon sequencing, screening PM for specific bacteria is frequently used to detect pathogenic airborne bacteria. Monitoring airborne *Coxiella burnetti*, the bacteria causing Q fever, gained attention in the Netherlands after a large Q fever outbreak. PCR analysis detected airborne DNA of this pathogenic bacteria in coarse PM (<10 um), in particular nearby goat farms during the season of goat kidding [49]. A similar phenotypic and genotypic approach have been used to characterize antimicrobial resistant *E.coli* collected in air samples of cattle farms [50]. Depending on the temperature and wind directions, airborne *E.coli* is able to dissimilate up to 150 m from the farm [51], thereby potentially affecting health of nearby residents. This was further supported by a study measuring the genomic fingerprint of *E.coli* up to 400m away from the stable, showing high similarities with corresponding indoor samples [51].

2.2.2. Cultivation methods

In addition, cultivation-based methods have been applied to detect pathogenic airborne bacteria in poultry farms, which in contrast to DNA based method, are able to detect viable microorganism like, *Enterococcusus spp, E. coli, Salmonella spp*, and *Aspergillus fumigates* [52]. It is important to remember that culture-based detection techniques to profile airborne pathogens are sensitive to the collection device used for the PM collection as illustrated for viable airborne *Salmonella spp* in broiler chicken farms [53]. Selecting the most suitable collection device for cultivable bacteria is of particular interest to estimate the actual infection risk of bacteria. For instance, it has been shown that the detection of *Campylobacter jejuni* DNA by molecular techniques did not reflect the viability of this pathogenic bacteria in airborne samples from a laying hen farm [54]. Therefore, molecular techniques are powerful in detection of airborne pathogenic bacteria, but do not always predict the risk for infection.

2.2.3. Antibiotic resistant bacteria

In addition to airborne pathogenic bacteria, species that harbor antibiotic resistant genes are also of special interest. *Methicillin-resistant Staphylococcus aureus* (MRSA) is emerging among persons occupationally exposed to pigs, cattle or poultry [55, 56]. Studies in ambient air identified low concentrations of this bacterium up to 150 m from pig farms [57]. It has been shown that the MRSA in nasal swabs of healthy volunteers that shortly visited a pig farm was highly correlated to the MRSA load in the air [58]. Studies with people working in pig and veal calf farm confirmed that increased MRSA

levels in dust correlated with nasal MRSA carriage [58]. Airborne transmission of MRSA is not restricted to only the nose, as classical cultivation in combination with mass spectrometry identified MRSA in various PM size fractions collected in pig farms, potentially depositing in all parts of the airways [59].

2.2.4. Infectious Airborne Fungi

Apart from (pathogenic) bacteria, airborne fungi also have been shown to be a potential health risk in or nearby farms [60]. Microbial load depends on animal type, production stage, housing method, humidity and temperature [14, 61]. Total counts of fungi in coarse PM in poultry houses depend on the production cycle, and varied largely ranging from 10^2 - 10^6 CFU/m^3. Classical cultivation identified *Aspergillus spp* in airborne samples, which could also be isolated from workers of that particular laying hen farm [62]. *Aspergillus* was also characterized as the most abundant genera in a study focusing on airborne fungi in pig farms, followed by *Scopulariopsis brevicaulis* and *Penicillium spp* [63]. Similar to airborne bacteria, fungal deposition in the lungs depends partly on fragment size. A study addressing various farms including a poultry and swine farm assessed size-resolved PM with respect to fungi and fungal derived fragments. Size fractions >1.8 µm contained most of the collected fungal contaminants, however also small size fractions (<1 µm) contained fungal fragments especially of *Alternaria* and *Botrytis* [64]. Lugauskas and colleagues [60] identified in air samples from a poultry and swine facility a variety of *Penicillium* and *Cladosporium spp* that have been characterized in literature as allergenic. Furthermore, *Eurotium spp* and thermophilic fungi have been cultured from air samples in a poultry house at a concentration level that might induce respiratory diseases [65].

3. Underlying mechanisms of the immune response to BioPM:

It has been suggested that exposure to BioPM can influence the type and intensity of the immune response which may contribute to [66] or protect against [67] respiratory disease such as asthma. The ability of different microorganisms or their components in ambient air to shape the immune response may provide potential explanations for the rising trends of asthma.

3.1. Immunological aspects of allergy and asthma

Asthma is a chronic inflammatory disorder of the airways that makes breathing difficult. Asthma symptoms include wheezing, breathlessness, chest tightness, and coughing [68]. Most asthma begins in childhood in association with sensitization of the airways to common allergens, especially those derived from animal feather or skin, fungi and

pollen, house dust mite, and cockroaches [69, 70]. Immunoglobulin (Ig)E bound to high affinity FcεRI receptors on mast cells, can interact with an antigen/allergen resulting in degranulation of these cells that release mediators (such as histamine, serine proteases) that are involved in the early asthmatic response [71]. Also, T helper (Th)-2 cytokines Interleukin (IL)-4, IL-5 and IL-13 are released in this process [72]. While IL-4 and IL-13 are important for IgE and mucus secretion, IL-5 plays a pivotal role in the recruitment and activation of eosinophils in the airways [73]. These activated eosinophils subsequently release inflammatory mediators (such as eotaxin-1, macrophage inflammatory protein 3 (MIP-3)) in the airways that could damage airway epithelial cells and therefore promote an increased airway hyperresponsiveness (AHR) [74]. Recent studies have shown that ambient PM is a major environmental factor that plays an important role in the initiation and exacerbation of asthma [75, 76]. Although the association between PM and asthma is well established, the mechanisms responsible for PM-induced inflammation and the initiation/worsening of asthma remain poorly understood.

3.2. Innate immune response to bioaerosol

Microorganisms present in the airways may elicit different innate immune responses by triggering different signaling pathways. Dendritic cells (DCs) are specialized antigen-presenting cells (APC) capable of presenting specific antigens to naïve T-lymphocytes including in this way antigen-specific T cells responses. Pattern-recognition receptors (PRRs), as one of the central components of innate immune system, are expressed by these APC cells, also have the potential to take up antigens/allergens, process them into small peptides and then present them for recognition by T cell receptors [77]. Toll-like receptors (TLRs), a family of PRRs, can be activated by microbial, fungal and viral products and their ligands, the so-called pathogen associated molecular patterns (PAMPs). These ligands include endotoxin/LPS (recognized by TLR4), peptidoglycan (PNG)/lipoproteins (TLR2 and TLR6), and flagellum (TLR5) [78], which are molecule structures that could be present in ambient air. Interaction between PAMPs from allergens and PRR in DCs, resulting in signals transduction and cytokine production, magnify their capacity to others direct T cell towards areas of the regional lymph nodes [79]. Small amounts of TLR ligands from microbes present in PM, when inhaled along with allergens, may provide the necessary adjuvant effect to drive the allergic response.

BioPM contents are hypothesized to mediate TLR activation, leading to a pro-inflammatory response in the airways [80-83]. BioPM and TLR responses have been studied in human cell models and mouse models. TLR2 [84] and TLR4 [85] genetic variation appear to play a role in airway responsiveness of human subjects to exposure to BioPM. Exposure to BioPM derived from a swine confinement facility has been shown

to up-regulate TLR2 expression in human airway epithelial cells [86] and BioPM-induced pro-inflammatory cytokine production was dampened when blocking TLR2 in epithelial cells [87]. Moreover, airway neutrophil influx and cytokine release are significantly reduced in TLR2-deficient mice after pig farm BioPM extract intranasally challenge. In these mice airway AHR was still partially measured and airway inflammatory responses were not completely abrogated [88]. The TLR4 signaling pathway was also suggested to be involved in respiratory inflammatory responses. An *in vitro* study showed that endotoxin components from dairy farm BioPM mediated inflammatory responses through the TLR4 pathway in human macrophages [89]. After one-day exposure to BioPM collected from pig farm, the acute neutrophil recruitment was reduced in TLR4-deficient mice compared to wild type mice. However, cytokine production and airway AHR were not altered [90]. Findings from these animal studies indicate that there are complex interaction between TLR2 and TLR4 signaling pathways in response to BioPM.

3.3. Oxidative stress and BioPM -induced inflammation

Recent findings indicate that oxidative stress (OS) may also be involved in the increased inflammatory response of the airway to air pollutants [91, 92]. OS is reflected by an imbalance between oxidant generation, including reactive oxygen species (ROS), and antioxidant defenses [93, 94]. Mild OS can be mitigated by the activation of the antioxidant defense system that is mediated by nuclear factor-like 2 (Nrf2), a transcription factor that is the master regulator of cellular antioxidant and detoxification enzymes. When this protective mechanism fails, numerous free radicals may occur leading to inflammation [95-97]. In addition, it has been shown that the increased level of OS is associated with the severity of asthma [98, 99]. Since the respiratory tract provides a huge surface area in contact with air pollutant, PM can be considered an important source to provoke airway inflammation and OS may play an important role in this process.

Th2 shifting by ambient PM was stronger in Nrf2 deficiency mice compared to wild-type mice, which indicates OS may contribute to allergic sensitization to PM exposure [100]. Epidemiological studies show that PM can exacerbate symptoms of pre-existing asthma and asthmatics are more sensitive to PM than healthy individuals [101]. An association was found between BioPM exposures and adverse health outcomes in subjects with a history of chronic respiratory health problems [102] or duration of farming [103]. The malondialdehyde (MDA) level, a marker of OS, is significantly increased in serum from mice after 1 and 7 days (4 h per day inhalation) exposure to ambient fine PM compared to non-exposed control mice [104]. When comparing to healthy individuals, asthmatic patients showed significantly reduced antioxidation in plasma, and neutrophils from

asthmatic patients showed an increased tendency to generate ROS in the presence of ambient fine PM [105]. Furthermore, a recent *ex vivo* study [106] has shown that vitamin D enhanced antioxidant responses to ambient PM, suggesting that antioxidant may protect the airways from ambient PM-induced inflammation on primary human bronchial epithelial cells (HBECs) from healthy and asthmatic donors, though vitamin D had a more profound effect on IL-6 gene expression of healthy donors than asthmatics. A possible explanation is the ability of PM to act as an adjuvant for allergic sensitization to non-specific environmental allergens [107-109]. This adjuvant effect of PM can be linked to the ability of these particles to generate OS, which is supported by the observation that thiol antioxidants such as N-acetylcysteine (NAC) and bucillamine are effective in inhibiting the adjuvant effect of diesel exhaust particles (DEP) in an ovalbumin (OVA)-induced animal asthma model [110, 111]. BioPM-induced OS is speculated to play an important role to mediate respiratory symptoms and diseases. A finding that seems to confirm this comes from a study in normal human bronchial epithelial cells exposed to swine confinement facility dust extract, in which several radicals (including 8-isoprostaine, superoxide and catalase activity) were significantly reduced when the cells had been pretreated with antioxidants [112].

3.4. Interaction between TLR, oxidative stress and inflammation

TLRs activation is arising as a potential link between OS and inflammation. It has been reported that H_2O_2-induced OS can upregulate the expression of TLR2/4 in peripheral blood mononuclear cells (PBMCs) isolated from healthy people, and this increase was abrogated by antioxidant treatments [113]. To our knowledge, there is limited information available on whether livestock BioPM interferes with the TLR activation in DCs and the generation of OS or contributes to asthma pathogenesis. Several theories have been investigated previously on PM from other sources. PM could directly induce the production of cytokines involved in the allergic and inflammatory response. It can also down-regulate TLR2 and TLR4 expression, leading to suppression of the Th1 response and favoring Th2 polarization. Th2 cytokines can further increase ROS production by airway epithelial cells, resulting in an amplification cycle. Both direct and indirect effects of redox-active PM on the immune system are mediated by mechanisms involving cellular OS [114]. Wang *et al.* [115] have shown that combustion-generated free radicals could induce oxidative stress in DC, up-regulate their surface molecule expression, promote Th17 cells, and all these effects involved TLR3 dependent activation of DC [116]. Moreover, combustible tobacco caused cell viability loss, oxidative stress increase and cytokine secretion decrease after TLR4 activation of PBMCs, and these effects were rescued by pretreatment with NAC [117]. These findings provide a better insight into how PM interferes with the function of TLR through the generation of OS.

Understanding the mechanisms that modulate immune responses to BioPM allows for the identification of the factors that regulate this response and to determine which are the risks for asthma exacerbation. Additionally, defining the mechanisms of response will represent promising new approaches to prevent BioPM-related respiratory diseases.

4. Aim & Study design

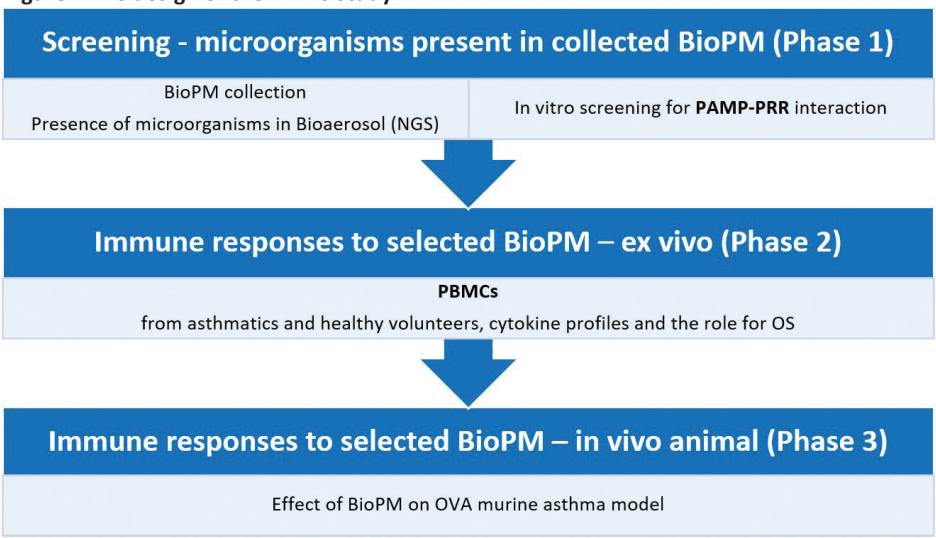
The work that comprises this thesis is part of the "Micro-organisms and their components present in ambient Air in relation to Respiratory Symptoms" (MARS) project performed by the Dutch National Institute for Public Health and the Environment (RIVM), Amsterdam Academic Medical Center (AMC), Michigan State University (MSU) and Utrecht University (UU).

This thesis aims to assess the effects of microorganisms present in PM in sensitive subjects, such as asthmatics. We will provide more knowledge on the microbial composition of BioPM emitted by livestock farms and whether these microorganism or thereof could induce airway inflammation in vulnerable patients. Furthermore, to establish a better understanding of the underlying mechanisms of BioPM resulting in respiratory symptoms. To address this broad aim and to fill some of the gaps identified previously, we established several models to investigate the association with health effects seen after BioPM exposure. The study design is depicted in **Figure 1**.

The specific aims of this thesis are:

- To characterize the microbial composition of indoor air derived from animal farms and to determine the effect that these microorganisms or their components have on innate immune receptors and cells;
- To determine how PBMCs from asthmatic patients and healthy subjects respond to fine (PM<2.5 μm) BioPM collected from livestock farms and the role of OS in this process;
- To study the effect that BioPM derived from farms have on airway inflammation using an OVA-induced murine model for allergic asthma.

Figure 1 The design of the MARS study.



5. Thesis outline

Following this introduction (**Chapter 1**), the next chapter of this thesis (**Chapter 2**) characterizes the microbial communities of size-resolved BioPM derived from six livestock farms and one non-livestock location, and determines the effect that these microorganisms or their components have on innate immune receptors and cells. **Chapter 3** shows the responses in PBMCs from both healthy donors and asthmatic people before and during loss of disease control, and whether antioxidant could abrogate these livestock BioPM-induced cytokine response intracellularly or extracellularly. In this study, the oxidative stress and inflammation effects of fine livestock BioPM were examined in primary human cells. Fine BioPM were also used in **Chapter 4 and Chapter 5** to study their effect on airway inflammation in animal studies. **Chapter 4** aims to examine the adverse effects of these BioPM for the potency to exacerbate allergic airway disease in OVA-induced allergic murine model and the difference between farm types, which may be associated with the differences in airborne microbiomes composition previously described. **Chapter 5** further hypothesizes the possible mechanisms/pathways that could be involved in the effect that BioPM have on airway allergic responses. Finally, **Chapter 6** provides a general discussion of the major findings and future perspectives.

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Chapter 2

Microbiome composition of airborne particulate matter from livestock farms and their effect on innate immune receptors and cells

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Abstract:

Patients with respiratory diseases in rural areas have been reported to have enhanced responsiveness to ambient particulate matter (PM). In addition to the physical and chemical components, ambient PM can contain microorganisms or parts thereof, referred here as BioPM, that can also contribute to the adverse health effects. This study aimed to characterize the microbial composition of BioPM originating from livestock, and to investigate whether these BioPM can trigger the activation of innate receptors and cells. Coarse (PM2.5-10 µm) and fine (PM<2.5 µm) BioPM samples were collected from indoor chicken, pig and goat farms using the versatile aerosol concentration enrichment system (VACES) connected to a Biosampler. The fungal and bacterial communities were assessed with an amplicon based approach using Next Generation Sequencing (NGS). In parallel, HEK-Blue cells expressing different pattern recognition receptors (Toll like receptors (TLR) 2, 3, 4, 5, 7, 8, 9 and NOD 1, 2) and a human monocytic cell line (MM6) were exposed to BioPM samples from these sites. Distinct airborne microbiota profiles associated with the corresponding animal farm were observed. Moreover, the various BioPM contained mainly ligands for TLR2 and TLR4 resulting in a concentration-dependent increase of pro-inflammatory cytokine secreted by MM6 cells. In addition, we show for the first time that only the pig-derived BioPM induced TLR5 activation. These findings suggest that animal farm specific BioPM trigger distinct inflammatory responses, which may contribute to airway diseases in humans.

Key words: airborne; BioPM; bacteria; fungi; TLR; inflammation

1. Introduction

Epidemiological studies indicate that particulate matter (PM) in ambient air is associated with adverse health effect including respiratory diseases [1-5], and that the physicochemical properties can affect the potency and type of response [1]. Both fine (<2.5 µm, Mass Medium Aerodynamic Diameter (MMAD)) particles and coarse (2.5-10 µm, MMAD) particles play a major role in triggering aggravated asthma or chronic bronchitis [6, 7]. Vehicular (road traffic) emissions are an important source of PM especially in urban areas. The impact of PM from other sources such as biomass burning and livestock farms are less well understood though it contributes to air quality as well [8, 9]. Working and resident at close proximity to areas with high density of livestock farms is associated with adverse respiratory health effects [10, 11], although opposite findings have also been reported [12]. Respiratory effects can be caused by inhalation of heterogeneous mixture of PM from livestock that contains pollutants like hydrogen sulphide, ammonium salts, volatile organic compounds, and endotoxins [13]. PM can contain microorganisms or parts thereof, which comprise of bacterial cells and cellular fragments, fungal spores and fungal hyphae, viruses, and by-products of microbial metabolism [14, 15], which is here referred to as BioPM. Although there are several studies describing the effect of the physical and chemical components of ambient PM on immune responses [16, 17], there is limited information on the microbial composition of BioPM, their effect on the immune response and hence the consequences for respiratory diseases.

A better insight in the composition of the air microbiome would therefore substantiate our understanding of the contribution of microorganisms or their components present in ambient air on respiratory diseases. Most of our understanding of airborne microorganisms has been obtained from the study of bacteria and fungi that can be cultured in selective culture media. However, cultivable microorganisms make up only a small fraction of the total airborne microbial composition [18]. Molecular typing techniques allow the detection of uncultivable microorganisms in the air, but are often restrict to a few (pathogenic) species. Therefore, recently developed next generation sequencing (NGS) technologies are promising, since it provides the opportunity to profile entire microbial communities from complex air samples, in a cost-effective and reproducible way [19].

Knowledge on which microorganisms are present in the different livestock farms BioPM and their effect on innate immunity is essential towards understanding how specific environmental exposure contribute to the occurrence or worsening of respiratory

diseases. Several mechanisms have been proposed to explain the pathogenesis of allergic inflammatory diseases, including the involvement of innate immune responses [20, 21]. The innate immune response is of crucial importance for the early containment of infection and the induction of adaptive immunity to pathogens [22]. However, exposure to external factors such as airborne microbes can influence the type and intensity of innate immune responses, which in turn can contribute to the generation of inflammatory diseases such as asthma [5].

One of the central components of the innate immune system is the family of pattern recognition receptors (PRRs) which are present either on the cell surface or intracellularly in various (innate) immune cells [23]. These PRR can recognize the presence of microorganisms by interacting with unique microbial molecular structures, the so-called pathogen-associated molecular pattern (PAMP). Activation of PRR by PAMPs results in a cascade of events leading to activation of transcription factors such as Nuclear Factor-ƙ-gene Binding (NF-ƙB) and subsequent cytokine production.

Exposure to BioPM derived from livestock farms may predispose workers and residents to respiratory diseases or worsen them. However, little is known about the ambient air microbial composition (external microbiome) and its effect on the immune response. In this study, we aimed to characterize the microbial composition of indoor air of animal farms and to determine the effect that these microorganisms or their components have on innate immune receptors and cells. NGS technology has been applied to profile entire microbial communities from complex BioPM samples and used dedicated cell lines to study immunological consequences.

2. Methods

2.1. BioPM sampling period and sites

Ambient indoor coarse (2.5-10 µm, MMAD) and fine (< 2.5 µm, MMAD) BioPM were collected at seven sites in the Netherlands during July 2016 to July 2017. The sampling sites include six indoor sites (inside or very near the exhaust ventilation pipes of two chicken farms, two pig farms, two goat farms) and a background non-livestock outdoor site (National Institution for Public Health and the Environment (RIVM)). All sites were located in the central region of the Netherlands. Per site, sampling was carried out for 2-6 days and for 6 hours per day (between 09:00-16:00 h) in order to collect sufficient material. The daily collected BioPM from each site were pooled in order to carry out *in vitro* studies. For the background non-livestock site, the maximum concentration of BioPM that could be collected after 6 days sampling was 0.1 µg/ml, which was

lower than what was collected for the animal farms (**Table1**). Indoor coarse BioPM samples collected at chicken farm 2, on June 2012 were used as reference BioPM for all experiments to correct for possible plate and day variation.

2.2. Sampling procedure

The Versatile Aerosol Concentration Enrichment System (VACES) was used to collect BioPM in fine size fraction and coarse collection for the *in vitro* studies. The BioPM was collected in water at a flow rate of 110 L/min. The procedure to use VACES to sample PM has been described extensively by Kim *et al.* [24]. In short, a single-nozzle virtual impactor was used to collect coarse fraction, whereas the fine fraction was collected by drawing air samples through two parallel lines. The fine size fractions go through a saturation-condensation system, which grows particles to 2-3 µm droplets, and then concentrate them by virtual impaction. The concentrated output flow from the virtual impactors are connected to a liquid impinger (BioSampler, SKC West Inc., Fullerton, CA) in order to yield highly concentrated liquid suspensions. The coarse particles do not require to be drawn trough a saturation-condensation system as they are able to penetrate directly into water. Before each use, MilliQ water was used to fill the impinge for the coarse mode.

In parallel, BioPM for bacterial profile studies were collected with Micro-Orifice Deposit Impactors (MOI™; 100-R) on filters (Teflo, R2PJ047/ R2PJ037, Pall, Port Washington, NY, USA) as previously described [25]. These precision cascade impactors are designed for sampling and collecting size-fractionated particle samples. The MOI has a sampling flow rate of 30 L/min and are provided with additional stages to size-fractionate aerosol particle samples. The 8-stage 100-R has a lower cut-size of 0.18 µm. Only four stages (< 1, 1–2.5, 2.5–5.62, 5.62-10 µm, MMAD) were used in this study.

The VACES, BioSampler and MOI were cleaned prior to use for each sample collection with ethyl alcohol (70%) and H_2O_2 (3%).

2.3. DNA isolation

DNA was extracted from environmental air samples that were collected on a filter with the MOI procedure, using magnetic beads. In short, filters were incubated for 1 h in a mixture of NucliSens lysis buffer diluted 2× in PBS. After centrifugation for 15 min at 1,000 *g*, the supernatant was collected and spiked with controls for both DNA extraction and qPCR amplification. Subsequent DNA extraction was performed with the NucliSens Magnetic Extraction Kit (bioMérieux, France) according to the manufacturer's protocol.

2.4. Library Preparation

Amplicon libraries were generated for respectively bacteria and fungi using a 2 step PCR approach. The primer sets for bacteria targeted the V4 (515F-813R) hypervariable region of the bacterial 16S-rRNA gene, while the primer sets for fungi targeted the ITS3-4 (ITS3F- ITS4R) hypervariable Internal transcribed spacer in the ribosomal RNA subunit of fungi (**S Table 1**). These primers contained the Illumina Nextera Adapters overhang at the 5' end, which are compatible with the Illumina Nextera XT indices. First PCR amplifications were carried out with the following 25 µl reaction mixture: 1X Phusion HF Buffer, 3% DMSO, 0.25 mM of each dNTP, 1 µM of each primers, 0.5 U Phusion High-Fidelity DNA Polymerase (Thermo Fisher Scientific), and 10ng template DNA. The following program was used for amplification: 95 °C for 3 min for initial melting; 20 cycles of 95 °C for 30 s, 55°C for 30 s, 72 °C for 30 s; 72 °C for 10 min. Amplification products were cleaned using Agencourt AMPure XP PCR purification beads (Beckman Coulter) according to the manufacturer's recommendations. DNA concentration and fragment size were measured on a QIAxcel using a DNA screening kit (Qiagen, Venlo, Netherlands). PCR2 amplification was carried out using the Nextera XT Index Kit according to the manufactures instructions to generate indexed paired-end libraries. In short, this PCR contained 1X Phusion HF Buffer, 3% DMSO, 0.25 mM of each dNTP, 1 µM of each Illumina P5 and P7 index primer, 0.5 U Phusion High-Fidelity DNA Polymerase (Thermo Fisher Scientific), and 5 µl PCR products from the first PCR. Thermal cycling was carried out at 95°C for 3 min, followed by 8 cycles of 95°C for 15 s, 55°C for 30 s, and 72°C for 30 seconds, followed by a final extension at 72°C for 5 minutes. These PCR reactions were again cleaned via a magnetic bead clean-up and run on a QIAxcel to measure size fragment and DNA concentration as described above.

2.5. Sequencing and data analysis

Prior to sequencing, amplicons of the individual samples were equimolar pooled and the PhiX Control v3 (Illumina) was added to the pool at 10% of the final concentration. Paired-end sequencing was performed on a MiSeq (Illumina, San Diego, CA, USA), using MiSeq Reagent Kit v3 (2x300 bp Paired-End Reads, 25 Gb output) according to the manufactures' instruction. Raw bcl files were processed using RTA v1.18 (MiSeq). De-multiplexing of the data based on the Illumina index reads was carried out using bcl2fastq v.2.17, which also converted the raw data to FASTQ files.

16S rRNA and ITS amplicon analysis was performed using the mothur software package version 1.35.1 [26] using the mothur Standard Operating Procedure pipeline for Illumina MiSeq data with minor modifications. Briefly, after forming contigs from the paired-end reads, PCR primers were trimmed off and any sequence with a homopolymer >8 bases,

with ambiguous base calls or reads >450 bp were eliminated from further processing. Chimeras were removed with UCHIME [27]. Finally, bacterial reads were classified to the species level using SILVA reference database release 128 [28]. The fungal sequences were assigned to phylum and genus-level phylogeny based on the fungal deposited in the GenBank database [29].

2.6. HEK-Blue cell lines and stimulation

To determine which human PRRs are activated by the BioPM, concentrations of the various farm BioPM samples ranging from 0.39 to 50 µg/ml and a background site BioPM (0.1 µg/ml) were incubated with different Human embryonic kidney (HEK)-Blue cell lines. Each cell line expressed a single PRR (TLR 2, 3, 4, 5, 7, 8, 9 or NOD 1, 2) (InvivoGen, San Diego, California, USA). Parental HEK-Null cells were also used to control for endogenous PRR activation. All cell lines contained a NFkB-inducible secreted embryonic alkaline phosphatase (SEAP) reporter gene. PRR signaling leads to the expression of the reporter SEAP gene, and the activity of the secreted enzyme can be detected in culture supernatants upon addition of the substrate Quanti-Blue (InvivoGen). The levels of SEAP activity are therefore, indicative of PRR activation. The HEK-Blue cells were cultured as previously described [30]. Briefly, HEK-PRR cells were incubated with increasing concentration of farm BioPM o/n in a volume of 200 µl DMEM medium, supplemented with 10% heat-inactivated fetal bovine serum (FBS (Gibco, Maryland, USA)) and 1% penicillin-streptomycin (Gibco, Maryland, USA), in 96-wells plates at 37 °C in humidified air containing 5% CO_2. The BioPM derived from the RIVM background site was used only at one concentration. Each HEK-PRR cell lines were also incubated with the indicated concentrations of their respective ligands, as positive controls: Pam3CSK4 (TLR2, 10 ng/ml), polyIC (TLR3, 1000 ng/ml), lipopolysacharide (LPS) (TLR4, 100 ng/ml), flagellum (TLR5, 100 ng/ml), imiquimod (TLR7, 1000 ng/ml), ssPolyU (TLR8, 1000 ng/ml), OND2006 (TLR9, 500 ng/ml), Ie-DAP (NOD1, 1000 ng/ml) and MDP (NOD2, 1000 ng/ml) (all from InvivoGen). PRR activation was expressed as OD value of SEAP activity induced by the BioPM normalized against the SEAP activity of the reference BioPM that was included in every experiment to correct for possible plate and day variation.

2.7. MM6 cell line and stimulation

To determine the effect of the different BioPM sources on activation of innate immune cells, a monocytic (MM6) cell line was used. The MM6 cells were cultured as previously described [30, 31]. Briefly, MM6 were maintained in RPMI 1640 medium containing 10% heat-inactivated FBS (Gibco, Maryland, USA) and 1% penicillin-streptomycin (Gibco). MM6 cells (5×10^5 cells/ml) were incubated in the same medium with increasing

concentration of farm BioPM collected from different animal farms (two chicken farms, two pig farms, two goat farms) and supernatants were collected after 24 hrs. Interleukin (IL)-6 was used as a readout for the activation of MM6 cells and measured using an ELISA (eBioscience, Vienna, Austria) according to the manufacturer's instructions. TLR ligands were added at the concentrations described above. Blocking polyconal antibodies against TLR2 (PAb2, 1 µg/ml), and TLR5 (PAb5, 1 µg/ml) and the TLR4 antagonists LPS from Rhodobacter sphaeroides (LPS-RS, 10 µg/ml) (all from InvivoGen) were added for 2 hours prior and during stimulation in order to examine the role of these TLRs on MM6 activation by the different BioPM. All experiments were performed on three separate days. The IL-6 production was expressed as pg/ml induced by the BioPM derived from various farms normalized to the IL-6 production induced by coarse BioPM samples collected at chicken farm 2 on June 2012 as reference BioPM for all experiments, to correct for possible plate/day differences.

2.8. Statistical analysis

GraphPad Prism 7.04 (GraphPad Software, Inc.) was used for statistical analysis. Unpaired t-test was used to compare SEAP activity or IL-6 induction between medium and positive controls, or when using the TLR blocking antibodies. Difference in the level of SEAP activity and cytokines induced by different BioPM concentration groups were analyzed with One-way Analysis of Variance (ANOVA), followed by post-hoc test (Dunnett's test). All the differences were considered to be statistically significant at $p \leq 0.05$.

3. Results

3.1. BioPM sampling characteristics

Characteristic features of the collected BioPM for each site and the sampling durations are shown in **Table 1**. For all BioPM size ranges, the lowest mass concentrations were measured at the RIVM background site (0.1 µg/ml), whereas the highest were measured at chicken farm 2 (17.3 mg/ml). For the *in vitro* experiments described below on measuring innate receptors and cell activation the BioPM from the different farms were tested at different concentrations.

3.2. Airborne microbiome profile in the stable

16s amplicon sequencing was applied to characterize the bacterial composition in size-resolved BioPM collected in stables of chicken, pig and goat farms as well as a non-farm related location. A total of 257 bacteria at the taxonomic level of genus were detected using the filters based collection system (**S Table 2A**). Profiles from both coarse and

fine fractions were incorporated into a principal component analysis (PCA), which demonstrated that bacterial profiles from farms grouped according to animal type, irrespective of the size fraction (**Figure 1A**). Although the microbiome profile from the chicken farms, clustered away from the other animal farms, a difference between the two farms was observed. To estimate the relative diversity at each sampling location, we calculated Shannon-Wiener diversity index at the species level classification for all size fractions. **Figure 1B** demonstrates a higher diversity in the stable of the different farms compared to a non-farming reference background, but no major differences between the animal farm types. To confirm that there was no bias in the collection method with respect to the observed bacterial communities in the BioPM, we compared the bacterial profile of BioPM collected using the filter based method and the VACES. In **Figure 1C**, no difference can be observed between the two collection methods. Since no major difference in bacterial composition was observed between the different fractions, the fine fractions of each farm was used for the comparison of bacterial communities between the farms. **Figure 2** shows the relative abundance for genus-level taxa that represent at least 5% of the bacterial community in one or more samples. The airborne bacteria at the poultry farms are dominated by *Lactobacillus* and *Staphylococcus,* whereas *Lactobacillus* and *Clostridium* are highly abundant in pig farms. *Pedobacter*, *Rhodococcus* and *Sphingomonas* are major constituents of airborne microbiome in goat farms only. Bacterial profiles at the species-level were also generated and are listed in **S Table 3**. Among these, several potential pathogenic bacteria were identified including *Streptococcus bovis, Serratia entomophila, Aerococcus viridans,* and *Corynebacterium xerosis.*

In parallel, a similar amplicon based technique was applied to profile the fungal community using Internal Transcribed Spacer (ITS)-profiling. In contrast to 16s profiling of the v4-variable region, the read length for ITS region was highly variable ranging from 281-634 bp. These fungal sequences were assigned to phylum and genus-level phylogeny, 90% of all the phyla belong to the Ascomycota and Basidiomycota. In total, 239 unique genus were identified in at least one of the samples. A complete list of all genus identified can be found in **S Table 1B**. In line with observations on bacterial composition, PCA analysis of the ITS data at the genus-level indicates that the composition of fungal community is also farm type related (**Figure 1 D**). The relative abundance for genus-level fungi that represent at least 5% of the community in one or more samples is given in **Figure 2B**. Dominant fungal taxa that are consistently present in both sites of a given animal-farm are *Ascochyta* and *Cryptococcus* (goat), *Eurotium* (chicken), and *Emericella* (pig). *Emericella* species that are highly abundant in indoor air of pig farms species are the sexual states of *Aspergillus* species.

Table 1. Mean value concentration of BioPM collected with the VACES device from different animal farms

Site	Date	Coarse (PM2.5-10) (μg/ml)	Fine (PM<2.5) (μg/ml)
Chicken 1	3,8 May	7850.0	3288.0
Chicken 2	11-13 July	17305.0	3782.5
Pig 1	3,5,11,19 Jan	986.0	745.0
Pig 2	4,5,6,11,13 Apr	673.0	154.0
Goat 1	3,9,14-16,28 Feb	520.0	400.0
Goat 2	15-17 May,12-14 Jun	618.0	95.0

Figure 1A

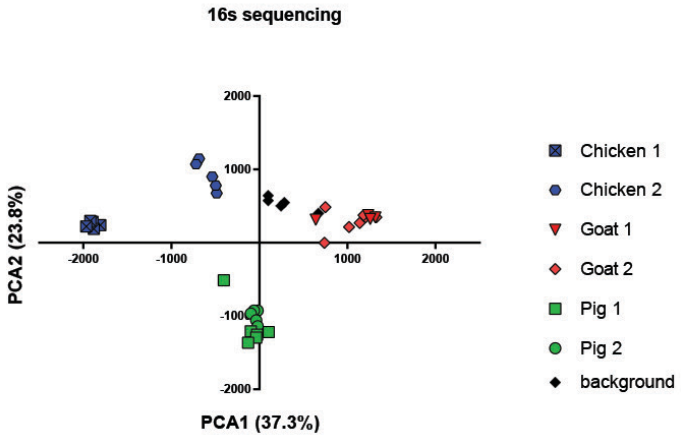


Figure 1B

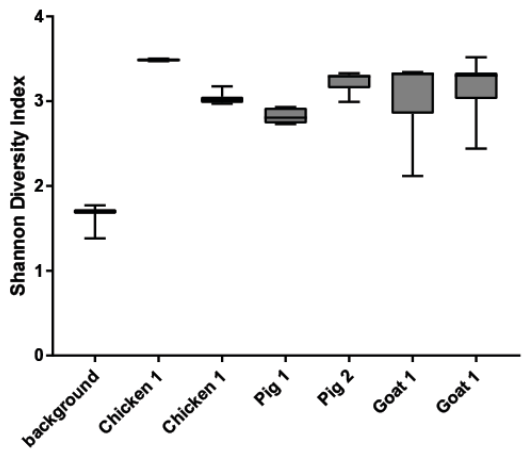


Figure 1C

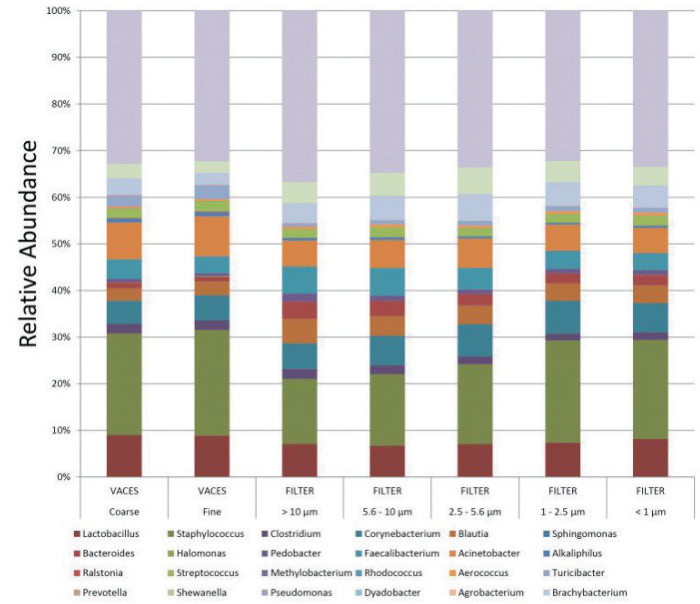


Figure 1D

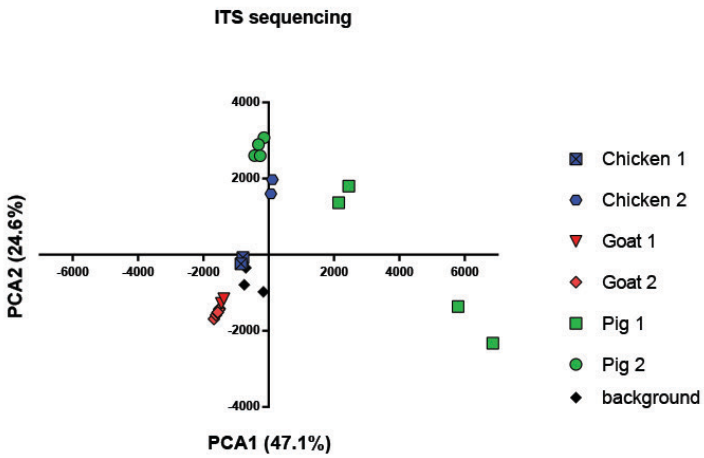


Figure 1. Composition of BioPM bacterial profile in different locations

A) Composition of the airborne bacterial profile in two chicken, two pig, goat farms and a non-farming reference location. Principal components analysis (PCA) plot characterizes distinct bacterial profiles associated with the type of animals present. Each point represents a sample, each color represents an individual farm. **B**) Boxplot showing the Shannon-diversity index at the species level for all the sampling locations. **C**) Comparing the relative abundance of bacteria at the genus-level for BioPM collected with the MOI and the VACES. **D**) PCA analysis on fungal profiles contained by ITS sequencing.

Figure 2A

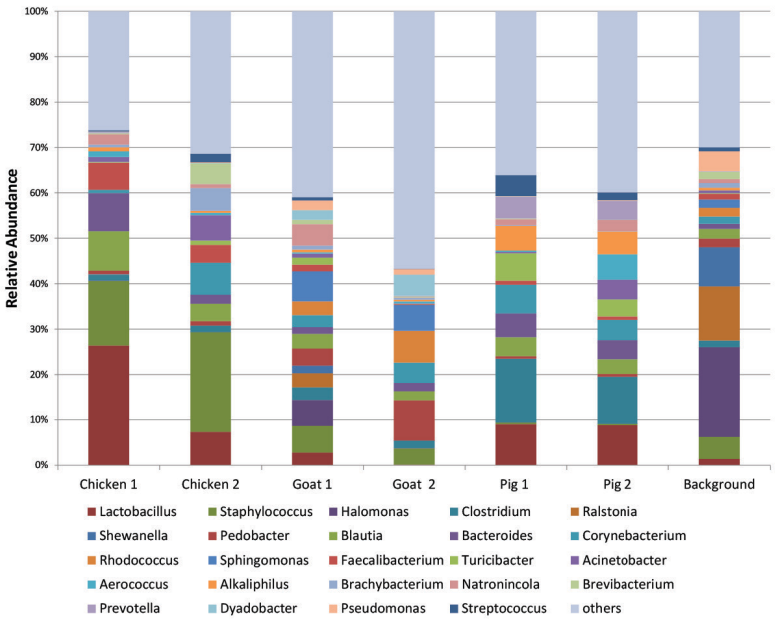


Figure 2B

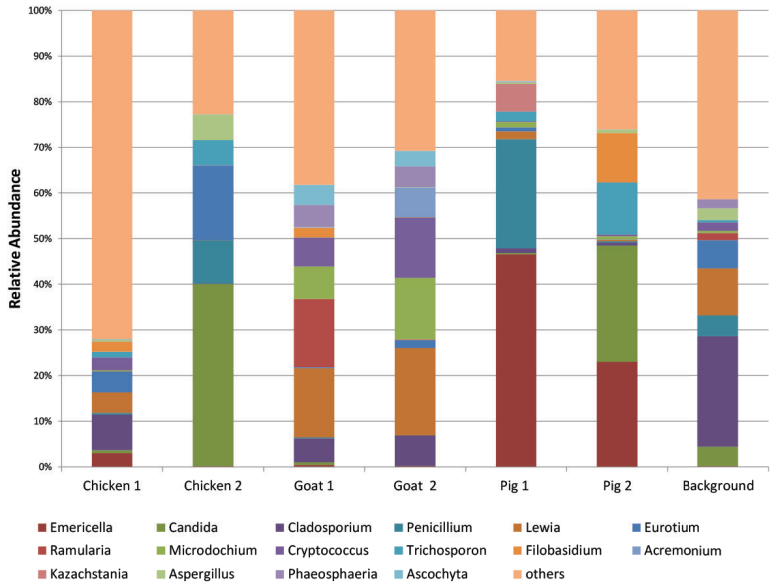


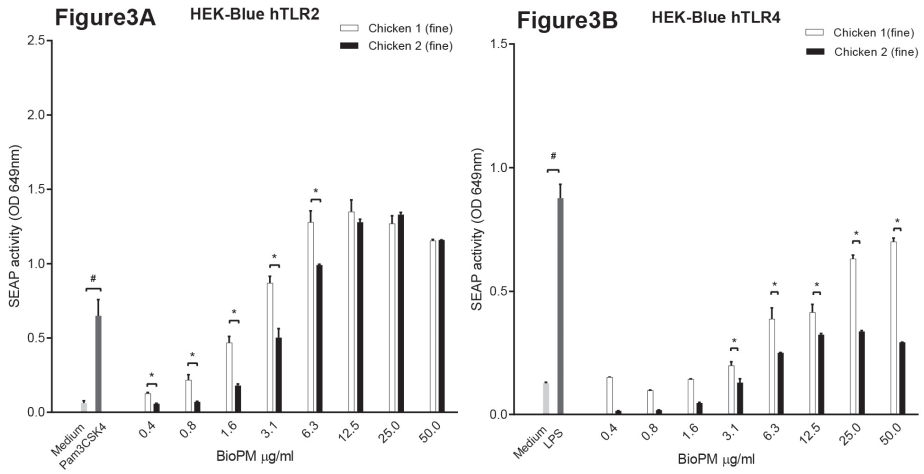
Figure 2. Comparison of the relative abundance of airborne microbiome

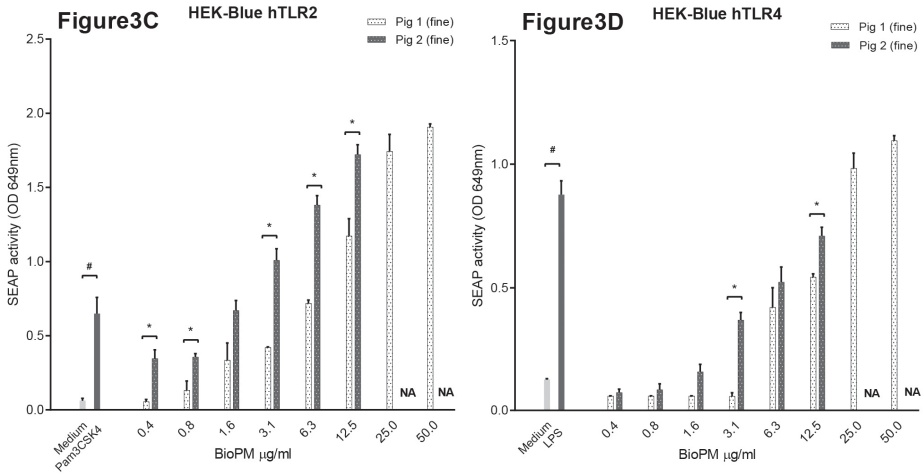
(**A**) Bacterial and (**B**) Fungal taxa at genus-level in the PM2.5 – 6.2 size fraction.

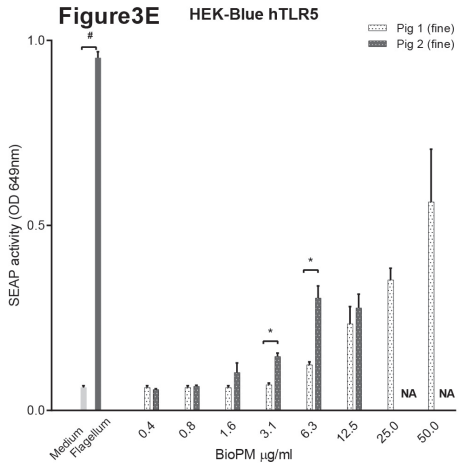
3.3. Activation of human TLR2, 4 and 5

To determine which PRRs could be activated by the different BioPM, a collection HEK-Blue cell lines expressing individual PRRs were used. All HEK-Blue cells responded as expected when stimulated by their respective PRR ligands (**Figure 3**). Furthermore, no endogenous PRR activation on the HEK-Blue cells was observed, as no SEAP activity was measured in the parental untransfected HEK-Blue-Null after stimulating with any of the PRR ligands or BioPM (data not shown).

Our findings indicated that all fine BioPM derived from the livestock farms activated TLR2 and TLR4 on HEK-Blue cells in a dose dependent manner. Only BioPM derived from the pig farms activated TLR5 as well (**Figure3 A-G**). When comparing the effect on PRR activation by the two animal farms tested, significant differences in SEAP activity was observed for BioPM derived from all farms. Comparable findings were obtained when using coarse BioPM derived from chicken/pig/goat farms (**S Figure1**). When comparing the effect of PRR activation by fine vs coarse BioPM from all animal farms no significance differences was observed except for a stronger activation of TLR2 and TLR4 by the fine compared to coarse BioPM in chicken farm 2 (**Figure 3 A, B** and **S Figure 1 A, B**).







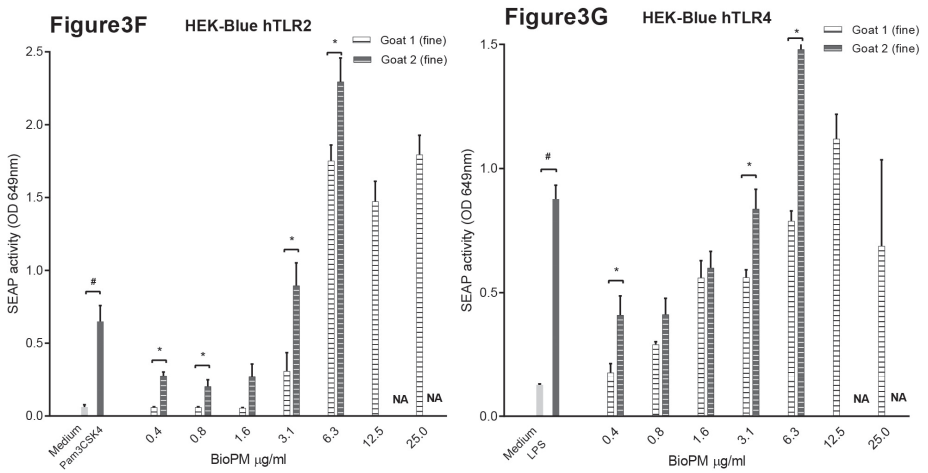


Figure 3. PRR activation by BioPM collected from different farms

HEK-Blue cells expressing different PRR were stimulated with fine BioPM collected from different sites: chicken farms with HEK-TLR2 (**A**) and HEK-TLR4 (**B**); pig farms with HEK-TLR2 (**C**), HEK-TLR4 (**D**) and HEK-TLR5 (**E**); goat farms HEK-TLR2 (**F**) and HEK-TLR4 (**G**). SEAP activity of HEK-Blue cells stimulated with the different BioPM, medium only or with the respective ligands (Pam3CSK4 (10 ng/ml), LPS (100 ng/ml), flagellum (100 ng/ml)) was determined in the supernatant after 24 hrs. Results are representative of three different experiments, showing mean values of triplicate determinations, with standard deviations represented by vertical bars.
P ≤ 0.05 (medium vs PRR ligands) * P ≤ 0.05 (farm1 vs farm2). NA: data not available

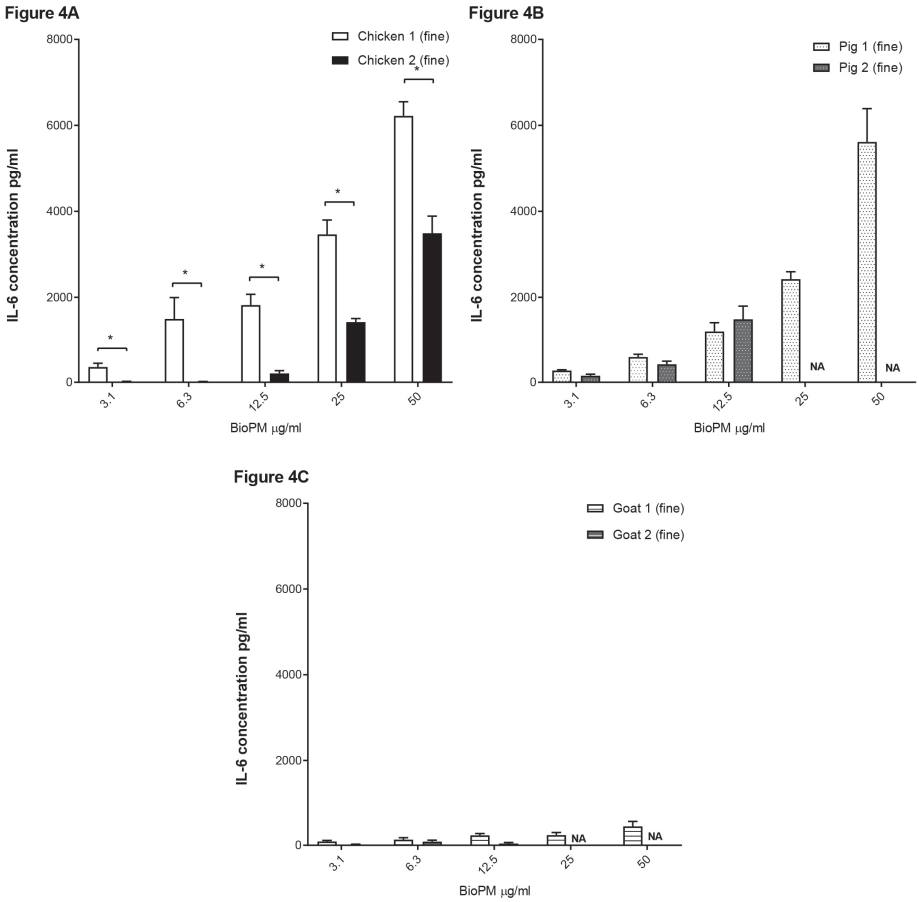


Figure 4. Effect of BioPM collected from different farms on the secretion of IL-6 by MM6 cells

The MM6 cells were plated in triplicate and stimulated with fine BioPM collected from different sites, chicken farms (**A**), pig farms (**B**), goat farms (**C**). IL-6 secretion was determined in the supernatant after 24 hrs stimulation with the different BioPM concentrations. Medium values for IL-6 were below the detection limit for all experiments. Results are representative of three independent experiments, showing mean values of triplicate determinations, with standard deviations represented by vertical bars.
P ≤ 0.05 (medium vs PRR ligands) * P ≤ 0.05 (farm1 vs farm2). NA: data not available

3.4. IL-6 production by MM6 cells

In addition to using the HEK-Blue cells expressing individual PRRs to screen for innate immune receptors activation by BioPM, the monocytic MM6 cell line expressing simultaneously multiple PRRs [32] was also used to measure innate immune cell activation.

Both fine and coarse fraction (**Figure 4 A and S Figure 2**) of the BioPM collected from all animal farms induce IL-6 secretion by MM6 cells in a dose-dependent manner. Significant difference was observed in IL-6 production induced by the fine BioPM derived from the two chicken farms. The level of IL-6 induced by the goat farms BioPM was lower compared to that induced by the other animal farms. Four samples (pig and goat farms, 25 and 50 µg/ml) were not included due to too their low BioPM concentration.

3.5. Blocking TLRs in MM6 cells

When using the HEK-TLR cell lines, all BioPM samples activated TLR2 and TLR4. In order to investigate the role of these TLRs in MM6 activation by BioPM, MM6 cells were treated with a blocking antibody against TLR2 or with the TLR4 antagonist prior and during incubation with BioPM derived from these animal farms. **S Figure 3A** shows that MM6 cells can be activated by Pam3SCK4, LPS, and flagellin, ligands for respectively TLR2, -4, and -5 activation. Furthermore, abrogation of IL-6 production by MM6 when using these TLR ligands in the presence of the corresponding blocking antibodies or antagonists is presented in this figure.

Results indicate that only blocking TLR4 interfered with the IL-6 production by MM6 cells upon stimulation with these BioPM (**Figure 5A-C**). For the pig farm derived BioPM, in addition to TLR2 and TLR4 we observed that these BioPM also activated TLR5. Therefore, in addition to the TLR4 antagonist and the antibody against TLR2, a blocking antibody against TLR5 was used. Here, we also observed that only blocking TLR4 interferes with activation of the MM6 cells was observed (**Figure 5B**). Comparable results were found when using either the coarse or fine fraction of all tested BioPM (**S Figure 3**).

Since antibodies against TLR2 and TLR5 did not significantly interfere with MM6 activation by BioPM and the TLR4 blocking did not completely abrogate the IL-6 production by these cells, we combined these blocking antibodies with the TLR4 antagonist. No additional decrease in IL-6 production by the monocytic cell line MM6 was observed (**S Figure 3**), indicating that engagement of TLR4 on these cells by its ligands present in the tested BioPM, dominates the observed IL-6 production.

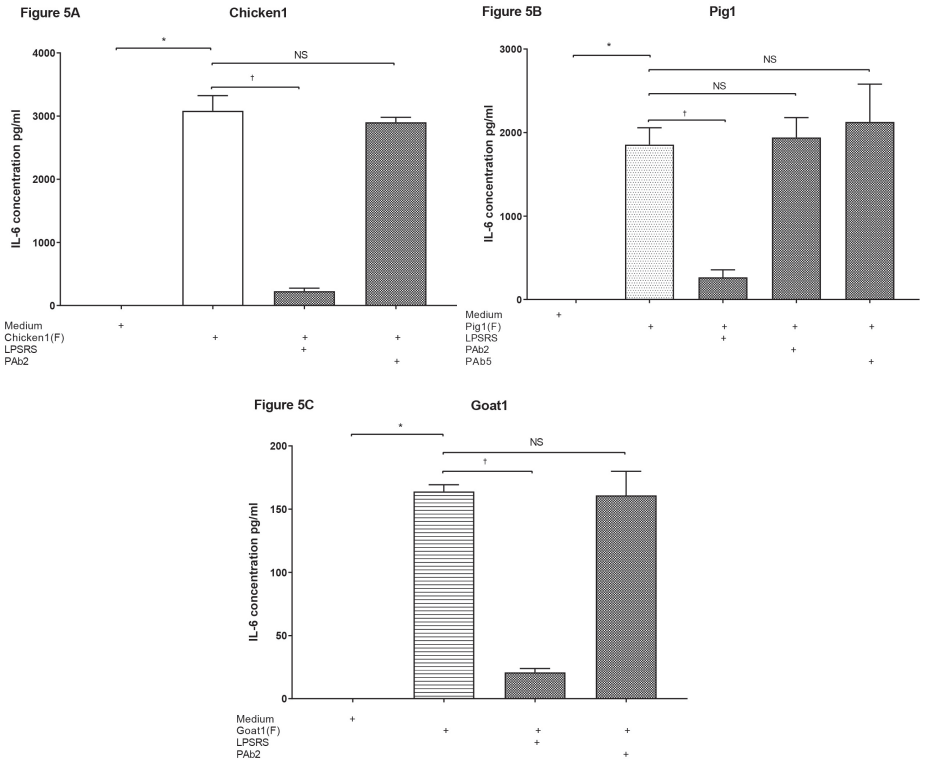


Figure 5. Specific blocking of TLR2, TLR4 or TLR5 receptors on MM6 cells using neutralizing antibodies or antagonist

MM6 cells were plated in triplicate treated with neutralizing antibodies against TLR2 and -5 (1 µg/ml) or antagonist LPSRS (10 µg/ml) 2 hours prior to stimulation with their fine BioPM collected from chicken farm 1 (**A**), pig farm 1 (**B**) and goat farm 1 (**C**). All BioPM concentration were 10 µg/ml. IL-6 secretion was determined in the supernatant after 24 hrs. Results are shown as mean values of triplicate determinations, with standard deviations represented by vertical bars.
* $P \leq 0.05$ (medium vs BioPM); † $P \leq 0.05$ (addition of blocking agent for receptors vs BioPM).

4. Discussion

In this study, we examined the microbial composition of size-resolved BioPM originating from indoor air derived from different livestock in the Netherlands and their effect on innate immune cells. Air microbiome analysis from the different animal farms indicated that the bacterial profiles are associated with the specific animal species, particularly for the pig and goat. The observed association was irrespective of size fraction. BioPM collected from farms contained mainly ligands for TLR2 and TLR4 irrespective the animal source. BioPM from the pig farms contained in addition ligands for TLR5. Despite the

fact that BioPM contain multiple TLR ligands, the activation of the monocytic MM6 cell line was mainly driven by TLR4 receptor signaling indicating that LPS from Gram-negative bacteria is likely to be a major contributor in immune activation by BioPM.

In the Netherlands, livestock density is among the highest in the world and the number of intensive livestock farms doubled within the first decade of the 21st century [33]. Livestock farms are known to emit large amount of PM and thereby contribute to air pollution [34-36]. Indoor PM concentrations in livestock farms have been shown to be up to 5 orders of magnitude higher compared to non-farming background locations. These PMs contain microorganisms and endotoxins which are suggested to induce respiratory infections and airway-related inflammatory responses in both farmers and residents living in close proximity [13]. However, there is very little information on which microorganisms are present in BioPM emitted from various farm animals and to what extends this affects innate immune receptors and cells.

Airborne fungal and bacterial communities may depend on season, humidity, collection method, size fraction, and environmental source [19, 37]. In line with previous studies [38], our results indicate that the microbial composition does not depend on the size fraction. With respect to the collection method, we found minor effects of the device used for sampling on the bacterial profiles only in goat farms. For this farm Staphylococcus *spp, Clostridium spp,* and *Corynebacterium spp* were more abundant in PM collected with sampling in water using the VACES compared to the dry air filter based method. For the other locations, we found comparable microbiome profiles between samples that were collected via filter and the liquid impinger methods. This implies that BioPM collected with the VACES is representative for the actual microbial community present in indoor air.

Interestingly, the composition of microbial communities in the BioPM collected in the present study is mainly reflecting the animal species. This is consistent with a previous publication [39] in which it was reported that different livestock (poultry and pig) were associated with a distinct airborne microbial community. In our study, the association between the bacterial profiles and the specific animal species was most evident for the goat and pig farms. Although the profile from chicken farms grouped away from the other animal farms, a difference between the two chicken farms was observed. These findings are in line with the significant differences in TLR2, TLR4 and MM6 activation observed when using these two chicken farm BioPM. A possible explanation for these findings is the two types of chicken farms used in this study. We collected indoor BioPM from chicken farm 1, which is a breeding farm whereas farm 2 is an egg-laying

farm. Remarkably, although both pig farm shows a highly comparable microbiome profile (**Figure 1A**), much more variation with respect to their mycobiome profile was observed (**Figure 1D**). Future studies determining the microbial composition of indoor BioPM should include larger number of the same type of animal farms to pinpoint drivers of these differences. Among the predominant bacterial taxa, we observed *Lactobacillus*, *Clostridium*, and *Prevotella* as in pig farms, and *Lactobacillus* in chicken farms. Although tracing exact source of the airborne bacteria was beyond the scope of this study, airborne bacteria in a dairy farm have reported to be predominantly derived from animal feces [38]. To the best of our knowledge, there is only one study describing the airborne fungal communities from an animal farm type, performed in an unbiased way using ITS sequencing [37]. In line with this study, we also found *Ascomycota* and *Basidiomycota* as dominant phyla in our pig farm BioPM samples, representing more than 90% of the fungal community. We also observed high variability of fungal communities at the genus level related to the animals present in the farms. Except for *Emericalla*, high variability between the two pig farms were observed, and this probably also explains the poor similarity of fungal taxa at the genus level described by Kumari *et al.* [37].

Among the different bacterial and fungal components, we observed that all BioPM samples contained potential zoonotic pathogens. Although we cannot exclude that the small size fractions only contain fragmented bacteria (or their DNA), it is tempting to suggest that airborne microbes in BioPM can infect the human respiratory tract. The potential pathogenic *Steptococcus spp, Corynebacterium spp, Clostridium spp* and *Aerocossus spp* were present in the BioPM samples in low abundance. The complete list of potential pathogens at the species levels (**S Figure 2**) shows 24 taxa that comprise at least 1% of the community. These are identified as infectious agents in human lung, skin or the urinary tract infections of (susceptible) individuals. This list should be taken with the precaution, since the taxonomy of an organism does not necessarily provide the information about the pathogenic level the organism [37].

Innate immunity relies on signaling by PRR, including NLRs and TLRs to alert the immune system of the presence of invading bacteria, viruses and fungi [40]. In this study, we aimed to screen for the microbial ligands in BioPM that bind to these receptors. Our results indicated that all BioPM derived from the livestock farms contains mainly bacterial and fungal ligands, evident from TLR2, TLR4 and TLR5 activation. In only a few studies the involvement of TLR2 and 4 in recognition of BioPM collected from various sources has been investigated. For example, TLR2 and TLR4 were activated by BioPM collected from urban air and cytokine productions induced by PM were inhibited

by TLR2 and TLR4 neutralizer in human airway macrophages [41]. TLR2 recognizes lipoprotein or peptidoglycan which is one of the main components of the cell wall of gram-positive bacteria. TLR4 is one of the well-characterized TLRs that recognizes LPS which is a common constituent of the cell wall of gram-negative bacteria [42]. Recently, it has been shown that PM2.5 may exacerbate allergic inflammation in the murine lung via a TLR2, TLR4 and Myeloid Differentiation factor 88 (MyD88) coupled signaling [43]. Previous *in vivo* findings have indicated that low level of LPS could cause TLR4-dependent Th2 response to OVA [44]. This suggests that the BioPM collected in our study could be an important risk factor for inflammatory and allergic lung diseases.

To our knowledge, we are the first to show that BioPM derived from pig farms activate TLR5. The most commonly known ligand for TLR5 is flagellin, which is a major component of flagellum found in certain bacteria [42]. Although blocking TLR5 did not alter the levels of IL-6 produced by the MM6 cell upon stimulation with the pig BioPM, we cannot discard and effect of TLR5 engagement on cell activation. Other cytokines produced by these cells or peripheral blood monocytes as well as their expression profile upon stimulation with the pig BioPM should also be investigated. In one study, the authors show that TLR5 agonists reduced development of airway hyperreactivity, eosinophilic airway inflammation and the production of Th2 type cytokines [45]. According to these findings TLR5 activation would have a protective role against airway inflammation. However, this finding remains controversial as other studies demonstrated that flagellin drives MyD88-dependent Th2 immune responses to indoor allergens in mice [46, 47]. Future studies on the role of TLR5 activation by BioPMs in respiratory diseases should be performed. When analyzing the airborne microbial composition, we could not find distinct bacterial populations in the pig farms that could explain the TLR5 activation by these samples. TLR5 expression is elevated in peripheral blood mononuclear cells (PBMCs) of children that live at pig farms, but not in children that live in other types of farms [48]. Whether this response is due to the abundance of airborne TLR5 agonists in the environment of pig farms remains to be investigated.

Our findings indicate that the BioPM size fraction did not influence IL-6 production by MM6. Other studies however showed that the coarse fraction of PM has similar or higher potency to induce release of the proinflammatory cytokines compared to the smaller fractions [49, 50]. This difference may be due to the source of the PM as well as the sampling technique, i.e. filters versus liquid based sampling. It is important to consider that coarse and fine fraction PM deposit in different regions of the respiratory tract. The coarse PM commonly deposit in the upper airway where asthma pathology occurs [51]. The use of monocytic cells derived from the upper and lower respiratory

tract would be an alternative approach to study whether the different BioPM size fractions influence the cytokine secretion profile of these cells.

To conclude on the effect of BioPM derived from specific animal farms on innate immune responses, future studies should include a larger number of livestock farms. In this study, the BioPM samples used were limited to two for each animal farm, which may not be representative for the entire type of livestock emissions. Moreover, the BioPM were collected only indoors whereas sampling in the surroundings of the farms can provide further information on the effect of BioPM on people living nearby. In the present study, we have used cell lines to study the innate immune response. It would be interesting to substantiate these observations using primary human PBMCs and airway macrophages. Finally, future in vivo studies should test the effect of fine and coarse PM on the airways. Although in vitro studies indicate that the different BioPM fractions induce comparable immune activation, in vivo responses may be different due to differences in regional deposition across the airway.

5. Conclusions

In summary, the results from this study indicate that distinct airborne microbiota profiles are associated with the corresponding animal farms and LPS is the most critical component of BioPM responsible for activation of innate immune cells. Whether the induced immune response has consequences for inflammatory responses upon inhalation of the different BioPM affecting (ongoing) respiratory diseases requires further investigation. The role of other ligands, such as lipoprotein and flagellum emitted from different animal farms on the airway should also be investigated. The knowledge generated is important for the development and implementation of measures to control microbiome air pollution from livestock farms.

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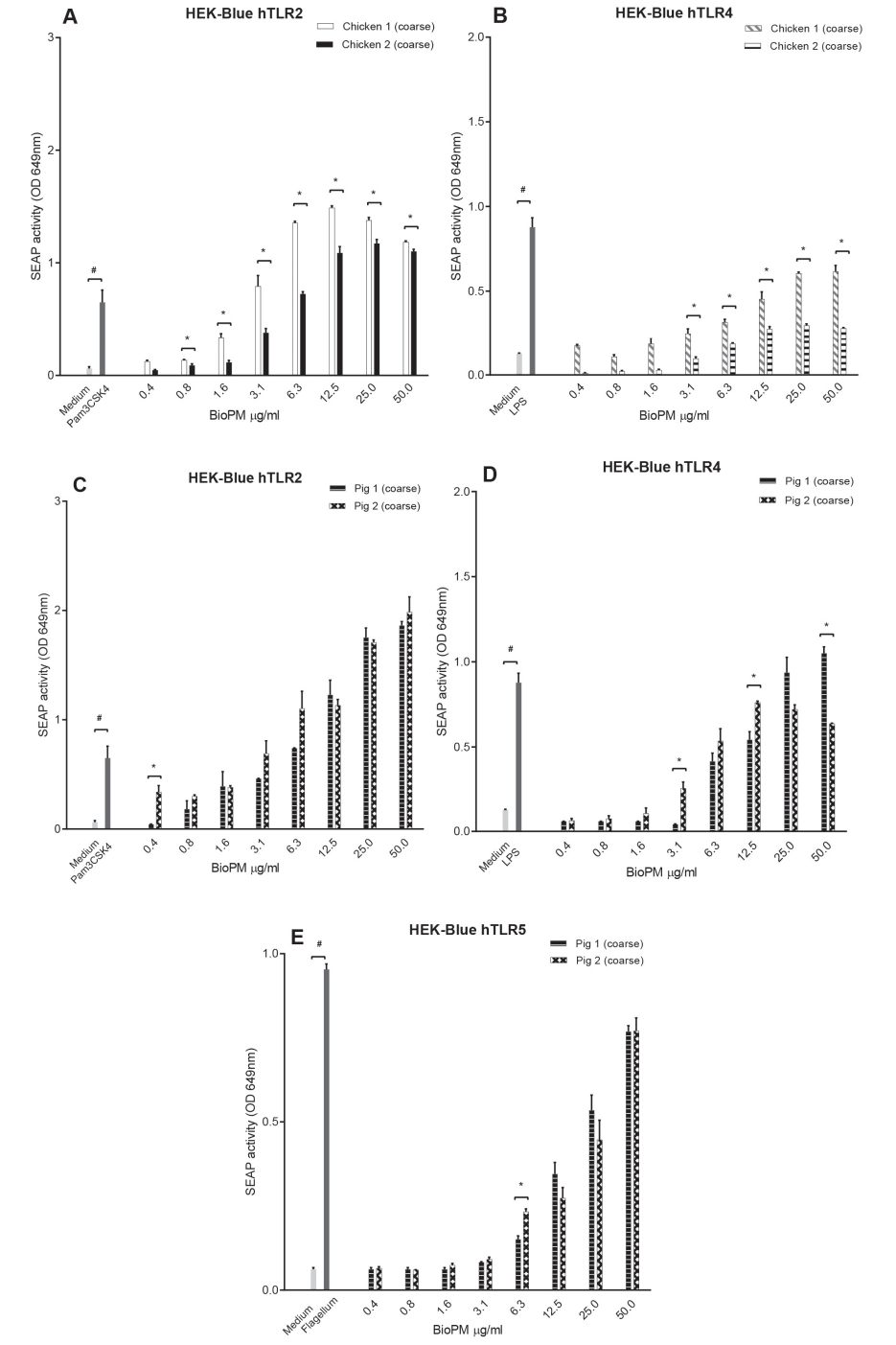
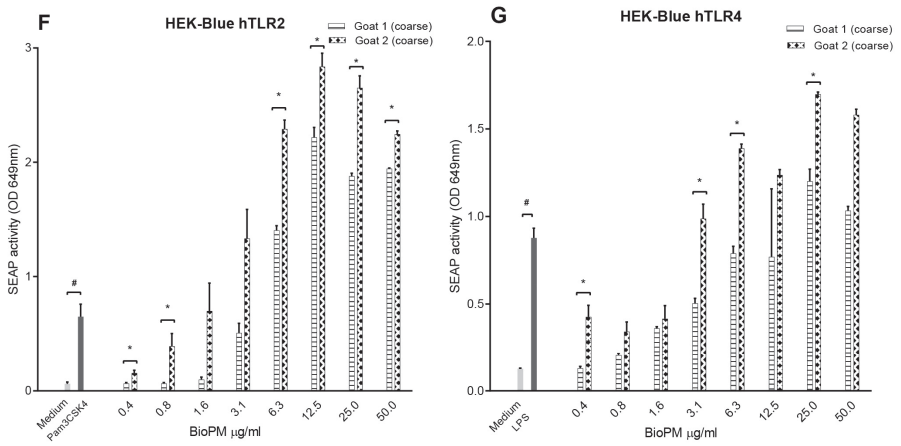
Supplementary

Supplemental figures:

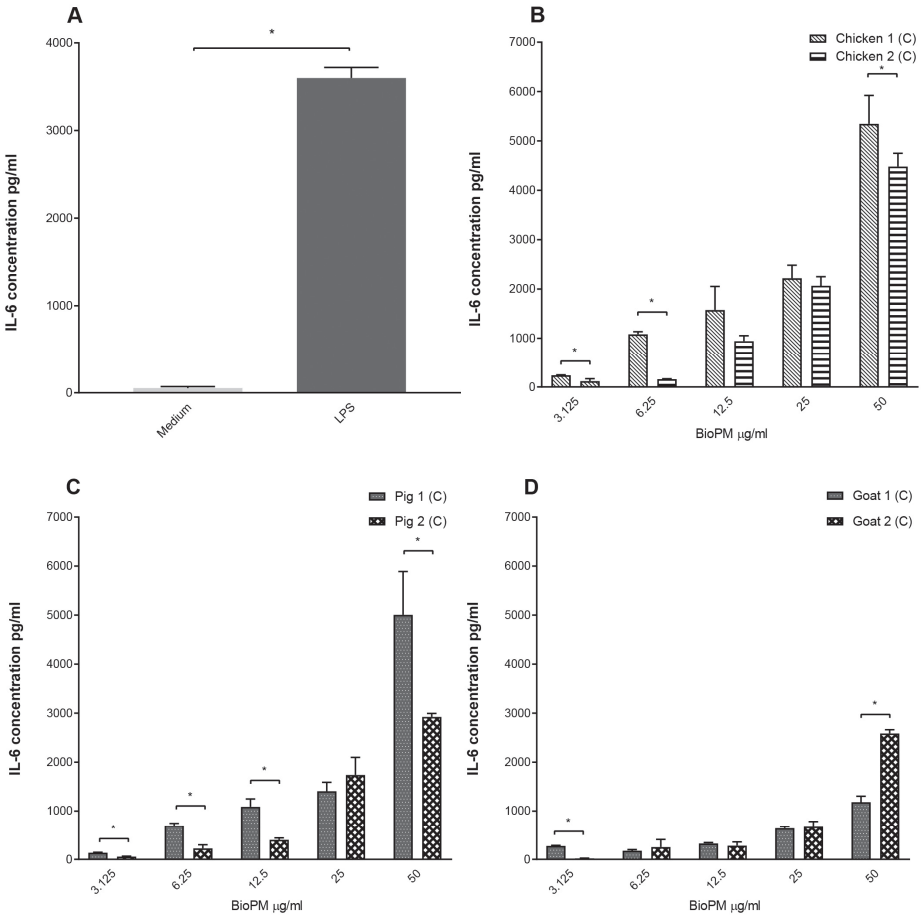




S Fig.1 PRR activation of BioPM collected from different farms

HEK-Blue cells expressing different PRR were stimulated with coarse BioPM collected from different sites: chicken farms with HEK-TLR2 (**A**) and HEK-TLR4 (**B**); pig farms with HEK-TLR2 (**C**), HEK-TLR4 (**D**) and HEK-TLR5 (**E**); goat farms HEK-TLR2 (**F**) and HEK-TLR4 (**G**). SEAP activity of HEK-Blue cells stimulated with the different BioPM, medium only or with the respective ligands ligands (Pam3CSK4 (10 ng/ml), LPS (100 ng/ml), flagellum (100 ng/ml)) was determined in the supernatant after 24 hrs. Results are representative of three different experiments, showing mean values of triplicate determinations, with standard deviations represented by vertical bars.

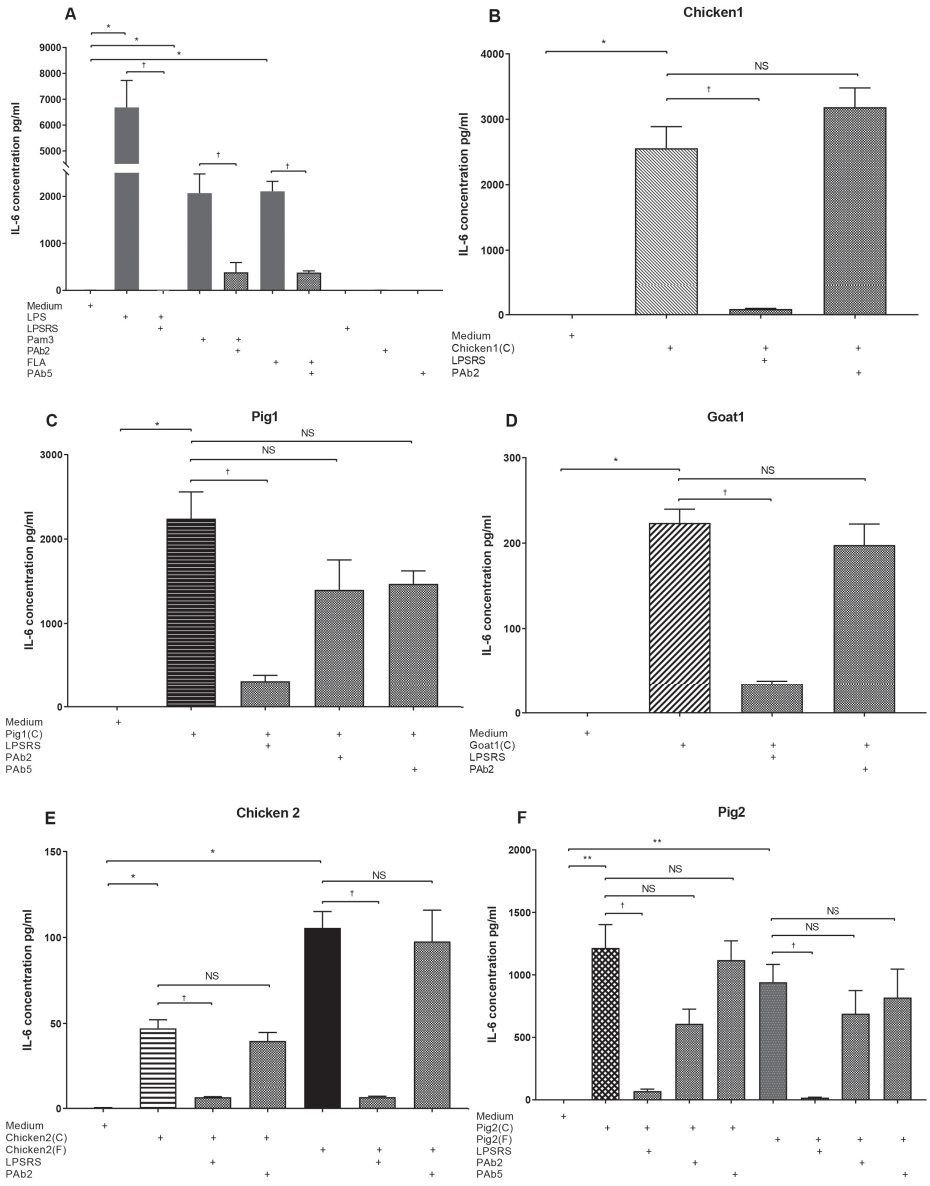
P ≤ 0.05 (medium vs PRR ligands) * P ≤ 0.05 (farm1 to farm2). NA: data not available

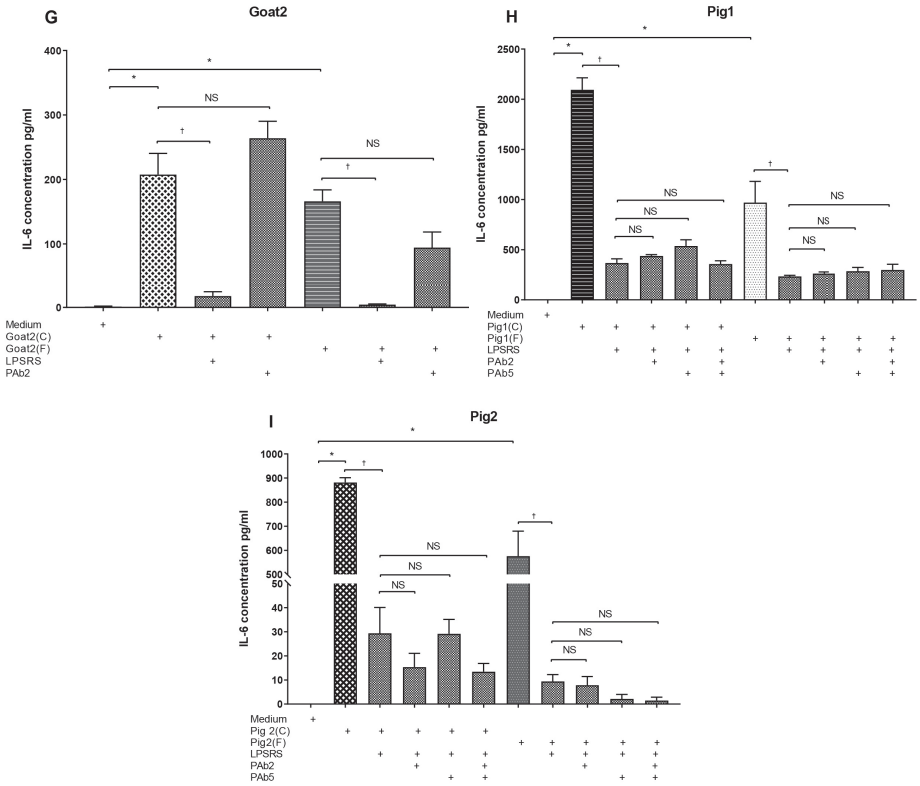


S Fig. 2 Effect of BioPM collected from different farms on IL-6 production by MM6 cells.

MM6 cells were plated in triplicate and stimulated with LPS (**A**), or coarse BioPM collected from different sites, chicken farms (**B**), pig farms (**C**), goat farms (**D**). IL-6 secretion was determined in the supernatant after 24 hrs. Medium values for IL-6 were below the detection limit for all experiments. Results are representative of three independent experiments, showing mean values of triplicate determinations, with standard deviations represented by vertical bars.

* $P \leq 0.05$ (PRR ligands vs medium or farm1 vs farm2). NA: data not available





S Fig. 3 Specific blocking of TLR2, TLR4 or TLR5 receptors on MM6 cells induced by BioPM collected from second location of all farms in presence of neutralizing antibody or antagonist

MM6 cells were plated in triplicate treated with neutralizing antibodies against TLR2 and -5 (1 µg/ml) or antagonist LPSRS (10 µg/ml) 2 hours prior to stimulation with their respective ligands (**A**), or chicken farm 1 coarse (**B**), pig farm 1 coarse (**C**), goat farm 1 coarse (**D**), BioPM collected from chicken farm 2 (**E**), pig farm 2 (**F**), goat farm 2 (**G**), pig farm 1 with multiple PRR blockers (**H**), pig farm 2 with multiple PRR blockers (**I**). All BioPM concentration were 10 µg/ml. IL-6 secretion was determined in the supernatant after 24 hrs. Results are shown as mean values of triplicate determinations, with standard deviations represented by vertical bars.
* $P \leq 0.05$ compare ligands to medium only; † $P \leq 0.05$ compare addition of blocking agent to ligand or BioPM only; NS: not significant

Supplementary Tables:

Suppl. Table 1. Primers used for library preparation

Name	Amplicon		Primer sequence (5'-3')[a]
515F	V4 region 16s rRNA	Forward	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG GTGCCAGCMGCCGCGGTAA
R806		Reverse	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG GGACTACHVGGGTWTCTAAT
ITS1	ITS region	Forward	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG GCATCGATGAAGAACGCAGC
ITS2		Reverse	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG TCCTCCGCTTATTGATATGC

[a] Underlines indicate Illumina adaptor sequences

Suppl. Table 2 A 2 B
Data and materials are available upon request of the author (Dingyu Liu, dingyu.liu@rivm.nl).

Suppl. Table 3. Selected (human) pathogenic bacterial species as defined by COGEM

			Poultry		Goat		Pig		non farming reference	
DSMZ	Overall	Species	mean[a]	SD	mean	SD	mean	SD	mean	SD
2	2	Streptococcus bovis	94.9	81.5	13.6	23.2	213.0	198.4	1.6	0.8
2	2	Comamonas kerstersii	6.3	4.5	8.4	21.2	3.6	3.1	359.6	51.9
-	2	Ralstonia pickettii	0.3	0.4	5.3	15.8	2.1	3.4	338.7	41.7
-	1	Serratia entomophila	155.6	125.7	13.8	25.4	9.1	7.3	0.0	0.0
2	2	Aerococcus viridans	33.0	18.0	21.1	3.7	112.2	122.2	0.0	0.0
2	2	Corynebacterium xerosis	1.8	1.6	21.6	25.6	63.8	25.0	0.0	0.0
2	2	Faecalibacterium prausnitzii	43.7	11.6	3.2	5.6	1.6	0.9	0.0	0.0
-	2	Bacteroides xylanisolvens	14.0	4.6	1.5	2.6	33.8	14.9	0.3	0.4
-	2	Facklamia hominis	3.7	1.9	21.9	13.3	32.5	26.9	0.0	0.0

-	2	Arthrobacter creatinolyticus	29.6	30.1	3.0	5.3	0.4	0.5	0.0	0.0
2	2	Acinetobacter lwoffii	28.0	7.1	2.8	1.7	9.2	11.0	3.3	4.6
2	2	Escherichia albertii	26.2	20.6	3.3	5.8	1.6	1.4	0.0	0.0
2	2	Clostridium histolyticum	21.7	16.8	6.8	4.1	26.0	16.5	0.6	0.1
-	2	Clostridium butyricum	0.0	0.0	0.1	0.1	21.6	9.4	0.0	0.0
2	2	Corynebacterium urealyticum	0.3	0.3	8.8	6.4	20.8	22.1	0.0	0.0
2	2	Bacteroides thetaiotaomicron	19.5	21.0	3.0	5.7	0.3	0.4	0.0	0.0
2	1	Clavibacter michiganensis	0.0	0.1	17.1	8.6	0.0	0.1	0.0	0.0
2	2	Corynebacterium testudinoris	1.0	0.6	0.5	0.4	16.2	14.1	0.0	0.0
2	2	Corynebacterium hansenii	0.7	0.3	5.0	5.7	15.7	7.9	0.0	0.0
2	2	Prevotella albensis	0.0	0.0	2.8	2.6	15.6	6.3	0.0	0.0
-	2	Johnsonella ignava	15.0	8.3	4.5	1.5	4.6	1.7	0.0	0.0
2	2	Fusobacterium gonidiaformans	14.9	16.0	1.3	1.6	4.3	4.0	0.4	0.5
-	2	Bacteroides vulgatus	14.8	8.5	2.6	4.9	1.4	1.7	0.0	0.0
2	2	Collinsella aerofaciens	0.0	0.0	0.1	0.2	10.6	5.9	0.0	0.0
2	2	Acinetobacter haemolyticus	8.9	8.0	0.7	1.3	9.9	11.4	10.5	14.9

[a] Average number of reads per 10.000. Bacterial species with prevalence >0.1% were included in the table

1 (**Class 1**): Microbial species covered by one of the following criteria:
II. The species has an extended history of safe usage under conditions without any physical restrictions, although there may be differences in virulence between strains within the species that should be taken into account.
III. The species belongs to one of the following classes but a specific strain within the species may lack the genes responsible for pathogenicity in humans and animals. The degree of attenuation of the particular strain may put it in an exceptional position. This implies that within species of categories 2, 3 and 4 specific strains may be down-regulated to class.
IV. Non-pathogenicity of the species has been convincingly demonstrated by *in vitro* and *in vivo* testing.
2 (**Class 2**):Species that can cause diseases in humans or animals, which are unlikely to spread in the human population and for which an adequate prophylaxis or therapy exists.

Chapter 3

Ex vivo innate responses to particulate matter from livestock farms in asthma patients and healthy individuals

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Under review

Abstract

Asthma patients suffer from periodic acute worsening of symptoms (i.e. loss of asthma control or exacerbations), triggered by a variety of exogenous stimuli. With the growing awareness that air pollutants impact respiratory disease, particulate matter (PM) receives increasing attention. Here, we investigated whether PM derived from livestock farms (BioPM) differentially affected innate immune and oxidative stress responses by peripheral blood mononuclear cells (PBMCs), as a surrogate for alveolar macrophages, from healthy individuals and asthma patients before and during loss of disease control. PBMCs produced IFNɣ, IL-1b, IL-10 and TNFa upon stimulation with BioPM collected from chicken, goat and pig farms (1 and 5 µg/ml), with that from pig farms inducing the highest cytokine levels. Overall, cytokine production was irrespective of the presence or state of disease. However, PBMCs from stable asthma patients upon exposure to the three BioPM showed more extreme TNFα responses than those from healthy subjects. Furthermore, PBMCs obtained during loss of asthma control that were exposed to BioPM from pig farms showed enhanced IFNɣ release, suggestive of a lowered threshold for IFNɣ production in these patients, and lowered oxidative stress levels upon pre-treatment with N-acetylcysteine (NAC) compared to stable disease. NAC, but not superoxide dismutase and catalase, also counteracted BioPM-induced cytokine release, indicative of intracellular generation of reactive oxygen species. BioPM, particularly that from pig farms, triggers enhanced inflammatory responses by PBMCs, which may worsen asthma and even more so during loss of asthma control.

1. Introduction

Asthma is a chronic inflammatory lung disease associated with reversible airway obstruction and increased responsiveness of the airways to a variety of stimuli (also known as bronchial hyperresponsiveness). Airway inflammation in asthma is heterogeneous, but increased levels of oxidative damage are seen in virtually all patients. Asthma patients may suffer from periodic acute worsening of symptoms, referred to as loss of asthma control or exacerbations, that can be triggered by several exogenous factors, including viruses and allergens [1].

Recently, air pollution from ozone, nitrogen dioxides and particulate matter (PM), including traffic- and livestock-related emissions, has received increasing attention as it exacerbates and even may induce asthma [2-4] and was shown to contribute to asthma mortality [5]. Traffic-related PM drives the transcription of inflammatory mediators relevant to asthma and is a potent inducer of oxidative stress [6], as many of its components may act as a source of free radicals. This is unlikely to be the case for PM collected from specific livestock farms (BioPM). BioPM, however, was shown to contain multiple Toll-like receptor (TLR) ligands and even microorganisms or parts thereof, with distinct microbiota profiles associated with corresponding animal species [7].

In this study, we evaluated whether BioPM triggers distinct innate responses by peripheral blood mononuclear cells (PBMCs) from clinically stable asthma patients as compared to healthy controls. As airway inflammation worsens during loss of asthma control, we have also collected PBMCs from those patients of whom stable samples were obtained, but now during corticosteroid withdrawal-induced loss of asthma control. This allowed us to determine whether the innate responses to BioPM in asthma were modulated compared to baseline. BioPM derived from chicken, goat and pig farms, which are considered major sources of BioPM in The Netherlands, were compared. In addition, we aimed to clarify whether BioPM exerts its effects via oxidative stress-dependent mechanisms.

2. Methods

2.1. BioPM sampling period, sites and procedure

Ambient fine (<2.5 μm, Mass Medium Aerodynamic Diameter) PM was collected at three livestock farms in The Netherlands from July 2016 to July 2017, including one chicken, one goat and one pig farm, all located in the central region of The Netherlands. Per site, sampling was carried out for two to six days and for six hours per day (between

09:00 and 16:00h) in order to collect sufficient material. The daily collected BioPM from each site was pooled in order to carry out the current study. Characteristic features of the collected BioPM for each site and detailed description of the sampling dates and procedures during the sampling collection is described elsewhere [7]. All BioPM were collected in demineralized water using a Versatile Aerosol Concentration Enrichment System as described previously [8].

2.2. Subjects

Allergic, mild to moderate asthma patients originated from a standardized prospective inhaled corticosteroid (ICS) interruption study [9-11]. The study design included a baseline visit and a loss of disease control visit. Following baseline measurements, patients were instructed to abruptly discontinue the use of ICS until loss of asthma control occurred (or for a maximum of eight weeks), which was defined as meeting two out of the three criteria mentioned below. Then, the second visit was scheduled. Criteria for loss of asthma control included: (1) morning peak expiratory flow <80% of baseline on at least two consecutive days, (2) wakening due to asthma on at least two consecutive nights and (3) use of more than eight puffs short-acting β_2-agonist per day on at least two consecutive days. The study was approved by the AMC Medical Ethics Committee (2011_082#B201152) and registered at the Netherlands Trial Register (NTR3316). All participants provided written informed consent. Healthy controls were recruited in accordance with a study protocol that was reviewed by the AMC Medical Ethics Committee (2015_074). The need for ethical approval was waived. Prior to sample donation, all donors gave informed consent. In the present study, we compared ten asthma patients at stable disease and during loss of control and ten healthy volunteers.

2.3. Processing and analysis of blood

Venous blood was collected in serum and heparin tubes. Total immunoglobulin E (IgE) in serum was determined by ImmunoCAP (Phadia AB, Uppsala, Sweden). PBMCs were isolated from heparin blood using standard density gradient techniques and stored in liquid nitrogen until further analysis.

2.4. PBMC stimulation

For stimulations, PBMCs were thawed, washed, counted on the Coulter counter (Beckman Coulter, Brea, CA, USA) and diluted to 10^6/ml culture medium. The optimal BioPM concentration (range: 0.01 to 50 µg/ml) was determined based on cell viability and cytokine production. For experiments described here, PBMCs were plated in the presence or absence of BioPM collected from chicken, goat or pig farms (1 or 5 µg/ml), with or without 1 hour pre-treatment with N-acetylcysteine (NAC) (Sigma-Aldrich, Saint

Louis, MO, USA; 1 or 10 mM) or a combination of superoxide dismutase (SOD) (Sigma-Aldrich; 100 µg/ml) and catalase (Boehringer Mannheim GmbH, Mannheim, Germany; 50 µg/ml) and incubated for 20 hours. Supernatant was collected for subsequent assays.

2.5. Viability

PBMC viability was determined using Cell Proliferation Reagent WST-1 (Roche Diagnostics GmbH, Mannheim, Germany) according to the instructions of the manufacturer.

2.6. Luminex

Interferon (IFN) γ, interleukin (IL)-10, IL-1β and tumor necrosis factor (TNF) α were measured using R&D Systems (Minneapolis, MN, USA) reagents according to the instructions of the manufacturer and read on a Bioplex 200 (Bio-Rad, Hercules, CA, USA). Possible interference of NAC with Luminex antibodies was excluded by direct addition of 10 mM NAC to the standards.

2.7. Mass spectrometry

Malondialdehyde (MDA) was determined by ultra-performance liquid chromatography-tandem mass spectrometry as described previously [12].

2.8. Statistical analysis

Statistical analysis was performed using GraphPad Prism 8.0 (GraphPad Software, La Jolla, CA, USA). Data are presented as mean ± SEM and were analyzed using paired or unpaired t-tests, F-tests to compare variances, RM one-way ANOVA or mixed-effects analysis where appropriate. P-values <0.05 were considered statistically significant.

3. Results

3.1. Clinical characteristics

Asthma patients were on average 28.3 ± 2.9 years of age and 70% of them was female. Clinical characteristics of asthmatics at stable disease and during loss of control are summarized in **Table 1**. The average time until loss of asthma control was 31.5 ± 4.8 days and this was associated with a significant increase in Asthma Control Questionnaire (ACQ) and Wisconsin Upper Respiratory Symptom Survey (WURSS) scores and a significant decrease in forced expiratory volume in one second (FEV_1) % predicted compared to baseline. Furthermore, loss of asthma control was accompanied by increased sputum eosinophils, whereas neutrophils remained unaffected. For the healthy controls, 30% had total IgE levels over 100 kU/L and were considered allergic. Monocyte percentages in thawed PBMCs did not differ between asthma patients and

healthy volunteers (14.87 ± 1.22 *versus* 12.37 ± 0.85; p=0.11) and were not affected by loss of asthma control (14.38 ± 0.83; p=0.51 *versus* stable asthma).

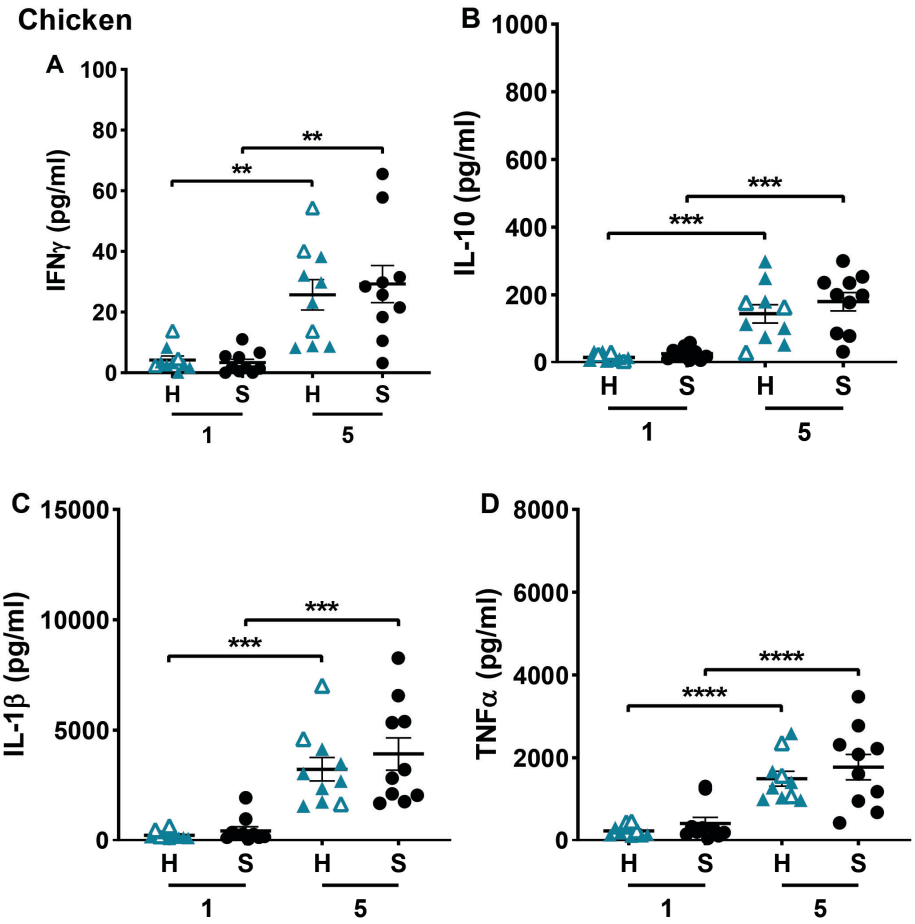
Table 1. Clinical characteristics of asthma patients at stable disease and during loss of control

	Stable	Loss of control	P-value
ACQ	6.30 ± 1.04	20.60 ± 0.88	**<0.0001**
WURSS	41.10 ± 8.97	60.10 ± 10.48	**0.04**
FEV_1 % predicted	103.0 ± 3.70	90.30 ± 5.89	**0.01**
FeNO (ppb)	39.40 ± 13.33	61.70 ± 15.40	0.24
Sputum eosinophils (%)	2.58 ± 1.45	13.46 ± 4.62	**0.03**
Sputum neutrophils (%)	41.90 ± 12.20	44.56 ± 8.30	0.65
Blood eosinophils (%)	3.39 ± 0.85	6.33 ± 2.15	0.07
Blood eosinophils (10^9/L)	0.22 ± 0.06	0.43 ± 0.17	0.13
Blood neutrophils (%)	55.19 ± 3.58	52.86 ± 3.23	0.19
Blood neutrophils (10^9/L)	3.80 ± 0.72	3.34 ± 0.32	0.35

Data (mean ± SEM) for the ten asthma patients of which PBMCs were used. Data for all patients included in the corticosteroid interruption study is provided elsewhere [12-14].
ACQ, Asthma Control Questionnaire; WURSS, Wisconsin Upper Respiratory Symptom Survey; FEV_1, forced expiratory volume in one second; FeNO, fraction exhaled nitric oxide

3.2. PBMC-derived cytokines from stable asthma patients versus healthy volunteers

For both groups, no cytokine levels were detected when PBMCs were cultured in medium only (not shown). After dose titration, 1 and 5 µg/ml BioPM collected from chicken, goat and pig farms were used to induce the production of IFNɣ, IL-10, IL-1β and TNFα by PBMCs from stable asthma patients and healthy volunteers in an apparent concentration-dependent manner (except for TNFα production induced by BioPM from the goat farm), with cell viability remaining >80% (**Figure 1** and **Figure S1** and **S2**). The source of BioPM determined the magnitude of cytokine production, with exposure to the pig farm generally inducing the highest cytokine production and exposure to the chicken farm the lowest (**Table S1**). The response to BioPM in healthy controls was not associated with allergic status, suggesting that the results for asthma patients are not allergy-related. No significant differences in response were detected between stable asthma patients and healthy controls. Yet, PBMCs from the asthma group compared to those from healthy controls showed more variability with, for the same patients, extreme (high and low) TNFα responses to the three BioPM (p<0.01, p=0.11 and p=0.07 for 1 µg/ml and p=0.13, p<0.05 and p=0.12 for 5 µg/ml BioPM from chicken, goat and pig farms, respectively).



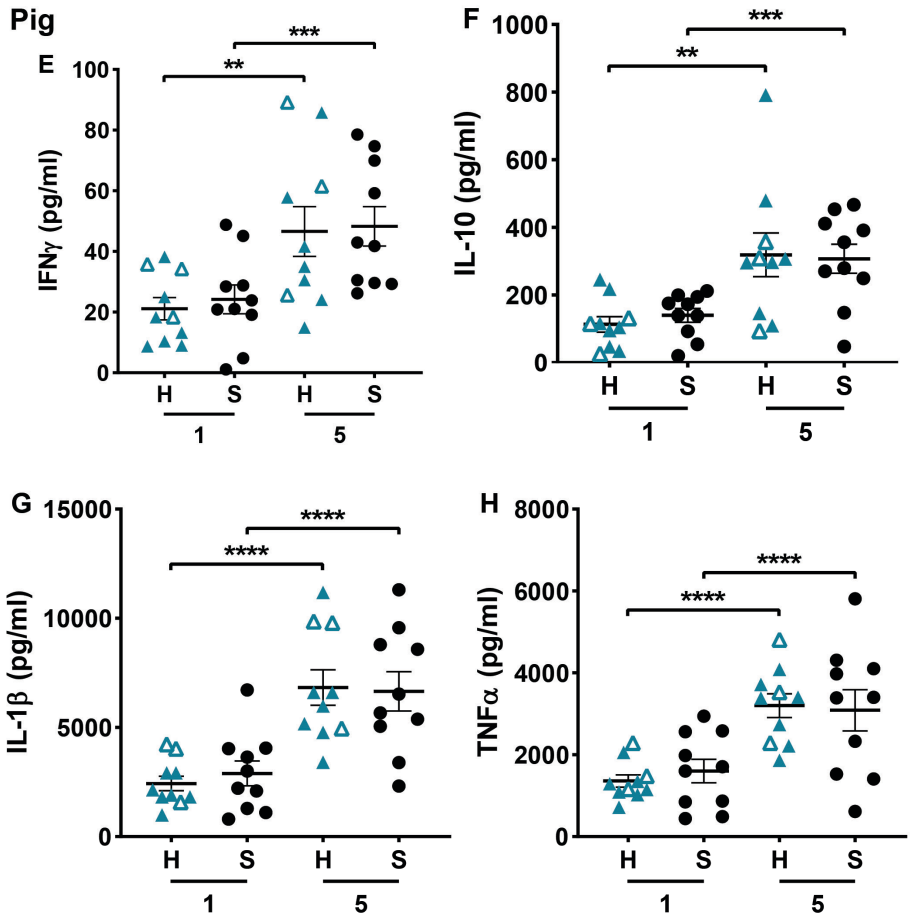
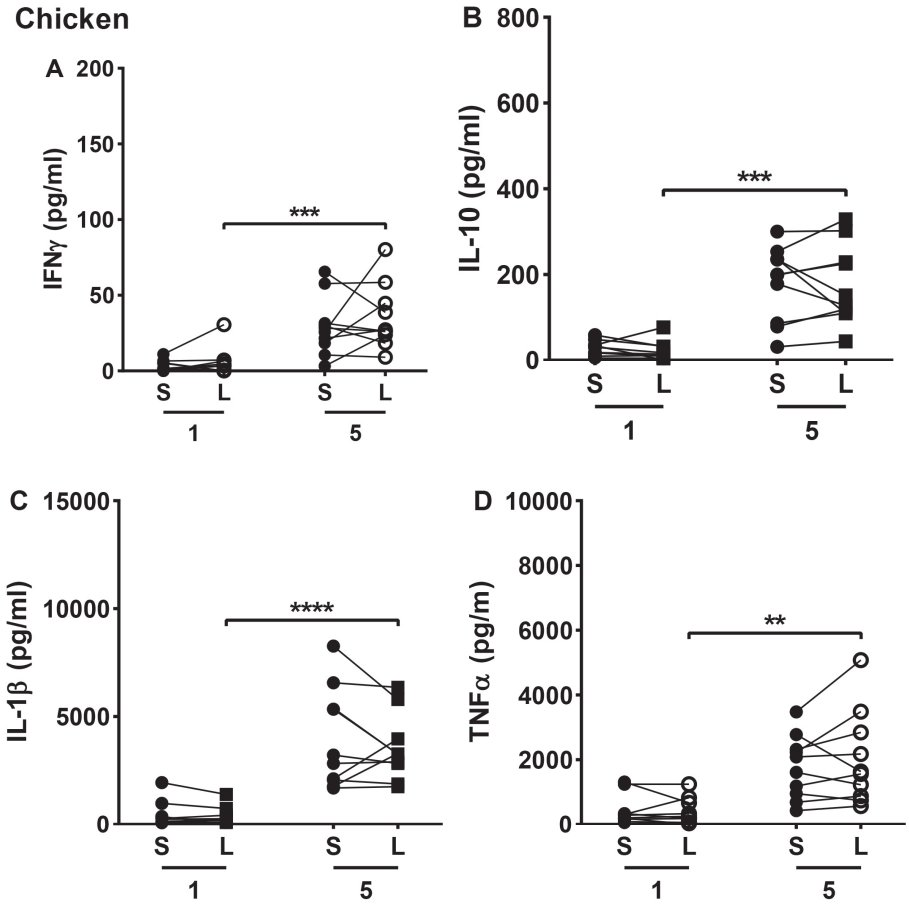


Figure 1. Cytokine production induced by BioPM (1 or 5 µg/ml) collected from chicken (**A-D**) and pig (**E-H**) farms in PBMCs from healthy volunteers (H; blue triangles; closed, non-allergic; open, allergic) and stable asthma patients (S; black dots); n=10. **p<0.01, ***p<0.001, ****p<0.0001.

3.3. PBMC-derived cytokines from stable asthma patients versus loss of control

We then questioned whether PBMCs would respond differently when obtained from patients experiencing loss of asthma control. Again, no cytokine levels were detected when PBMCs were cultured in medium only and BioPM induced cytokine production in a concentration-dependent manner by PBMCs from patients during loss of asthma control (with the exception of IFNɣ and TNFα production induced by BioPM from the goat farm) (**Figure 2** and **Figure S3**). PBMCs from asthma patients during loss of control demonstrated enhanced IFNɣ levels compared to stable disease after exposure to

1 µg/ml BioPM collected from the pig farm, but no other differences in BioPM-induced cytokine production were found between the two groups.



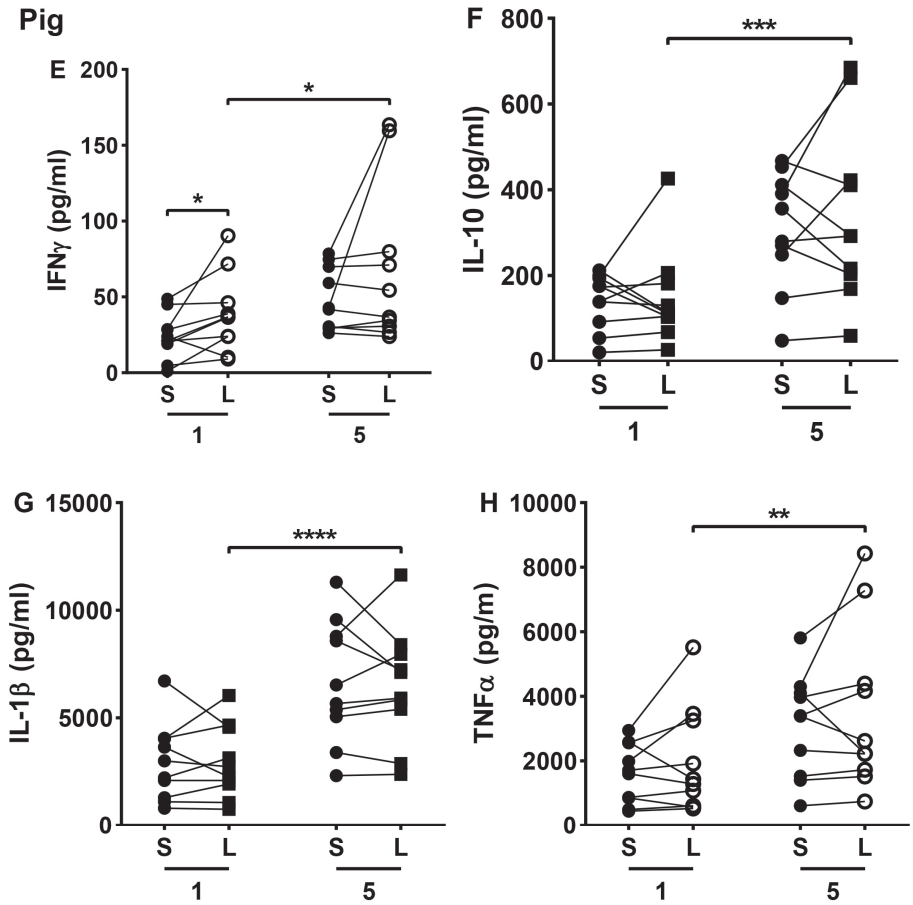


Figure 2. Cytokine production induced by BioPM (1 or 5 µg/ml) collected from chicken (**A-D**) and pig (**E-H**) farms in PBMCs from asthma patients at stable disease (S; closed dots) and during loss of control (L; open dots); n=10. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Significance for stable disease 1 *versus* 5 µg/ml is not shown.

3.4. Effect pre-treatment with antioxidants

Pre-treatment of PBMCs with the antioxidant NAC attenuated cytokine production induced by BioPM from all three farms in all three groups (**Figure 3** and **Figure S4**). NAC at a concentration of 10 mM was generally more efficient than 1 mM, with 1 mM in some cases not having any effect or even resulting in a minor increase in cytokine production. NAC by itself did not induce cytokine release (not shown). The effect of NAC appeared to be most pronounced for IL-10, where cytokine production was almost completely abolished when PBMCs were pre-treated with 10 mM NAC. In general, again no differences between healthy controls and asthma patients during stable disease and

during loss of control were found, although 5 µg/ml pig farm BioPM-exposed PBMCs from asthma patients were less responsive to pre-treatment with 1 mM NAC in terms of IL-10 production. The effect of NAC was, especially at 1 mM, most pronounced in the chicken farm, which inversely parallels the magnitude of cytokine production (**Table S2**).

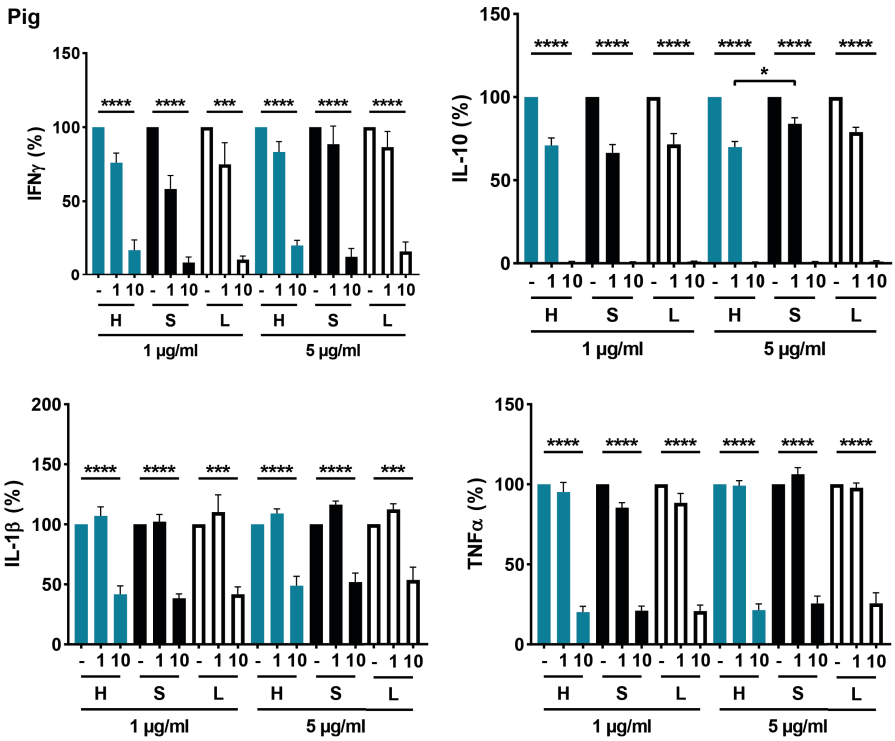


Figure 3. Effect of pre-treatment with NAC (1 or 10 mM) on cytokine production induced by BioPM (1 or 5 µg/ml) collected from pig farms in PBMCs from healthy volunteers (H; blue) and asthma patients at stable disease (S; black) and during loss of control (L; white); n=10. *p<0.05, ***p<0.001, ****p<0.0001.

In order to determine whether BioPM generates reactive oxygen species (ROS) intracellularly, e.g. by activation of mitochondria, or extracellularly, via NADPH oxidases or due to the presence of free radicals, we used the antioxidant combination of SOD and catalase. As SOD and catalase are large molecules that will not directly enter cells, this allowed us to discriminate between intracellular and extracellular ROS. In contrast to NAC, the combination of SOD and catalase did not inhibit BioPM-induced release of cytokines (**Figure 4**). In fact, the combination of SOD and catalase, without additional exposure to BioPM, resulted in enhanced cytokine production compared to unstimulated PBMCs (not shown). Why SOD and catalase induced cytokine production

by itself remains unclear, but since both commercially acquired enzymes were purified from tissues this increase could possibly be related to contaminants.

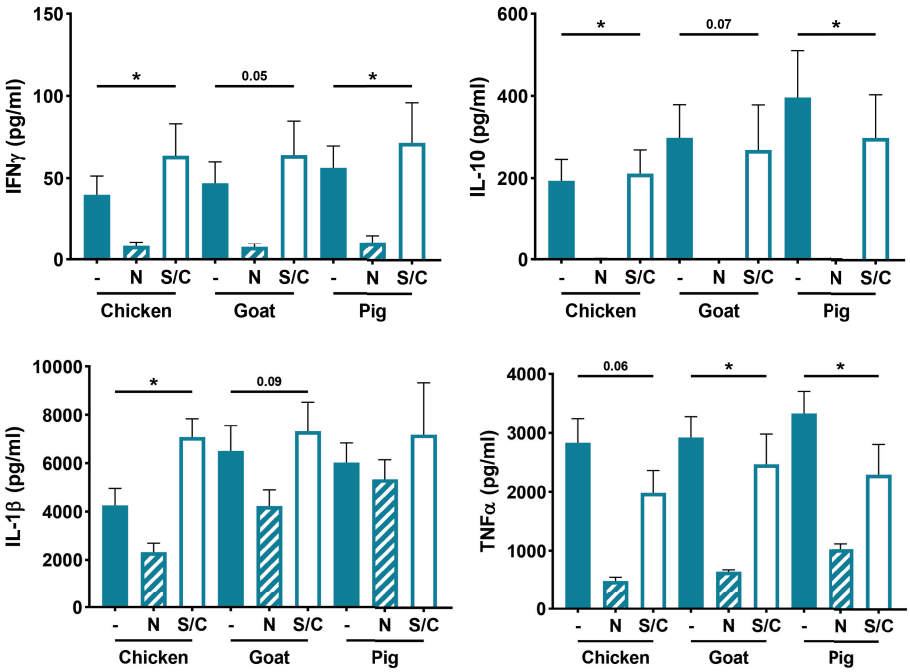


Figure 4. Effect of pre-treatment with NAC (N; striped; 10 mM) or SOD combined with catalase (S/C; white; 100 and 50 µg/ml, respectively) on cytokine production induced by BioPM (5 µg/ml) collected from chicken, goat and pig farms without pre-treatment (blue) in PBMCs from healthy volunteers; n=4. *p<0.05.

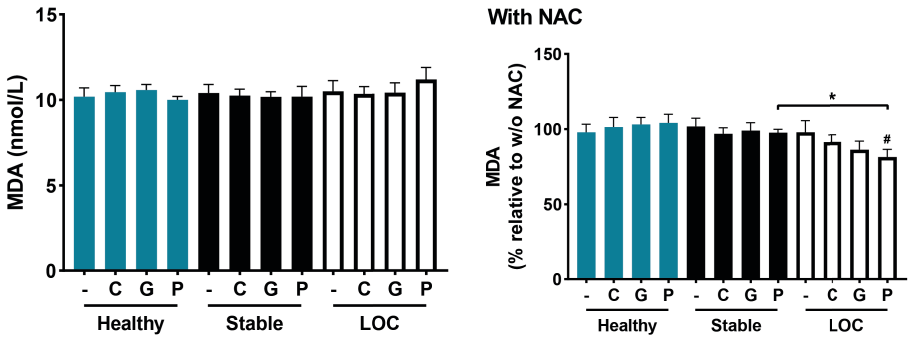


Figure 5. Effect of BioPM (5 µg/ml) collected from chicken (C), goat (G) and pig (P) farms and pre-treatment with NAC (10 mM) on oxidative stress levels in PBMCs from healthy volunteers (blue) and asthma patients at stable disease (black) and during loss of control (LOC; white); n=5. *p<0.05, #p<0.05 compared to no pre-treatment (100%).

3.5. Oxidative stress

Oxidative stress assessed as MDA in culture medium from PBMCs was not affected by exposure to BioPM and no differences were detected between groups or farms, although pig farm BioPM tended to slightly increase MDA levels in the loss of asthma control group (**Figure 5**). Pre-treatment with 10 mM NAC was able to lower levels of MDA in PBMCs from asthma patients during loss of control after exposure to BioPM collected from pig farms, but not in PBMCs from any of the other groups or after exposure to BioPM collected from chicken or goat farms. This was also the only condition being significantly lower compared to the stable disease counterparts.

4. Discussion

There is increasing evidence that PM from livestock affects respiratory diseases including asthma [13-15]. Whereas traffic-related PM effects are claimed to be mediated by ROS, this is less likely for BioPM. In the present study, we demonstrated that BioPM derived from chicken, goat and pig farms induced cytokine production by PBMCs from healthy and asthmatic individuals (potency rank: pig>goat>>chicken) with an apparent dose-dependency. These inflammatory events were abrogated by pre-treatment with NAC, but not SOD and catalase, suggestive of a mechanism (partly) related to intracellular ROS generation. No marked differences in inflammatory response to BioPM were detected between PBMCs from healthy controls and asthma patients. However, PBMCs obtained during loss of asthma control demonstrated an enhanced IFNγ response upon exposure to BioPM from the pig farm, which was paralleled by oxidative stress.

We measured increased levels of IFNγ, IL-10, IL-1β and TNFα upon stimulation with BioPM, which parallels earlier findings where levels of IL-1β in PBMCs and TNFα in serum from healthy volunteers were significantly higher after exposure to dust from swine confinement buildings, indicating that these cytokines may be associated with regulating the inflammatory response after inhalation of BioPM [16]. Furthermore, work with human U937 macrophages showed increased TNFα mRNA levels and other pro-inflammatory marker genes after exposure to PM collected from dairy farms [17]. TNFα is particularly of interest in the context of asthma as it was reported to determine the severity of hyperresponsiveness [18] and contribute to an exaggerated inflammatory response by bronchial epithelial cells from most asthma patients in the presence of IL-17 [19]. Here, the BioPM-induced TNFα response tended to be more variable with some extremes (high and low) for PBMCs from asthma patients compared to those from healthy subjects, although no differences were observed when comparing the asthma population as a whole. Nevertheless, as this extreme TNFα production was

found for the same patients with all three BioPM and not for the three other cytokines, this indicates that this is a genuine finding. We did not find any other differences in cytokine production between stable asthma patients and healthy volunteers, which is in agreement with blood responsiveness upon *ex vivo* stimulation with EHC-93 urban dust [20] and inflammatory responses upon *in vivo* exposure to ambient particles collected in Los Angeles [21]. On the other hand, it contradicts several other studies that found attenuated reactions to diesel exhaust in asthmatic individuals compared to controls [22, 23], which may relate to the specific nature of this PM. Similarly, levels of the oxidative stress marker MDA were comparable between stable asthma patients and healthy controls, yet not affected by exposure to BioPM. Previous studies, however, have shown that high pollution/PM induced oxidative stress levels in exhaled breath condensate and urine in healthy young adults and schoolchildren [24, 25], although this increase in oxidative stress was similar between non-asthmatics and asthmatics [26].

The lack of exaggerated inflammatory and oxidative stress events in PBMCs from asthma patients as a whole to BioPM was surprising as subjects with pre-existing respiratory disease are more susceptible to traffic-related PM-induced injury. This discrepancy between traffic-related PM and BioPM is presumably due to the presence of free radicals and/or chemical components in the first. The microbiota profiles present in our BioPM were identified previously, where it was also demonstrated that blocking of TLR4 interfered with cytokine production by MM6 cells stimulated with BioPM [7]. It is thus likely that mainly liposaccharides are responsible for the observed BioPM-induced inflammatory responses, although future studies should determine whether free radicals are truly absent in our BioPM. Clinical consequences may therefore depend on the source of PM, that may not only differ in composition but may, dependent on size, also deposit at different regions in the lung.

To the best of our knowledge, this is the first study that includes *ex vivo* BioPM exposure during loss of asthma control. Yet, the vast majority of BioPM-induced inflammatory responses was similar to stable asthma, whereas previous studies postulated greater effects of PM in more severe disease [27]. We did, however, detect increased IFNγ levels during loss of asthma control after 1 but not after 5 µg/ml pig farm exposure, which suggests a lower threshold for IFNγ induction by PBMCs from these patients. Enhanced IFNγ production by PBMCs was also seen for asthmatic children, but despite similar seroprevalence not in non-asthmatic children, in an *in vitro* response to *Chlamydia pneumoniae*, which has been associated with asthma exacerbations [28]. Still, the pathophysiological effect of enhanced IFNγ responses is unknown, although we have

shown before that the IFN response during rhinovirus-induced loss of asthma control correlates with eosinophilic inflammation and drop in FEV_1 [29].

Pre-treatment with the antioxidant NAC suppressed BioPM-induced cytokine production in all groups and counteracted oxidative stress in pig farm BioPM-exposed PBMCs from patients during loss of asthma control. These effects are therefore probably related to defective antioxidant defenses as suggested previously in mice exposed to wildfire PM [30]. Induction of antioxidant defenses using vitamin supplementation has previously been demonstrated to attenuate the impact of air pollutants in children with asthma [31] and in mouse models of ovalbumin-induced experimental asthma [32, 33]. The complete inhibition of IL-10 release by pre-treatment with 10 mM NAC was remarkable. It is not unlikely that the production of IL-10 is more sensitive to oxidative stress than that of other cytokines measured, as was also reported before [34]. The combination of SOD and catalase did not abolish cytokine release, indicating that ROS generation occurred mainly intracellular, though validation in a larger cohort is necessary. This would to a certain extent exclude the presence of a free radical source in BioPM and further supports BioPM-induced cell activation by TLR ligands and dysfunctional or increased mitochondrial respiration, leading to excessive ROS production and inflammatory events. The association between PM exposure and damaged mitochondria has been described before in healthy subjects [35].

One of the strengths of this study is that we compared PBMCs from the same asthmatic patients during stable disease and loss of control, which allowed us to directly determine any effects induced by acute worsening of the disease. However, we do realize that this study also has several limitations, including small sample sizes and the use of PBMCs instead of purified monocytes, monocyte-derived macrophages or airway macrophages. Whereas airway macrophages, as an important target for (Bio)PM, would have been the preferred cells of choice for this study, their collection before and during loss of control was not contemplated due to ethical constraints. The use of PBMCs over monocyte-derived macrophages in this study was based on the potential loss of imprinting by *in vitro* maturation of monocytes towards macrophages and the potential loss of monocytes during their purification. Furthermore, PBMCs demonstrated differential gene expression upon diesel exhaust inhalation in healthy volunteers, including inflammatory and oxidative stress pathways [36, 37], indicating that PM effects are not limited to the airways but can also be found in the circulation. In fact, it has been suggested previously that fine PM elicits systemic effects rather than respiratory symptoms [38, 39].

In summary, BioPM induced cytokine release by PBMCs from healthy and asthmatic subjects via a mechanism partly related to ROS that is mainly generated intracellularly. At large, we found no significant differences between the responses of PBMCs from controls and asthma patients, although for asthma all three BioPM induced extreme TNFα responses in the same patients. Therefore, some patients may respond in an exaggerated manner to BioPM, which likely contributes to enhanced inflammation and possibly may lead to enhanced loss of asthma control. Interestingly, PBMCs from patients during loss of asthma control showed enhanced IFNγ and oxidative stress responses upon stimulation with BioPM from the pig farm, indicating increased susceptibility to this particular livestock. Future research should include BioPM from a larger number of different animal farms and focus on the actual components responsible for inflammation and oxidative stress upon exposure to farm-derived BioPM. Our findings also support that individuals should be made aware of the potential effect when working in or living near animal industries.

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Supplementary

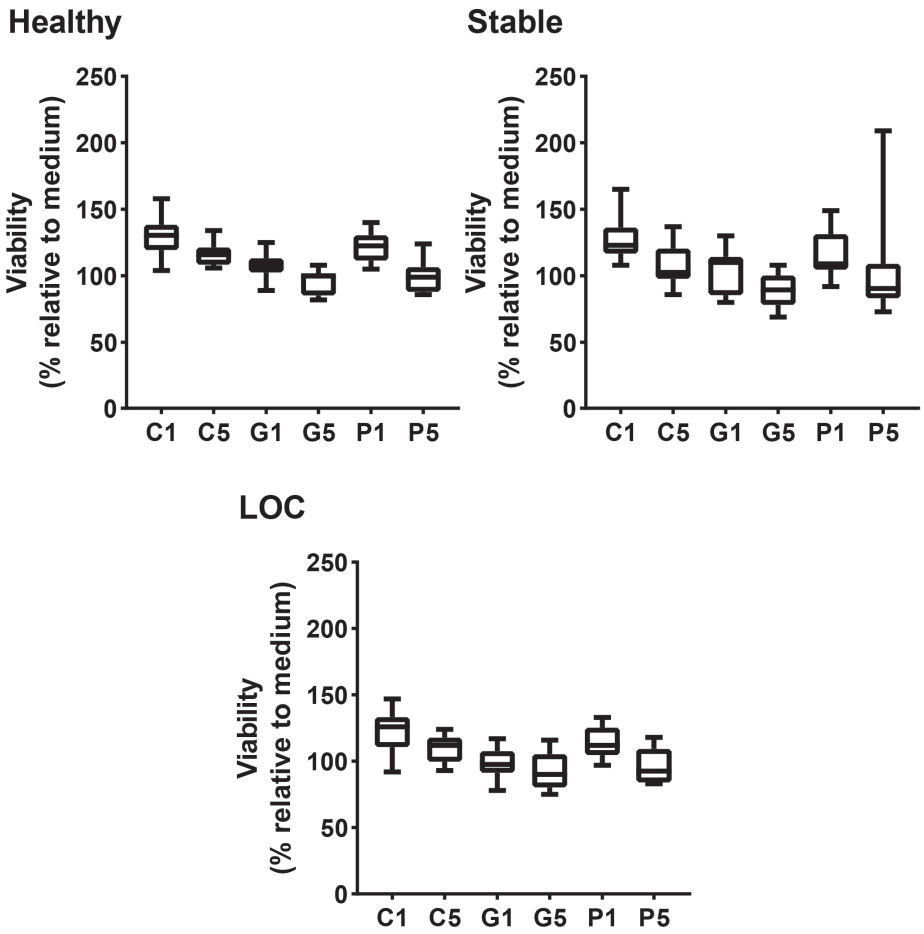


Figure S1. Viability of PBMCs from healthy volunteers and asthma patients at stable disease and during loss of control (LOC) upon exposure to BioPM (1 or 5 µg/ml) collected from chicken (C), goat (G) and pig (P) farms; n=10.

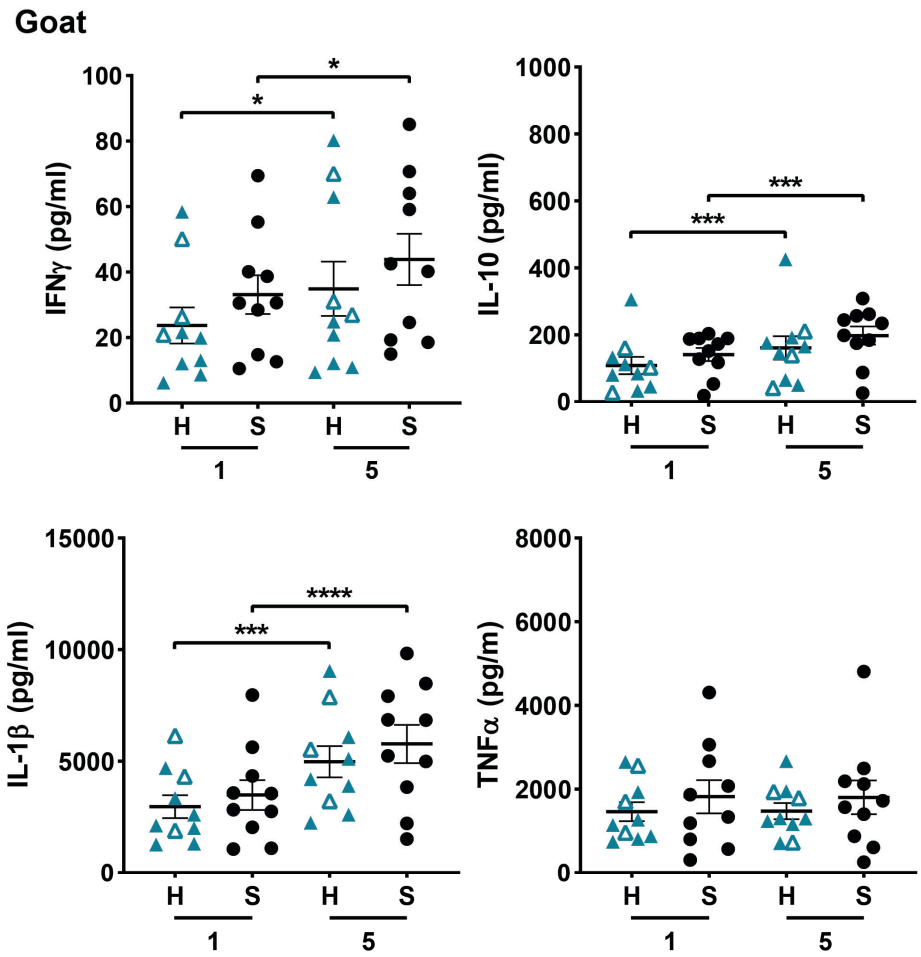


Figure S2. Cytokine production induced by BioPM (1 or 5 µg/ml) collected from the goat farm in PBMCs from healthy volunteers (H; blue triangles; closed, non-allergic; open, allergic) and stable asthma patients (S; black dots); n=10. *p<0.05, ***p<0.001, ****p<0.0001.

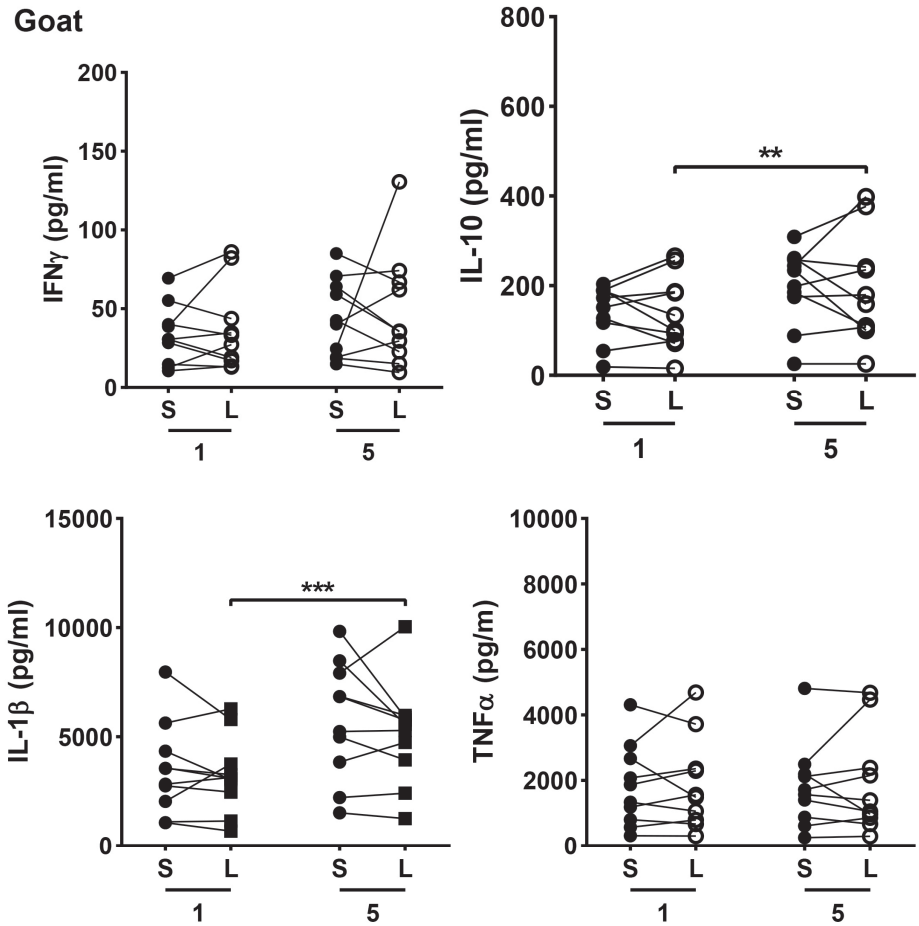
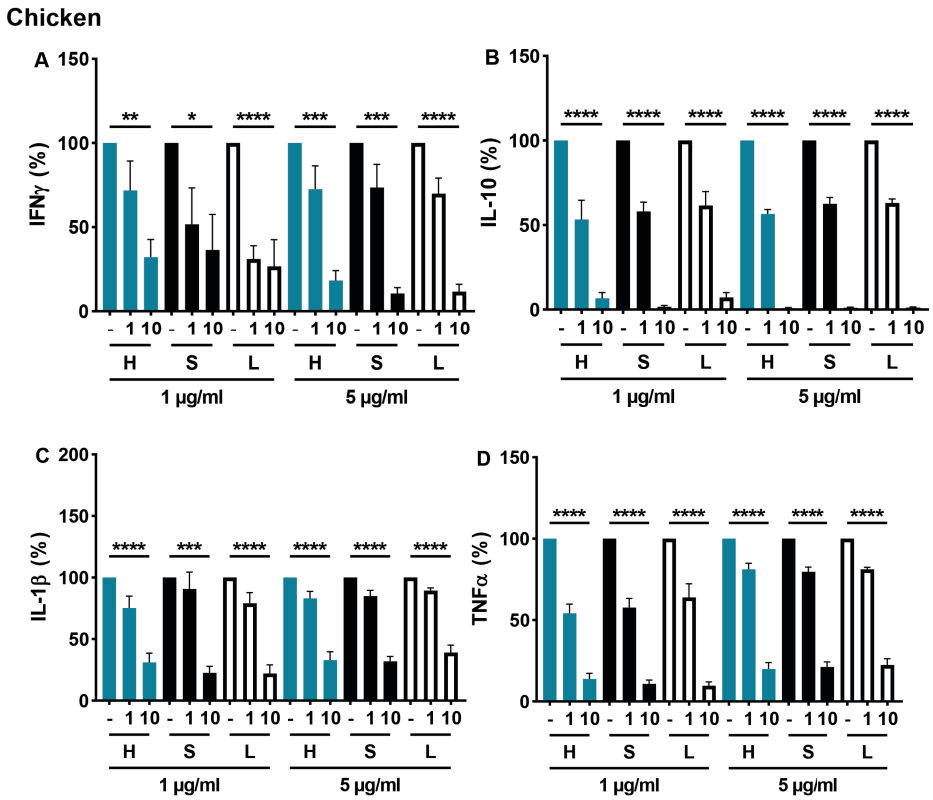


Figure S3. Cytokine production induced by BioPM (1 or 5 µg/ml) collected from the goat farm in PBMCs from asthma patients during stable disease (S; closed dots) and during loss of control (L; open dots); n=10. **p<0.01, ***p<0.001



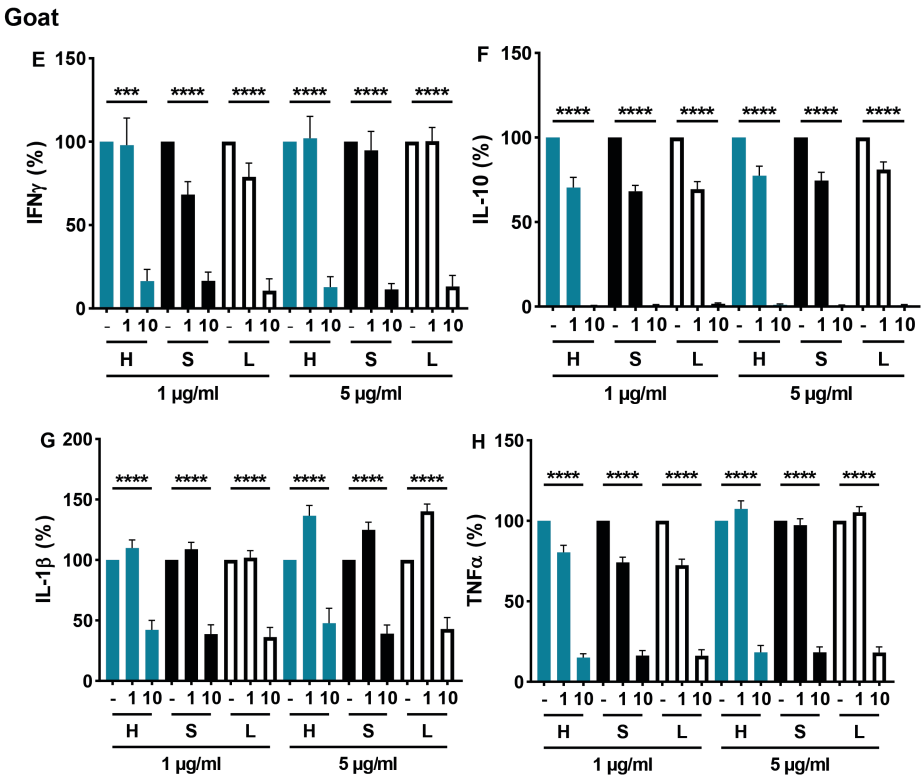


Figure S4. Effect of pre-treatment with NAC (1 or 10 mM) on cytokine production induced by BioPM (1 or 5 µg/ml) collected from chicken (**A-D**) and goat (**E-H**) farms in PBMCs from healthy volunteers (H; blue) and asthma patients during stable disease (S; black) and during loss of control (L; white); n=10. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Table S1. Cytokine production in PBMCs from all volunteers induced by BioPM (1 or 5 µg/ml) collected from chicken, goat and pig farms

	[BioPM]	Chicken	Goat	Pig	P-value
IFNɣ	1	4.41 ± 1.10	31.28 ± 3.91	28.05 ± 3.55	**<0.0001**
	5	30.10 ± 3.41	42.34 ± 5.31	54.32 ± 6.58	**<0.0001**
IL-10	1	20.75 ± 3.35	129.4 ± 13.68	133.7 ± 15.21	**<0.0001**
	5	166.4 ± 15.86	184.3 ± 19.06	322.6 ± 32.65	**<0.0001**
IL-1β	1	347.6 ± 78.14	3242 ± 326.4	2753 ± 276.7	**<0.0001**
	5	3565 ± 332.8	5283 ± 434.4	6658 ± 478.0	**<0.0001**
TNFα	1	345.8 ± 65.94	1727 ± 207.3	1644 ± 201.6	**<0.0001**
	5	1765 ± 189.0	1726 ± 216.1	3275 ± 321.6	**<0.0001**

Data are represented as mean ± SEM; n=30

Table S2. Effect of NAC (1 or 10 mM) on cytokine production in PBMCs from all volunteers induced by BioPM (1 or 5 µg/ml) collected from chicken, goat and pig farms

	[BioPM]/[NAC]	Chicken	Goat	Pig	P-value
IFNɣ	1/1	54.18 ± 10.52	81.71 ± 6.77	70.20 ± 6.16	0.06
	1/10	32.22 ± 8.99	14.57 ± 3.65	12.13 ± 2.72	**0.02**
	5/1	72.02 ± 6.97	99.06 ± 6.22	86.13 ± 5.71	**0.006**
	5/10	13.50 ± 2.71	12.48 ± 3.15	16.31 ± 3.02	0.21
IL-10	1/1	57.51 ± 4.92	69.35 ± 2.66	69.63 ± 3.06	**0.03**
	1/10	5.20 ± 1.50	1.00 ± 0.22	0.88 ± 0.14	**0.007**
	5/1	60.71 ± 1.78	77.67 ± 2.86	77.58 ± 2.15	**<0.0001**
	5/10	1.06 ± 0.19	0.91 ± 0.18	0.82 ± 0.18	0.30
IL-1β	1/1	81.74 ± 6.31	106.9 ± 3.47	106.5 ± 5.60	**0.002**
	1/10	25.40 ± 3.76	39.11 ± 4.37	40.65 ± 3.24	**0.001**
	5/1	85.82 ± 2.50	133.9 ± 4.11	112.6 ± 2.25	**<0.0001**
	5/10	34.64 ± 3.23	43.22 ± 5.55	51.45 ± 4.92	**<0.0001**
TNFα	1/1	58.39 ± 3.75	75.67 ± 2.25	89.73 ± 2.96	**<0.0001**
	1/10	11.47 ± 1.62	15.87 ± 1.77	20.70 ± 1.91	**0.0001**
	5/1	80.70 ± 1.59	103.4 ± 2.46	101.1 ± 2.03	**<0.0001**
	5/10	21.19 ± 2.06	18.27 ± 2.07	24.21 ± 2.90	**0.007**

Data are represented as percentages relative to without NAC; mean ± SEM; n=30

Chapter 4

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

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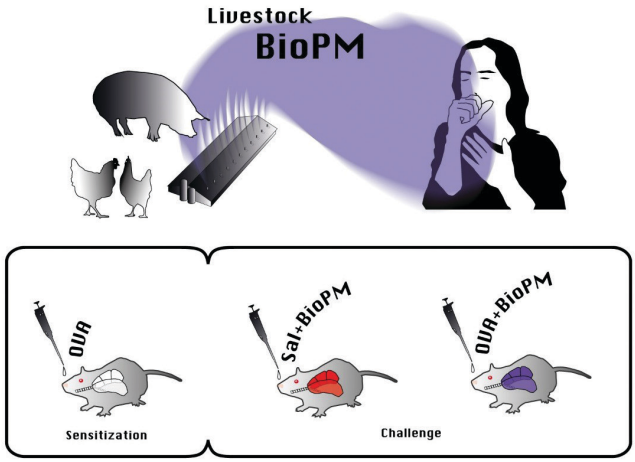
Abstract art



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, Th2, Th17, Murine, Ovalbumin

1. 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 is 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 farmers [5] and people living in an area with a high livestock density [3, 6].

Asthma, characterized by bronchial hyperresponsiveness, 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.

2. Materials and Methods

2.1. 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 (Pig 1 (commercial farm), Pig 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 hours 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 (Pig 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].

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

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 (**Figure 1**).

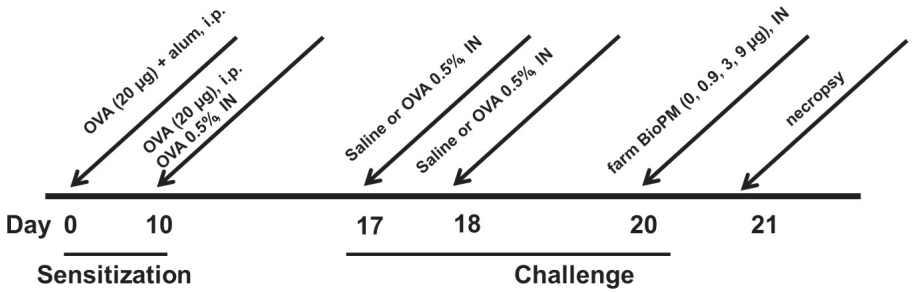


Figure 1 Study design. Mice were intraperitoneally sensitized with 20 µg OVA with alum on day 0, followed by intraperitoneal administration of 20 µg OVA and intranasal administration of 0.5% OVA on day 10. On days 17 and 18, OVA-sensitized mice were challenged intranasally with 0.5% OVA or saline alone. 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 hours after the last intranasal challenge.

2.3. Intranasal instillation, necropsy, lavage collection and tissue preparation

Mice were aneuthanized 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.

2.4. 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% 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.

2.5. 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 RT 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-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 used when the following criteria were met: at least 3 out of 6 animals per group showed cytokine levels >10 pg/ml.

2.6. 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.

2.7. 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 two chicken farms was pooled to increase sample size, the same was done for the two 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.

3. Results

3.1. Pulmonary histopathology

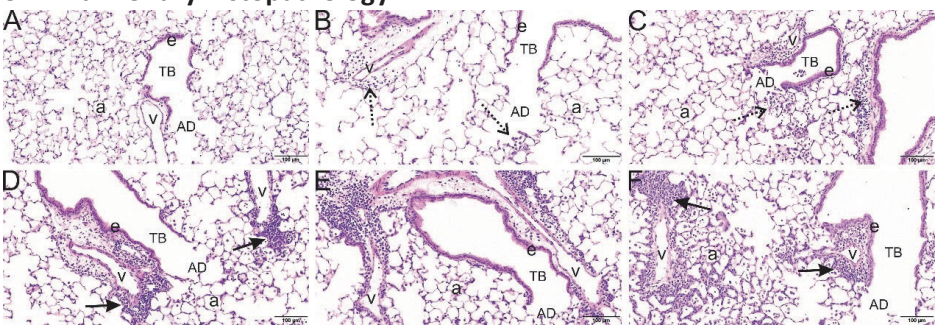


Figure 2. Light photomicrographs of lung tissue sections from mice intranasally challenged with (**A**) saline (vehicle control; 0 µg BioPM), (**B**) saline and 9 µg of Chicken 1 BioPM, (**C**) saline and 9 µg of Pig 1 BioPM, (**D**) OVA (0 µg BioPM), (**E**) OVA and 9 µg of Chicken 1 BioPM, or (**F**) 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.

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 nonallergic 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 challenged, saline-instilled control mice (0 ug BioPM) **(Fig 2 D)**. Morphometric determination of the pulmonary densities of AB/PAS-stained mucosubstances in bronchiolar epithelium **(S Fig 2 A-D)** or major basic protein-laden eosinophils **(S Fig 3 A, 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)**.

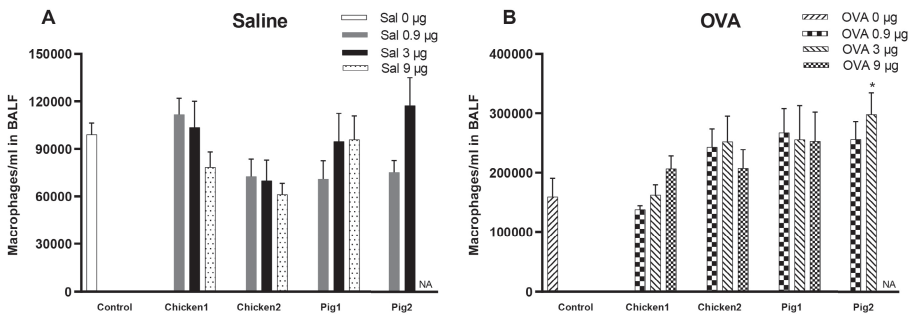
Compared to saline treated control animals, mice that were challenged only with OVA and no BioPM had a significant increase in BALF total cells (1.1×10e5 ± 2.2×10e4

versus 3.1×10e5 ± 1.5×10e5 in BALF of non-allergic and allergic mice, respectively). This increase in total cells was due to the dramatic increase in eosinophils and neutrophils.

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 5 H, 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)**.

3.2. Inflammatory cells in BALF



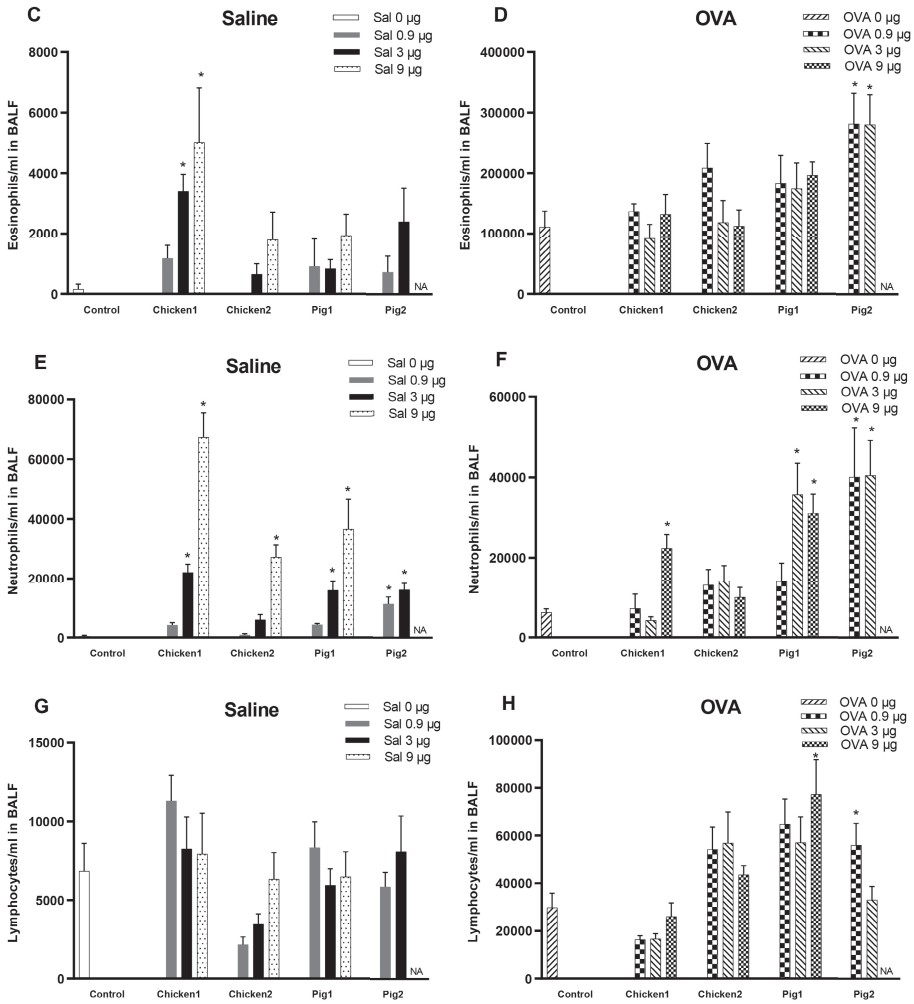


Figure 3. Macrophages (Sal (**A**), OVA (**B**)), eosinophils (Sal (**C**), OVA (**D**)), neutrophils (Sal (**E**), OVA (**F**)) and lymphocytes (Sal (**G**), OVA (**H**)) cell counts in BALF.

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 ($p < 0.05$)

3.3. OVA-specific Immunoglobulin levels in blood plasma

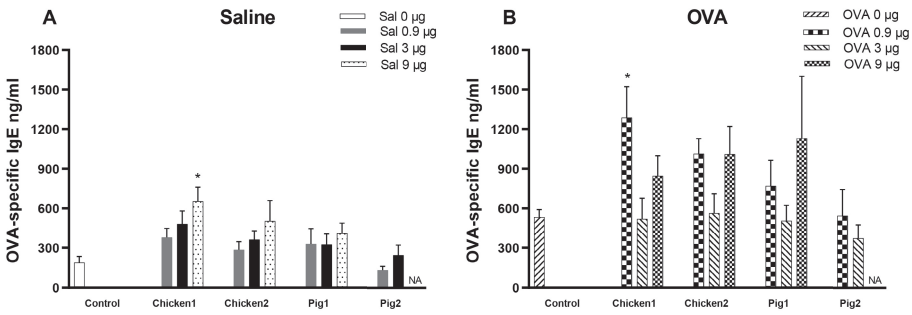


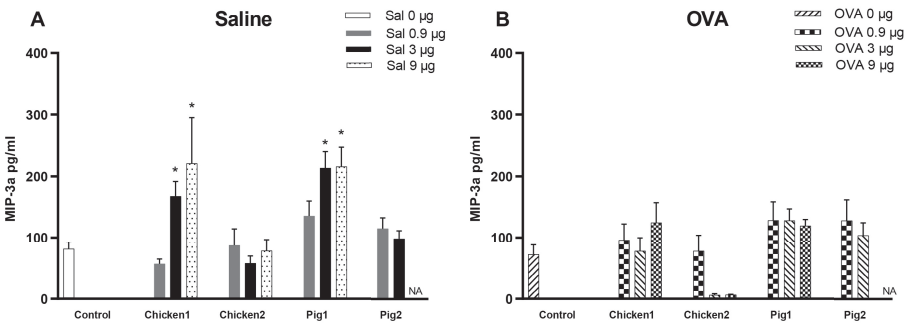
Figure 4. OVA specific IgE production (Sal (**A**), OVA (**B**)) in plasma.

Note: Samples of 9 µg 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 ($p < 0.05$)

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 µg 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)**.

3.4. Cytokine and chemokine expression in BALF



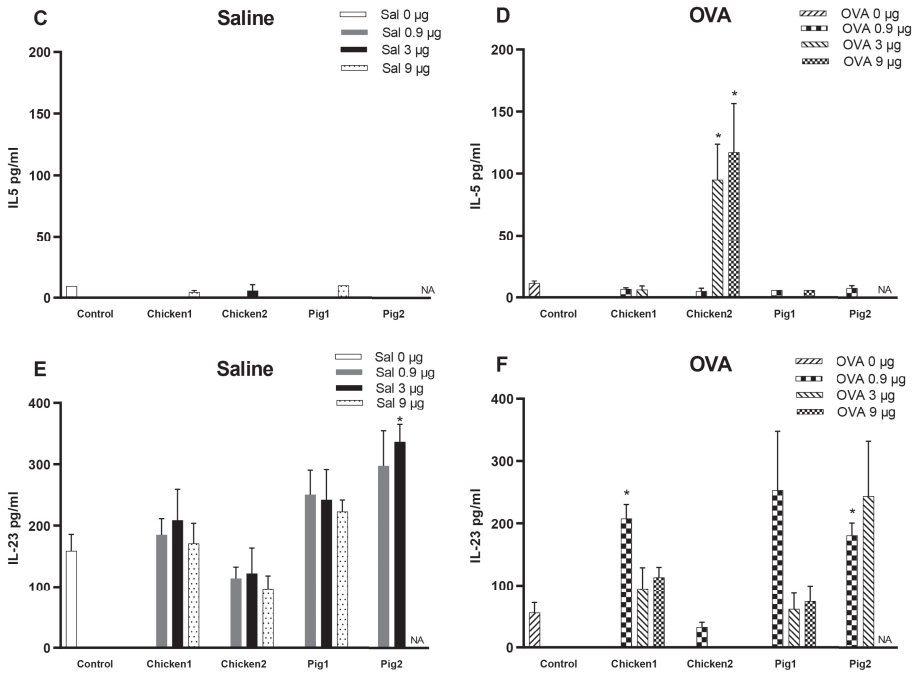


Figure 5. Production of cytokines and chemokines in BALF. MIP-3a (Sal (**A**), OVA (**B**)), IL-5 (Sal (**C**), OVA (**D**)) and IL-23 (Sal (**E**), OVA (**F**)) were analyzed by a Luminex.

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 ($p < 0.05$)

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

4. 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].

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 stables [34] 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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Supplementary

Lung Morphometry

Histologic slides containing the lung G5 and G11 tissue sections were scanned and digitized with a slide scanner (VS110, Olympus America, Center Valley, PA), and evaluated via stereological methods with newCAST software (VisioPharm, Hoersholm, Denmark). For quantification of major basic protein (MBP)-positive eosinophils, digitized images of the lung G5 and G11 lung sections were selected as regions of interest and 40% of the lung tissue was captured at 400x magnifications by systematic random sampling. Percentage of MBP-positive cells in the total lung tissue and in three discrete regions of 1) the parenchyma (alveoli and alveolar ducts), 2) perivascular and peribronchial interstitial spaces, and 3) other regions (inside blood vessels, bronchiole airspaces and pleura, were estimated using Stepanizer stereology software with a point grid by dividing number of points hitting areas positive for MPB ($a_{(p)}$ postive) by the total number of points falling on all lung tissue (MBP-positive and –negative; $a_{(p)}$ reference tissue). For each region, percent density of eosinophils was calculated with the equation:

$$\text{Eosinophil Density (\%)} = \frac{(\text{No. positive cells}) \times a(p)_{\text{positive cell}}}{(\text{No. reference tissues}) \times a(p)_{\text{Ref tissue}}} \times 100$$

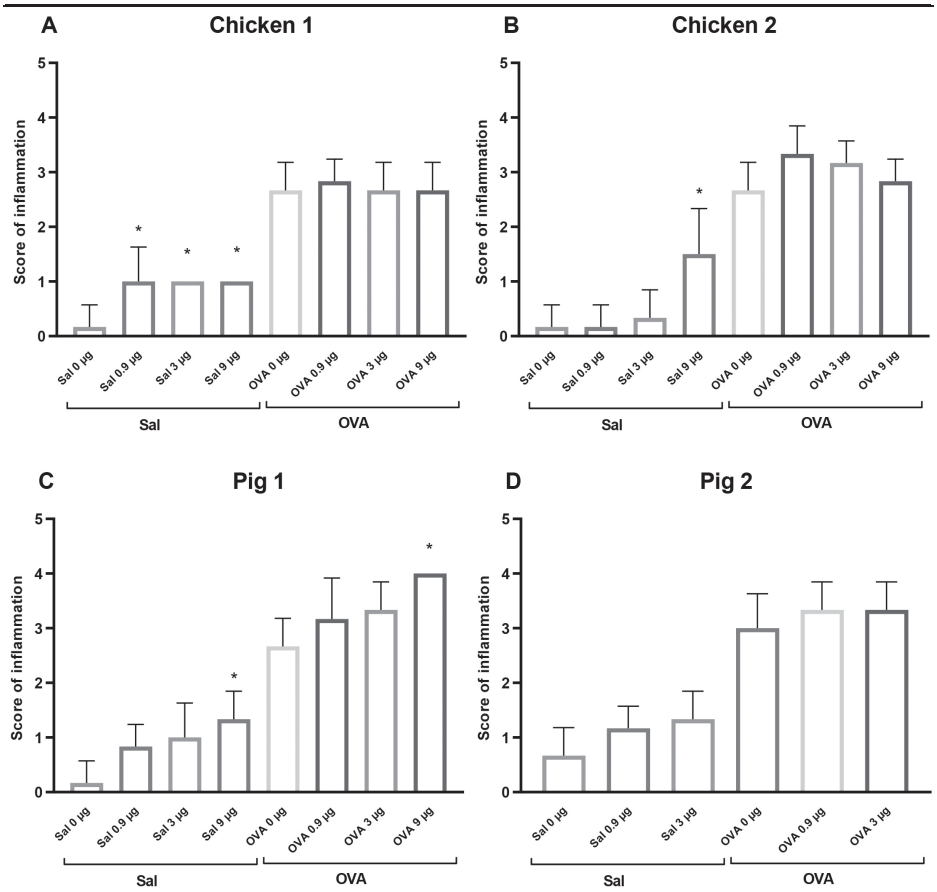
For quantification of AB/PAS-positive mucosubstances in the bronchiolar epithelium, all bronchiolar epithelium lining G5 and 11 along the main axial airway were selected and captured at 400x magnifications. A point intercept grid was placed over the sampled images to estimate density of mucosubstances per basal lamina. The number of points hitting AB/PAS-positive mucosubstances (Pm) was counted. The density of AB/PAS-positive mucosubstances ($\hat{V}_m$) was estimated by multiplying the total number of Pm by the area/point (a/p) and dividing them by the number of points hitting the reference space (n) as shown in the equation.

$$\hat{V}_m = \frac{\sum P_m \times a/p}{n}$$

The surface density of the basal lamina ($\hat{S}_{BL}$) in the selected images was estimated by counting the number of intercepts (*I*) of the line probe with the basal lamina of the lateral wall divided by the length per point (*l/p*) and the number of points falling on the reference space (*n*) as described:

$$\hat{S}_{BL} = \frac{2 \times \sum I}{l/p \times n}$$

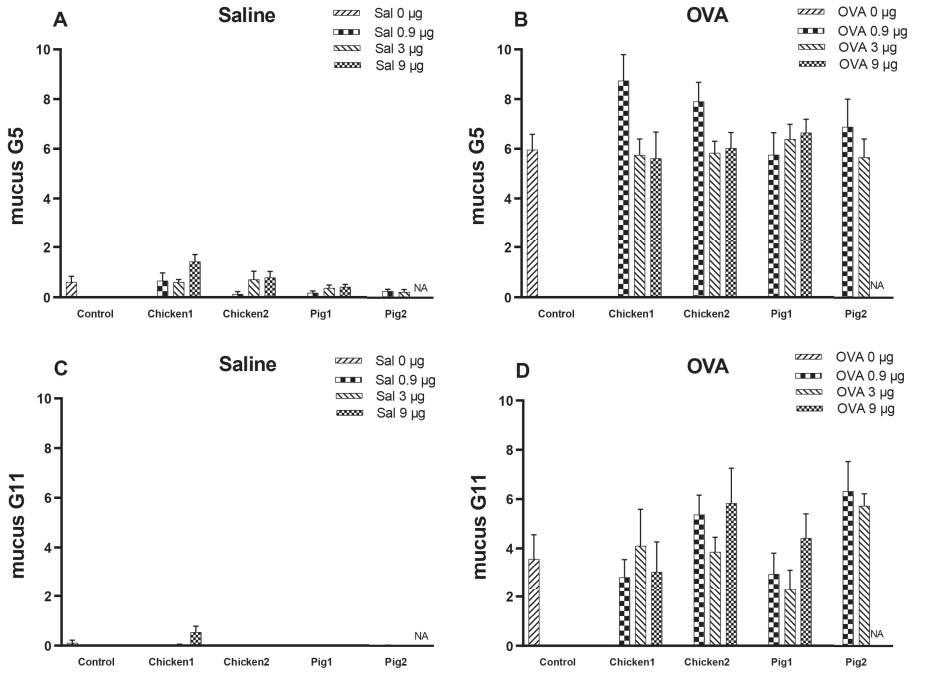
The positive density per basal lamina of the bronchiolar epithelium was then estimated by dividing $\hat{V}_m$ by $\hat{S}_{BL}$.



S Fig 1. Semi-quantitative scoring results from airway inflammation after exposure to BioPM from Chicken 1(**A**), Chicken 2 (**B**), Pig 1(**C**) and Pig 2 (**D**).

Note: Samples of 9 µg BioPM from Pig 2 were absent due to low BioPM contration. Graph represents mean (SEM).

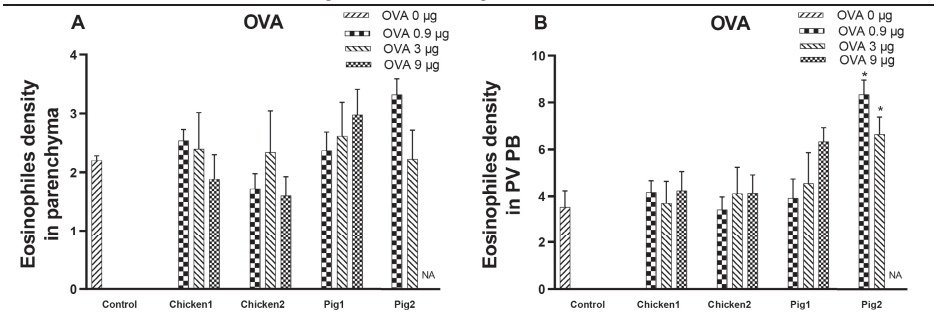
Airway epithelial mucus production



S Fig 2. Airway epithelial mucus production at the level of axial airway generation 5 (Sal (**A**), OVA (**B**)) and distal airway generation 11 (Sal (**C**), OVA (**D**)). Increase in airway epithelial mucus, as a feature of allergic airway disease, was analyzed on lung tissue at the G5 and G11 of the intraepithelial AB/PAS stained mucosubstances in saline and OVA-challenged mice.

Note: Samples of 9 µg BioPM from Pig 2 were absent (NA: data not available) due to low BioPM concentration. Graph represents mean (SEM).

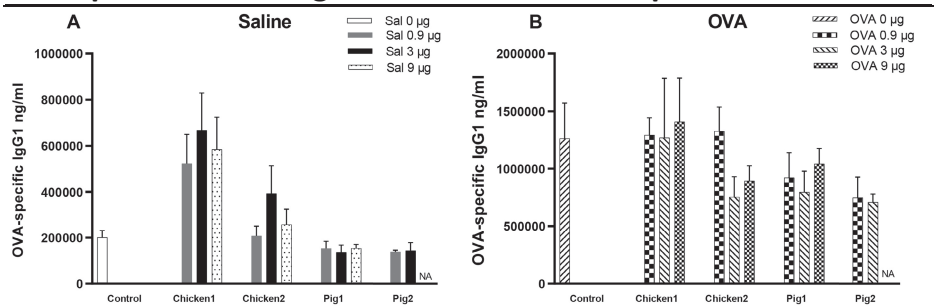
Immunohistochemistry of eosinophils



S Fig 3. Immunohistochemistry of airway eosinophils. Photomicrographs of parenchyma (**A**) and peri-bronchiolar/vascular (PB/PV) area (**B**) surrounding the axial airway in OVA-challenged mice.

Note: Samples of 9 µg BioPM from Pig 2 were absent (NA: data not available) due to low BioPM concentration. Graph represents mean (SEM). * significantly different from respective 0 µg group ($p < 0.05$)

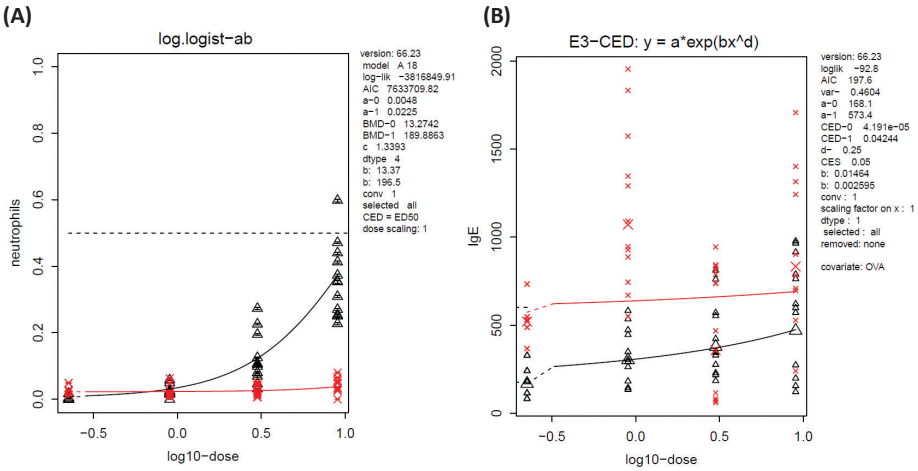
OVA-specific Immunoglobulin levels in blood plasma



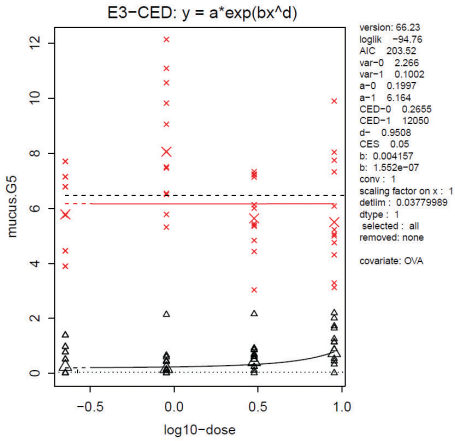
S Fig 4. OVA specific IgG1 production (Sal (**A**), OVA (**B**)) in plasma.

Note: Samples of 9 µg BioPM from Pig 2 were absent (NA: data not available) due to low BioPM concentration. Graph represents mean (SEM).

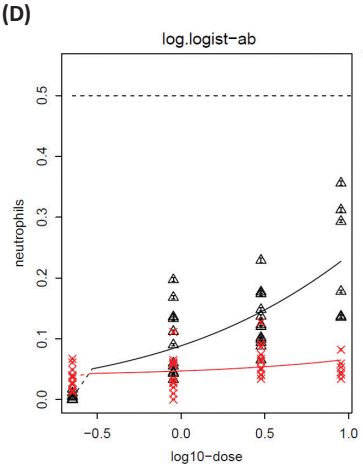
Dose-response models and benchmark dose



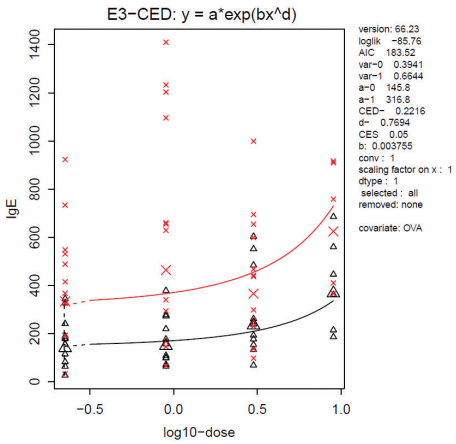
(C)



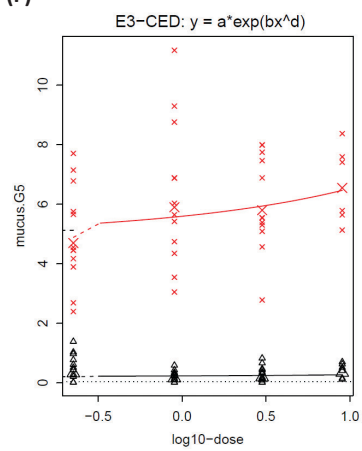
(D)



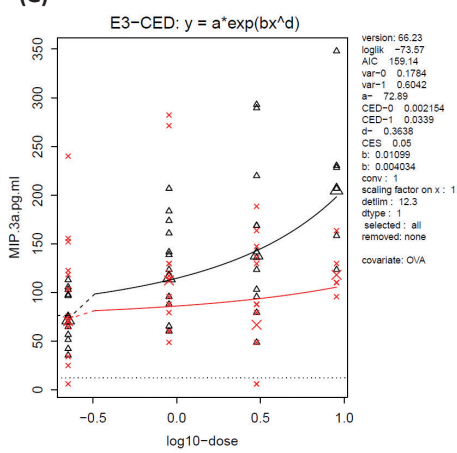
(E)

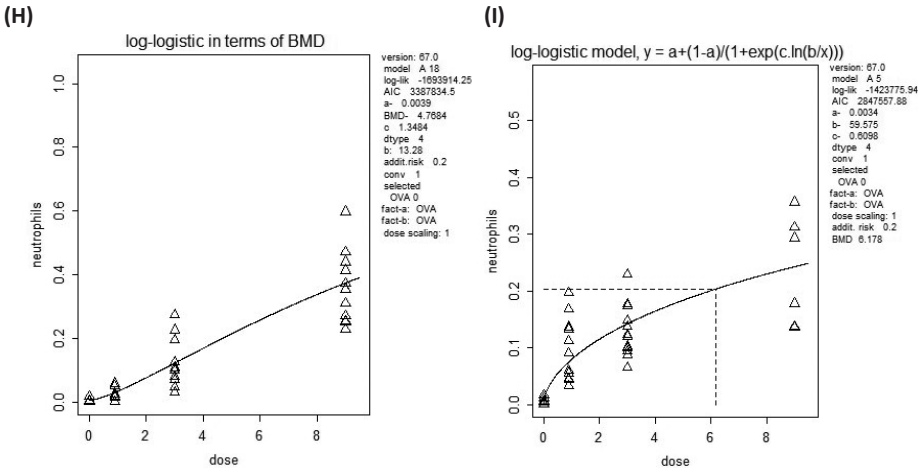


(F)



(G)





S Fig 5. BMDs calculated by dose-response modeling

Does response curves for the percentage neutrophils (**A**), OVA-specific IgE (**B**) and mucus (**C**) induced by **chicken** farms BioPM in the saline group (black triangles) and OVA group (red crosses). Does-response curves for the percentage neutrophils (**D**), OVA-specific IgE (**E**), mucus (**F**) and MIP-3a (**G**) induced by **pig** farms BioPM in the saline group (black triangles) and OVA group (red crosses). BMDs were calculated for the percentage neutrophils in the mice that were exposed to chicken farms BioPM (**H**) and pig farms BioPM (**I**).
Note: Samples of 9 μg BioPM from Pig 2 were absent due to low BioPM concentration.

When comparing to saline-challenged mice, the groups that were OVA-challenged showed considerably higher airway allergic response with the exception of the percentage neutrophils and MIP-3a **(S Fig 4 A, G**).

Clear dose-related increases in the percentage neutrophils were observed in mice that were exposed to chicken and pig farms BioPM in saline-challenged mice (indicated by black triangles) **(S Fig 4 A, D)**. A dose-dependent increase in MIP-3a and OVA-specific IgE production were detected after treatment with pig farms BioPM only **(S Fig 4 E, G)**. We also found a mild tendency of increased mucus secretion in saline-challenged mice after administration of chicken farms BioPM **(S Fig 4 C)**, whereas a similar effect appeared in OVA-challenged mice (indicated by red crosses) after exposure to pig farms BioPM **(S Fig 4 F)**.

Subsequently, the curves that showed an apparent increase were analyzed to assess the BMD associated with their respective BMR. The percentages of neutrophils were increased by BioPM from chicken and pig farms exposure in saline-challenged mice, with BMD of 4.8 and 6.2 μg, respectively (**S Fig 4 H, I**). No other BMD could be derived.

This dose-response analysis using the BMD suggests that the percentage neutrophils is a very sensitive parameter that is influenced by exposure of livestock farm BioPM.

S Tab 1. Cytokine/chemokine analysis in BALF
Cytokines and chemokines production in BALF of saline-challenged mice (**A**) and OVA-challenged mice (**B**).

A Sal-challenged

	Th1 or promote Th1			Th2 or promote Th2				Th17			Other
	IL-2	IL-28B	IL-27	IL-25	IL-5	IL-13	IL-31	IL-23	MIP-3a	IL-17A	IL-10
Chicken 1	-	+	-	+	-	+	+	+	↑	-	-
Chicken 2	-	+	-	+	-	+	+	+	+	-	+
Pig 1	-	-	-	+	-	-	+	-	↑	-	-
Pig 2	+	+	+	+	-	-	+	↑	+	-	+

Note: "-" indicates non-detectable; "+" indicates detectable; "↑" indicates enhancement after BioPM exposure

B OVA-challenged

	Th1 or promote Th1			Th2 or promote Th2				Th17			Other
	IL-2	IL-28B	IL-27	IL-25	IL-5	IL-13	IL-31	IL-23	MIP-3a	IL-17A	IL-10
Chicken 1	-	+	-	+	-	+	+	↑	+	-	+
Chicken 2	-	+	-	+	↑	-	-	+	+	-	+
Pig 1	-	-	-	+	-	-		-	+	-	-
Pig 2	+	+	+	+	-	-	↑	↑	+	-	+

Note: "-" indicates non-detectable; "+" indicates detectable; "↑" indicates enhancement after BioPM exposure

S Tab 2. LPS levels

Sites	Fine (μg/ml)	Total EU/ml in 500 μg/ml BioPM
Chicken 1	3288.0	0.1110
Chicken 2	3782.5	0.0965
Pig 1	745.0	2.5503
Pig 2	154.0	2.3377

Note: EU: endotoxin unit

Chapter 5

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.

Key words: BioPM, goat farm, allergic airway disease, Th2, Th17, genomic pathways, murine, ovalbumin

1. 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 [1, 2]. 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 [3].

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 [4-6]. Epidemiological studies demonstrate that asthmatics are more sensitive to airways responses from PM exposure when compared to healthy individuals [7]. The effect of allergens from indoor air are more strongly linked to asthma prevalence and severity than outdoor allergens [8].

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 [9]. The number of dairy goats increased rapidly from 2000 to 2019 in the Netherlands [10]. Animal farming operations produce a complicated mixture of airborne PM, that is able to elicit pro-inflammatory respiratory responses [11]. We define BioPM as a complex biological mixture of 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 [12]. Q fever outbreaks, a zoonotic disease resulting from a respiratory infection with *Coxiella burnetti*, occurred in the Netherlands between 2007 and 2020 [13]. The association between ambient *C. burnetti* DNA and residential areas near goat farms has been found in an epidemiological study [14]. 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 [15]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 [16].

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).

2. Materials and Methods

2.1. 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 09:00-16:00 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 [17]. Detailed description of the sampling dates, procedures during the sampling campaign and BioPM microbial composition are described elsewhere [18]. No relevant contributions from other sources such as combustion (traffic) is anticipated as BioPM is sampled immediately from stables.

2.2. Experimental protocol

Female BALB/c mice (female mice are more susceptible than male mice to develop allergic airway inflammation [19, 20]), 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°C-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.

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.

Table 1 Experimental groups. 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.

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	

2.3. 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 [21]. 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.

2.4. 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)-3, 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 , TNFα 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 3 detectable (>10 pg/ml) individuals out of 6, which is the animal number per group.

2.5. 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 1:16000.

2.6. 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.

2.7. 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 which 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) [22] 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.

2.8. 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/) [23]. 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.

2.9. 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 [24].

3. Results

3.1. Pulmonary histopathology

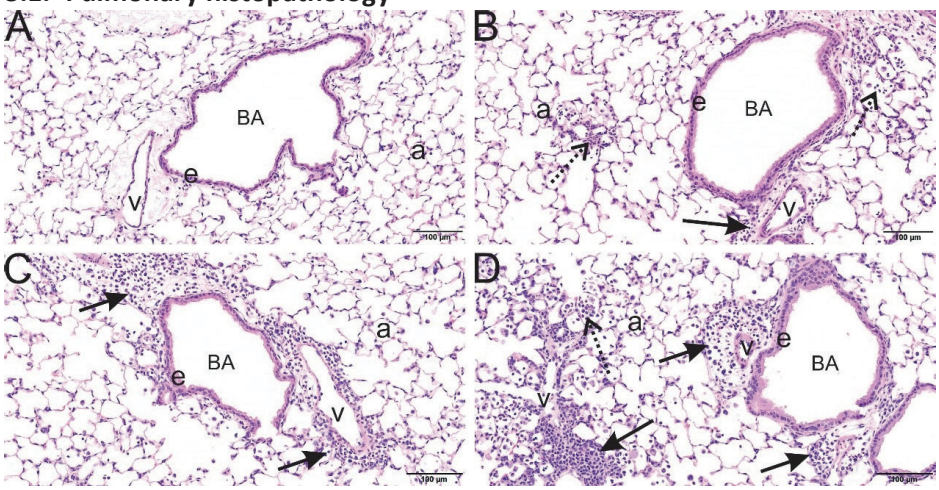

Fig 1. Light photomicrographs of lung tissue sections from mice intranasally challenged with (**A**) saline (vehicle control; 0 µg BioPM), (**B**) saline and 9 µg of goat farm BioPM, (**C**) OVA (0 µg BioPM), or (**D**) OVA and 9 µg of goat farm BioPM.

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.

Control mice that received only intranasal instillations of saline (0 µg BioPM, **Fig 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) (**Fig 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 (**Fig 1 A and 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; **Fig 1 C**) than the minimal type 1 immunity/inflammation in mice that received intranasal BioPM but no OVA challenge (**Fig 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 (**Fig 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 (**Fig 2 A and C**), but no significant changes with subsequent BioPM instillations in OVA-challenged mice (**Fig 2 C and D**).

3.2. Allergen-induced airway epithelial remodeling (mucus cell metaplasia)

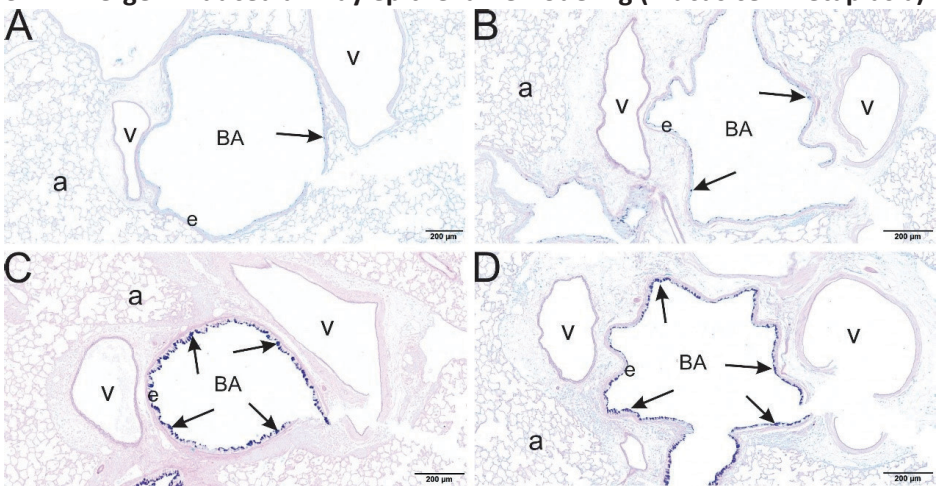

Fig 2. Light photomicrographs of large-diameter bronchiolar airway (BA; proximal axial airway in left lung lobe) from mice intranasally challenged with (**A**) saline (vehicle control; 0 µg BioPM), (**B**) saline and 9 µg of goat farm BioPM, (**C**) OVA (0 µg BioPM), or (**D**) OVA and 9 µg of goat farm 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.

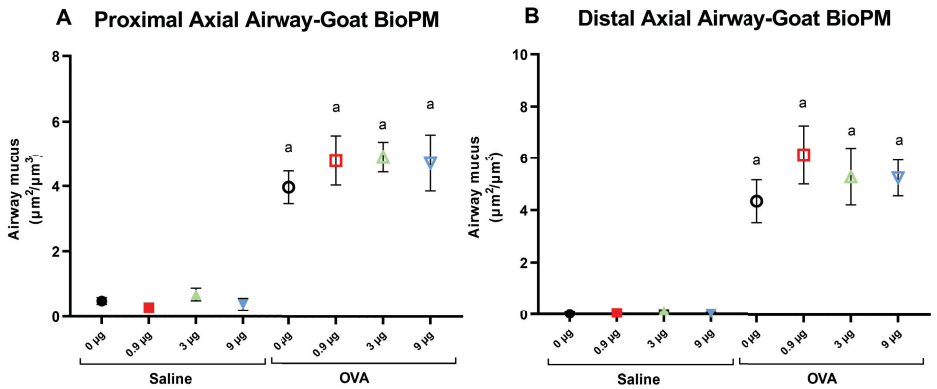

Fig 3. Airway epithelial mucus storage in Proximal (**A**), and in Distal (**B**) axial airway. Increase in AB/PAS stained epithelium to indicate intraepithelial mucosubstances as a feature of allergic airway disease, was analyzed in proximal and distal bronchial airways of mice challenged with Saline or OVA, as indicated. Graph represents mean (SEM)

"a": significantly different from respective saline group ($p < 0.05$).

Table 2 Gene expression analysis of mucus related genes

Gene	OVA/Sal vs OVA/OVA			OVA/Sal 0 vs 3 µg			OVA/OVA 0 vs 3 µg		
	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 non-BioPM exposed mice; FDR, false discovery rate, "-": data is not available

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 (**Fig 3 A and 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* were 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 [25, 26]. 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 (**S Fig 1**).

3.3. Airway inflammatory cell influx in BALF

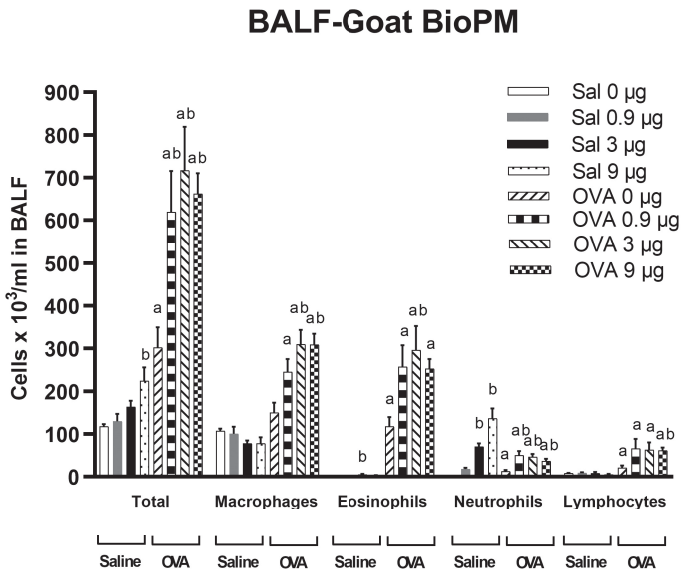

Fig 4. Differential macrophages, eosinophils, neutrophils and lymphocytes cell counts in BALF

"a": significantly different from respective saline group ($p < 0.05$)
"b": significantly different from respective 0 µg group ($p < 0.05$)

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 (**Fig 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 increases were only found with 3 µg BioPM for eosinophils and with 9 µg BioPM for lymphocytes (**Fig 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 (**Fig 4**).

3.4. OVA-specific Ig levels in blood plasma

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 (**Fig 5**).

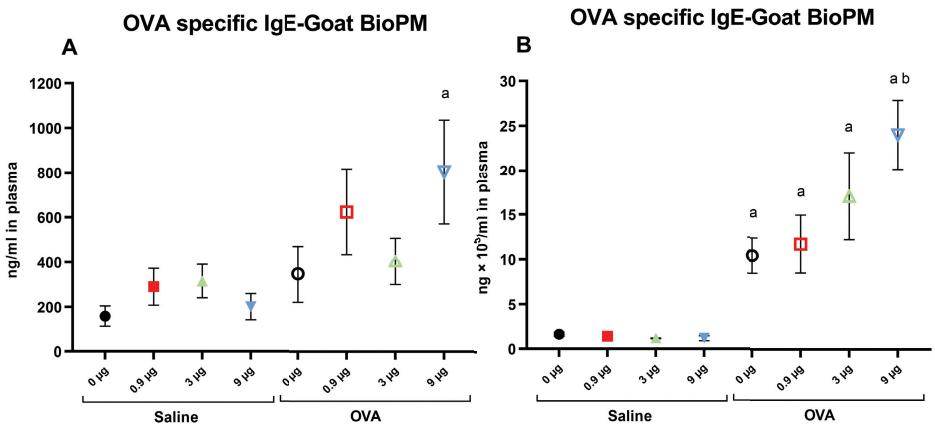

Fig 5. OVA specific IgE (**A**) and IgG1 (**B**) production. The levels were analyzed by an ELISA

"a": significantly different from respective saline group ($p < 0.05$)
"b": significantly different from respective 0 µg group ($p < 0.05$)

3.5. Cytokine and chemokine expression in BALF

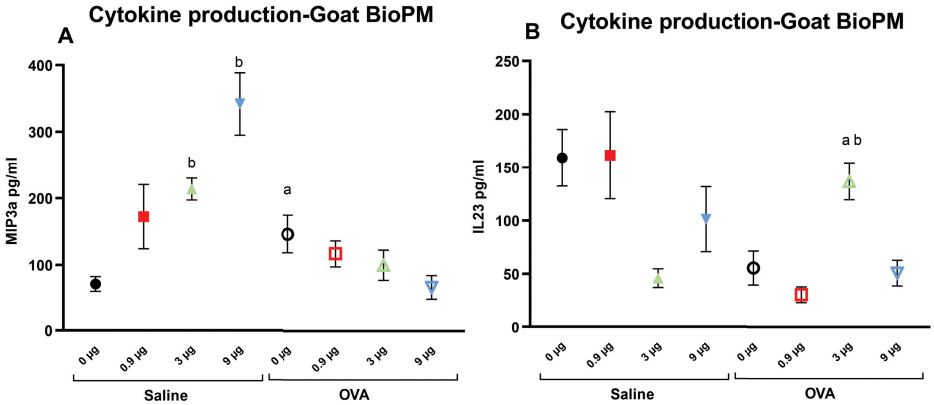

Fig 6. Production of BALF cytokines and chemokines. MIP-3a (**A**) and IL-23 (**B**) were analyzed by a Luminex.

"a": significantly different from respective saline group ($p < 0.05$)
"b": significantly different from respective 0 µg group ($p < 0.05$)

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 (**Fig 6**).

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

Gene/ Protein	Function	Gene expression OVA/Sal vs OVA/OVA			Cytokine Sal/OVA	Gene expression OVA/Sal			Cytokine OVA/Sal	Gene expression OVA/OVA			Cytokine OVA/ OVA
						0 vs 3 µg			0 vs 3 µg	0 vs 3 µg			0 vs 3 µg
		log2 fold change	P value	FDR		log2 fold change	P value	FDR		log2 fold change	P value	FDR	
IL-2	Th1 cytokine	0.14	0.66	-	+	-0.03	0.84	-	+	0.02	0.90	-	+
IFNL3 / IL-28B	Th1 cytokine	0.08	0.48	-	+	-	-	-	+	4.86E-03	0.93	-	+
IL-12A/ IL-12p70	Th1 cytokine	-0.1	0.53	0.69	-	0.19	0.21	0.39	-	-0.05	0.81	0.90	-
IFN-γ	Th1 cytokine	0.89	0	-	-	0.42	0.05	-	-	-0.12	0.59	-	-
IL-27	Th1 cytokine	0.09	0.78	-	+	-0.12	0.54	-	+	-0.21	0.26	-	+
IL-15	Th1 cytokine	-0.2	0.18	0.34	-	0.15	0.30	0.48	-	0.26	0.15	0.32	-
IL-25	Th2 cytokine	-0.08	0.5	-	-	-0.02	0.74	-	-	-	-	-	-
IL-5	Th2 cytokine	1.16	0	-	+	0.59	2.00E-03	-	+	0.15	0.46	-	+
IL-13	Th2 cytokine	2.1	2.05E-10	5.48E-09	-	0.40	8.11 E-03	-	-	0.11	0.61	0.77	-
IL-31	Th2 cytokine	-	-	-	+	-	-	-	+	-	-	-	+
IL-4	Th2 cytokine	1.06	0	-	-	0.41	0.05	-	-	0.61	5.82E-03	-	-
IL-33	Upstream Th2 instruction	0.71	5.50E-07	7.67E-06	-	0.52*	3.31E-08	5.03E-06	-	0.25	0.10	0.26	-
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	-	+	-0.11	0.60	-	+
IL-17F	Th17 cytokine	0.75	6.00E-03	-	-	0.16	0.07	-	-	0.08	0.61	-	-
IL-23a/ IL-23	Th17 cytokine	0	0.99	-	+	0.13	0.37	-	+	0.13	0.35	-	+
IL-21	Th17 cytokine	2.18	1.41E-11	-	-	8.39E-03	0.94	-	-	-0.05	0.80	-	-
IL-22	Th17 cytokine	-	-	-	-	0.02	0.62	-	-	-	-	-	-
IL-6	Acute phase response cytokine	0.74	0.02	-	-	0.67	1.41 E-06	-	-	0.05	0.83	-	-
IL-1β	Acute phase response cytokine	0.4	0.05	0.14	-	0.63*	2.76E-05	9.60 E-04	-	0.43*	0.02	0.09	-
Tnfrsf1a /TNFα	Acute phase response cytokine	-0.31	0.98	0.99	-	-0.31*	5.24 E-04	7.13 E-03	-	-0.33*	7.73E-07	1.58 E-04	-
Lta/ TNF-β	Acute phase response cytokine	1.6	8.02E-11	2.30E-09	-	0.30	0.14	0.30	-	0.09	0.65	0.80	-
CD40LG/ CD40L	Acute phase response cytokine	0.5	0.03	0.09	-	0.28	0.14	0.30	-	-0.07	0.74	-	-
IL10	T-reg cytokine	2.76	2.51E-19	2.83E-17	-	0.20	0.18	-	-	0.04	0.86	-	-
Csf2/ GM-CSF	Growth factor	0.46	0.02	0.05	-	0.70*	1.58E-05	6.54 E-04	-	0.17	0.37	0.57	-

"*": significantly changed ($p < 0.05$) compared non-BioPM exposed mice; FDR. false discovery rate; "-": data is not available/ not detectable; "+": detectable; "↑": enhancement after BioPM exposure

3.6. 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 **S 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 (**S 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 (**S Table 2**). The top 20 annotation clusters and their associated genes are listed in **S 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 **Table 5** and **Table 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 (**S 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 4 Pathway enrichment analysis for all DEGs

Pathways (upregulated)	OVA/Sal 0 vs 3 µg	OVA/OVA 0 vs 3 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 PM treatment group

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

Gene	OVA/Sal 0 vs 3 µg			OVA/OVA 0 vs 3 µg		
	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 non-BioPM exposed mice; FDR. false discovery rate

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

Gene	OVA/Sal 0 vs 3 µg			OVA/OVA 0 vs 3 µg		
	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 non-BioPM exposed mice; FDR. false discovery rate

4. 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.

In this study, the enhancement of OVA-specific IgE/IgG1 and eosinophil recruitment were observed in plasma and BALF, respectively, following farm BioPM challenge in allergic (OVA-challenged) animals. The presence of IgE and IgG1 antibodies have been shown to prime the vessel wall for eosinophil extravasation, driven by IL-4 [27]. IL-13 was found to be necessary to initiate mucus thickening [28]. Ambient fine PM has been reported to be associated with an acute increase of airway inflammatory cells, Th2 cytokines (IL-4, IL-13) production and IgE expression in an OVA mouse model [29]. 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 chemokine/cytokines can recruit and prime eosinophils and Th2 allergic response [30]. 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*) [31]. Moreover, the expression of TNFα, a cytokine that contributes to the enhancement of allergic inflammation [32], was lower after OVA + BioPM treatment. Repeated exposure to pig farm dust extract potentiated the production of eotaxin1 (CCL11), MCP-5 (CCL12) and TNFα protein levels in mice when asthmatic symptoms were present, although the contents of this extract dust are not well characterized [33]. 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 [34]. 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 [35]. The role of ILCs in the effects of air pollutants on classic innate immune cells and lung ILCs are recognized [36]. 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 [37], were significantly increased [38]. 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), although non-allergic mice responded with a 2-fold greater BALF neutrophil counts 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 [39]. 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 is in line with 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 [40, 41], 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*) [42], 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 [43] and IL-17 has been proposed to be involved in neutrophilic inflammation induction and. airway remodeling in severe asthma [44]. Asthmatic patients with more severe forms have been reported to have neutrophilic inflammation and a mixed Th1 and Th17 cytokine profiles [45, 46]. Nonetheless, the neutrophilic response and Th17 cells were increased when Th2 cytokines were suppressed [47], 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. For instance, the lung tissues can be stained targeting for the presence of Th17 cells or neutrophils.

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 [48]. 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 [49]. 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 [18]. 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 [50, 51].

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 [52]. 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 pre-treatment 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) measuement to assess the lung function, is a typical test used to help diagnose (the severity of) airway hypersensitivity, a hallmark of asthma [53]. Lung function reduction has been attributed to effects on airway remodeling. Inflammation, increased IgE/IgG1 level, and mucus are strongly associated with the development of airway hypersensitivity [54, 55]. In our study, we did not evaluate the lung function of the animals. However, we did measure markers that are 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 [56].

The effects of BioPM derived from indoor chicken and pig farms modified allergic airway responses in a mouse model of allergic airway disease as shown in our previous publication [57]. Variation in the responses between farms was observed and this may be linked to the different microorganism compositions of the various farms. By contrasting the agricultural sources of BioPM that may affect the airway allergic responses could gain 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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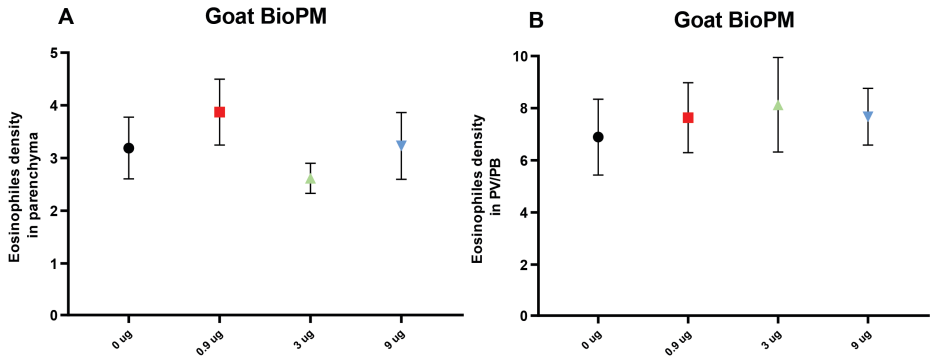
Supplementary

Immunohistochemistry of eosinophils



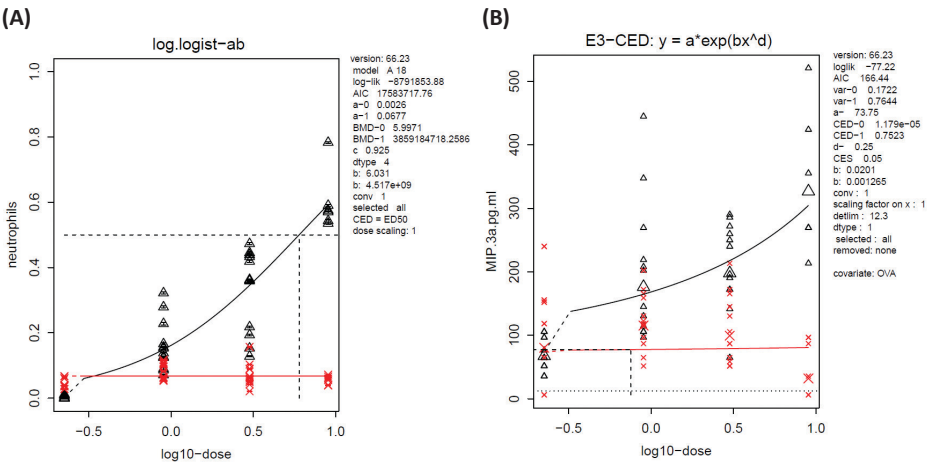
S Fig 1. Immunohistochemistry of airway eosinophils. Photomicrographs of parenchyma (**A**) and peri-bronchiolar/vascular (PB/PV) area (**B**) surrounding the axial airway in OVA-challenged mice.

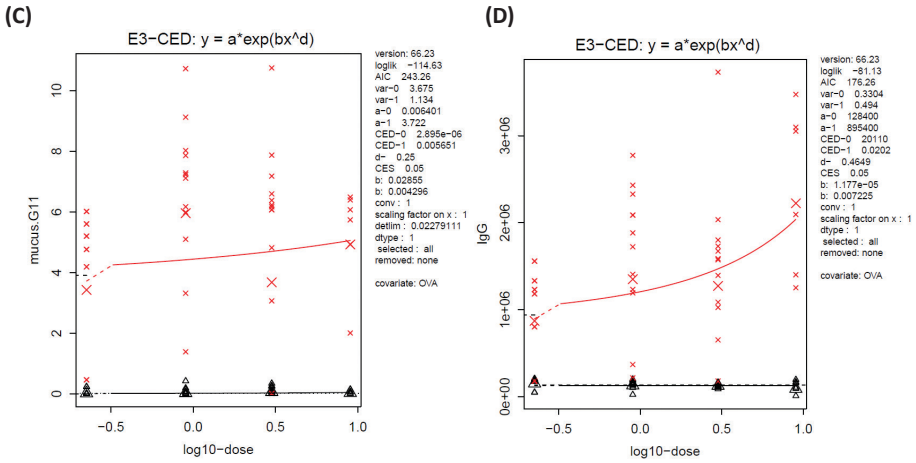
Pathway enrichment analysis

S Table 1 The number of DEGs and clusters analyzed by DAVID

	Upregulated DEGs			Downregulated DEGs		
	Sal 0 vs 3 μg	OVA 0 vs 3 μg	Sal vs OVA	Sal 0 vs 3 μg	OVA 0 vs 3 μg	Sal vs OVA
Genes	1662	1318	2576	2277	1795	2397
Clusters	204	147	344	316	303	276

Dose responses





S Fig 2. Dose-response curves

Dose-response curves for the percentage neutrophils (**A**). MIP-3a (**B**). mucus (**C**) and OVA-specific IgG1 (**D**) induced by goat farms PM in the saline group (black triangles) and OVA group (red crosses). Data from two goat farms were pooled to increase sample size.

Chapter 6

Summary
General Discussion
Future Perspectives

1. Summary

Exposure to livestock farm BioPM has been shown to have a range of effects to promote or inhibit different facets of allergic respiratory diseases. The main aim of this thesis was to investigate whether microorganism or their components present in indoor ambient air of livestock farms contribute to the initiation and/or worsening of respiratory symptoms in asthmatic patients.

In **Chapter 2**, we characterized the microbial profiles of livestock farms BioPM and their effect on innate receptors and on the cytokine production by monocytic cells. The bacterial and fungal communities from livestock airborne BioPM were found to be grouped according to animal farm. BioPM derived from all farms contained mainly ligands for TLR2 and TLR4, only the pig farm BioPM activated TLR5. BioPM from all farms induced a concentration-dependent increase in IL-6 production by monocytes, which was mainly dependent on TLR4 activation.

Chapter 3 describes BioPM-induced oxidative stress (OS) and inflammation when using PBMCs from healthy individuals and asthmatic patients before and during loss of disease control. BioPM from all farms induced the enhancement of an inflammatory response (IL-1β, IL-10, TNFα and IFNγ), which were abrogated by pre-treatment with the antioxidant NAC, indicative of a mechanism (partly) related to oxidative stress. However, we found no marked differences in OS and inflammatory response to BioPM between PBMCs from healthy controls and asthmatic patients.

Chapter 4 shows how BioPM from chicken and pig farms could modify airway allergic responses by using an OVA-induced experimental mouse model. The differences between farms were also described. All farm BioPM elicited a neutrophil influx in BALF of non-allergic mice. BioPM derived from pig farm 2 induced the highest inflammatory cellularity in BALF in allergic mice than BioPM from other farms. BioPM from all farms elicited Th17 cytokine (IL-23) production except BioPM from chicken farm 2, which induced an increase of Th2 cytokine (IL-5). These results indicate that inhalation exposure of BioPM from chicken and pig farms may result in enhancement of airway allergic response in mice following exposure to OVA that involves Th17 and Th2 responses. The different effects among the farms may be associated with the differences in airborne microbiomes composition reported in **Chapter 2**.

We studied further the effect of goat farms BioPM on airway allergic response using the airway allergic murine model and described the possible underlying mechanisms in

Chapter 5. Neutrophilic and eosinophilic responses were enhanced in non-allergic and allergic mice after exposure to goat farm BioPM, indicating enhanced airway allergic inflammation in both saline and OVA-challenged mice. RNAseq analysis of lungs from mice exposed to BioPM indicate that neutrophil chemotaxis and oxidation-reduction processes were the most apparent genomic pathways in non-allergic and allergic mice, respectively.

2. General Discussion

The general discussion in this chapter focuses on the following perspectives:

1. Is BioPM exposure a cause of respiratory diseases? In other words, whether microbial-rich indoor BioPM originating from livestock farms trigger and/or worsen airway inflammation and the potential mechanisms that explain the effect.
2. BioPM-related acute and chronic inflammatory response.
3. The relation between BioPM exposure and lung microbiomes in host, in order to predict the impact of microbial community on respiratory diseases.
4. Take home message from the three exposure models.

2.1. The effects of exposure to livestock BioPM

Farming, a major ambient pollution source in many countries worldwide [1], is often related to respiratory diseases [2, 3]. Epidemiological studies reported the association between ambient *Coxiella burnetti* and one of the largest Q fever outbreaks in residential areas near goat farms in the Netherlands [4-6]. Exposure of BioPM from livestock farms could be a potential risk to occupational farmers and residents living nearby. Inhalation of microorganism and their components may provoke respiratory inflammation, including a wide range of immune response [7, 8]. Moreover, BioPM farming exposure could worsen the respiratory symptoms in individuals with pre-existing respiratory diseases [9]. Our work addressed a neglected field, the effect of BioPM from livestock farms on healthy and enhanced inflammatory responses during asthma *in vitro* and *in vivo*.

Our data shows that BioPM from chicken, pig and goat farms induced increasing IL-6 production by monocytes which was mainly dependent on TLR4 activation (**Chapter 2**). Furthermore, the exposure of BioPM caused the enhancement of neutrophilic response as Th17 disorder and eosinophilic response as Th2 disorder in both control and allergic mice, indicating the severity of allergic responses (**Chapter 4&5**). The involvement of TLRs activation, Th2 and Th17 responses are hypothesised to be the major mechanism underlining inflammatory responses to exposure of livestock BioPM. It has been reported that TLR2 and TLR4 may contribute to polarize Th1/Th2 imbalance following treatment of ambient PM2.5, leading to acute inflammatory response in healthy mice [10]. Residual oil fly ash induced a greater degree of lung injury and the activation of downstream signal molecules in wild type mice than TLR4 knock-out mice [11]. In addition to chemical agents, microbial components in BioPM might

play a contributory role in allergic and inflammatory response [12, 13]. As discussed in **Chapter 2**, BioPM is a complex mixture, containing microbial-rich components which differ between farms. TLR2 and TLR4 were activated by BioPM collected from all farms, indicating the presence of ligands (lipoprotein or peptidoglycan, LPS) for these TLRs. Importantly, organic dust extract from swine facility could significantly affect the cell-surface marker expression and cytokine production when monocytes are differentiated into macrophages [14]. There is even a potential synergist effect among these components (LPS and peptidoglycan) compared to each ligand alone [14]. *In vivo*, neutrophil recruitment and cytokine expression were decreased in TLR4-deficient mice compared to wild type mice following exposure to swine barn air, although AHR was not differed between intact and mutant mice [15]. Besides TLRs, the innate immune system includes other PRRs, such as NOD-like receptors (NLRs). Two NLRs (NOD1 and NOD2) are intracellular molecules that can also sense bacterial components and drive the activation of mitogen-activated protein kinase (MAPK) and NF-κB [16, 17]. Although either NOD1 or NOD2 were activated by BioPM as described in **Chapter 2**, it has been shown that NOD2 expression was upregulated by organic dust extract, induced NF-κB signaling in monocytes/macrophages, and the inflammatory events were enhanced in NOD2 knockout mice compared to control mice [18]. Together, these observations imply that various innate immune receptors are involved in the BioPM-induced inflammatory responses.

Results in **Chapter 3** describes that the effect of BioPM may involve a mechanism (partly) related to OS when using PBMCs from both healthy and asthmatics following exposure of livestock farms BioPM. Ambient PM has been shown to contribute to skew Th2 cytokines production in Nrf2-deficient mice compared to wild-type mice, suggesting that the OS generation of PM may be a factor to amplify the allergic effect [19]. When comparing to the healthy group, no significant differences in inflammatory response and oxidative stress were observed when using PBMCs from asthmatics exposed to farm BioPM in *ex vivo* studies (**Chapter 3**). Whereas in **Chapter 5**, in the presence of BioPM, the oxidation-reduction related pathways were recognized in allergic mice, but not in non-allergic mice. Exposure to ambient PM resulted in an increased tendency of ROS in blood neutrophils from asthmatics compared to controls, leading to exceeding OS and hence tissue injury [20, 21]. Pfeffer *et al.* [22] found that ambient PM-induced inflammation were inhibited by antioxidant supplementary (vitamin D) in human bronchial epithelial cells from healthy and asthmatic donors, although antioxidant was more profound in IL-6 gene expression in healthy donors than asthmatics.

2.2. Acute and chronic inflammatory response

Acute inflammatory responses related to exposure to BioPM from swine confinement facilities- have been recognized, which is in agreement with our findings in **Chapter 4**. Single exposure to pig farm dust extract potentiated the recruitment of neutrophils and increased the production of TNFα, IL-6, keratinocyte chemoattractant and MIP-2 in BALF from non-allergic mice [23]. It is evident that there are comparable findings from mice and human studies concerning the responses to BioPM from swine confinement facilities. For instance, a significant increase by neutrophil recruitment and TNFα, IL-8 in both systemic and airway inflammation appeared in healthy subjects following single exposure to swine barns [24, 25].

The association between repeated exposure to farming dust from swine confinement facilities and inflammatory responses in livestock workers has been investigated *in vivo* using animal models and *in vitro* with human derived immune cells [23, 26, 27]. The inflammatory response from exposure to farm BioPM may attenuate over time. It has been shown that there is a direct link between lung disease and respiratory diseases in farmers exposed to livestock-derived microorganisms, including Gram-negative and positive bacteria as well as fungi or their fragments [14, 28], as discussed in **Chapter 4**. Nonetheless, the mechanisms to explain this chronic inflammatory responses are not well understood. The prevalence of asthma was low in Amish children compared to Hutterites, despite their genetic similarity. This differences could be explained by the microbial variety that are present in different farms and farming practices [29]. The protective effects of dust extracts from Amish farms was abrogated in MyD88 and Trif knock-out mice, indicating the innate immune pathway is critical. In addition, the adaptive immune system plays an important role in mediating a chronic inflammatory response. Th17 inflammatory responses were induced in mice and agricultural workers following repeated exposure to mixed dust extracts from cattle and pig farms as well as bulb and onion industries in the Netherlands [30]. Interestingly, there was a higher prevalence of reporting rhinitis, hay fever and asthma in New Zealand children compared to reference group [31]. Overall, the differences between exposure timing/duration/dosage, farm type, region and individual susceptibility are likely responsible for determining the promotion or protective effect of microorganisms against allergic responses. Understanding how immune responses influences the respiratory disease process and whether certain microbial agents are potentially affecting both the development and modulation of the immune responses needs to be addressed in future studies.

2.3. Lung microbiota

In recent years, studies on the interactions between ambient PM and lung microbiota as well as the underlying mechanisms leading to respiratory diseases did receive increasingly attention [32]. It is not clear whether there is a stable microbial community in lung or if it changes in response to environmental exposures. The exposure of biomass fuel and motor vehicle exhaust PM could modify the lung microbial composition in rats [33]. Moreover, the composition of airway bacterial microbiota differed between patients with asthma and control subjects [34, 35]. We have shown that different livestock farms have specific airborne microbiome communities in **Chapter 2**. Profiling the composition of the microbiome in non-allergic and allergic mice and how microbial communities shift with BioPM exposure may establish the impact of BioPM on the lung microbiome. It may explain the roles of microbiome components in responding to inhaled BioPM in people with asthma or occupational farmers and residents living near livestock farms.

2.4. Take home message from the three exposure models

Findings from our *in vitro* studies indicate that BioPM from chicken farms elicited higher IL-6 production than other farms BioPM, whereas BioPM from pig and goat farms enhanced greater inflammatory and allergic responses than chicken farms BioPM in *ex vivo* and *in vivo* studies. Monocytic cells (MM6) that we used in *in vitro* study are known to express TLR2, 4 and 5 [36]. However, BioPM-induced responses are complex and cannot be easily be elucidated when using only a cell line. TLR2 and TLR4 are involved in mediating inflammatory responses in PBMCs isolated from mice after ambient PM2.5 exposure [37]. In addition, other receptors that are expressed by PBMCs, such as TLR3, TLR7 [38], GCRβ [39] or other unknown receptors, could also interact with BioPM and mediate inflammatory responses. *In vivo* experiments (**Chapter 4 & 5**) are more evident to investigate the effect of BioPM exposure to the lung in whole organism. Studies on determining the effect of BioPM exposure *in vivo* may provide deeper insight to biological processes than *in vitro* studies using only cell lines. Nonetheless, the *in vitro* findings described in **Chapter 2** gave us leads on whether BioPM could induce any responses at all by immune cells and what outcomes to expect in the following *ex vivo* and *in vivo* studies. The combination of all three exposure models gives an overall picture.

3. Future Perspectives

There are several parameters to reflect concerning the severity of air pollutions in air quality guidelines, including PM10 and PM2.5 mass concentrations, nitrogen/sulfur dioxide and ozone [40, 41]. The association between health effects and PM mass concentrations, size, chemical components, oxidative potential or microorganism has been demonstrated in previous studies [42-44]. However, most toxicological studies concentrate on the effect by the PM physical and chemical components [45, 46], whereas the effects of microbial components have been largely ignored. The components of air pollution might not equally contribute to the inflammatory responses and health effects [43]. It is also difficult to distinguish the independent effects of this complex PM mixture. Future studies may consider examining the contrasting and comparable effects that physico-chemical and microbial components may have on health, which probably will provide a better understanding of PM-induced respiratory disease in the future.

As mentioned in **Chapter 3**, no marked differences in inflammatory response to BioPM were observed between asthmatic patients and health donors. However, more variations (not significant) in TNFα production were seen in PBMCs from asthmatics induced by all farm BioPM comparing to healthy people. Future studies may include larger number of donors or more severe asthmatic people. In addition to the classical Th2 immune response, the important role of innate immune system is increasingly recognized in asthma pathology [47]. Macrophages, are part of the first line of defense that interact also with allergens. The present data are largely descriptive and the relevance of using PBMCs as a surrogate for macrophages to investigate the systemic inflammation in individuals responding to BioPM. Macrophages, cataloged as M1 (induced by TNFα or INFγ) and M2 (induced by IL-4 or IL-13) phenotypes, parallel Th1 and Th2 cells with involvement in allergic response, have their specific functions [48, 49]. To get a better understanding of the immunological mechanisms involved, future studies may consider immunostaining alveolar macrophages from asthmatic patients exposed to BioPM to try to identify M1 or M2 based on the distinct expression of interferon regulatory factor 5 or YM1 [50]. It was also demonstrated that macrophages and airway epithelial cells can interact with each other mediating a systemic inflammatory response [51]. Exposing ambient PM to lung epithelial cells could activate high level of intracellular ROS, OS effective genes, and oxidative damage [52, 53]. The co-culture cell model of human bronchial epithelial cells and macrophages should be considered to mimic synergistic effect elicited by livestock farm BioPM.

Both OVA and HDM are widely used allergens to elicit airway inflammation, airway remodeling and airway hyperresponsiveness (AHR). However, both of them have pros and cons. While OVA is not a common aeroallergen in humans, egg allergy is the second-most common food allergy in humans and OVA is one of the major egg white antigens [55]. As such, both mice and humans can become sensitized to OVA, produce IgE, and experience mast cell-dependent pathologies. We are using OVA to study the downstream, common type 2 immune pathways, however they may be initiated, and not to understand any physical interactions between farm BioPM and OVA. Furthermore, there is little to no evidence that HDM produces markedly different type 2 immune pathologies than does OVA in rodents, or that it better replicates human allergy [56, 57]. Also, HDM, contains high level of LPS, which will interfere with the allergic response as discussed in Chapter 4. Removal of LPS seems to result in other artifacts resulting in a loss of allergic potency of HDM [58]. An option is to expose mice to dual or more allergen for mimicking human exposure to multiple allergen as it really occurs. Dual chronic allergen exposure to OVA and HDM resulted in the enhancement of airway inflammatory responses, AHR and airway remodeling when compared with either OVA or HDM in mice [59]. Another possibility is to use pollens to induce allergy symptoms.

We have applied only a single and relatively high dose of BioPM to study the acute response in the present animal experimental studies, whereas the people living nearby farms are probably exposed to lower levels but over a larger period. Therefore, repeated low dose PM exposure may be an option to investigate the chronic respiratory disease. Mobile inhalation exposure in field to determine the relationship between PM exposure and health effects is visible, although the results are not reproducible because the PM concentration and components always vary [60]. In the future *in vivo* studies could be performed in which PM can be administered by inhalation. PM dosimetry (such as deposition fraction, dose) in lung and tracheobronchiolar area of mice can be estimated by using the computational Multiple-Path Particle Dosimetry model (MPPD) [61]. The outcome may provide dose-response data that is useful for risk assessment. Although the outcomes of this thesis have implications for people living near farms, it needs to be stated that asthma is a heterogeneous disease and the cell or murine model may not be entirely representative for allergic airway disease in humans. Clinical studies are needed to verify if it is similar to the present results.

The outcomes of MARS contribute to the understanding of the pathogenesis of asthma, in particular on the impact that air contamination from different sources may have on asthmatic patients and the implications for reducing such exposures. The present studies may provide information to define health related exposure limits as well as to

set health based regulations for farmers and residents. In addition, the information may guide policies to reduce the most harmful airborne pollutants, which will contribute to disease prevention and the reduction of harmful emissions from husbandry. It is necessary to take measures to reduce BioPM exposure. For instance, replacing bedding material more often or adding additional ventilation. Wearing N95 mask when working at the stables can largely reduce the inhalation level of BioPM and dampen the acute adverse respiratory symptoms, however, this is still not routinely used [62].

Asthma can be controlled by medication or by avoiding specific exposures, however, it is difficult to treat because of different factors. People with asthma are at high risk from inhaling BioPM. The therapeutic approaches are very limited for occupational farmers and residents living nearby, especially for susceptible people with pre-existing respiratory diseases. Exploration and identification of complex pathways or mediators in this thesis may provide the potential therapeutic strategy to prevent/treat BioPM-caused respiratory disease. The mechanisms underlying BioPM-induced respiratory diseases are not well understood, but it has been showed that LPS and lipoprotein/peptidoclycan are important causative BioPM components. Therefore, TLR4 [63] and TLR2 [64, 65] pathways could be targeted as options for temporary disease control. In addition, several studies document the beneficial effects of multivitamins intake, such as vitamin C and E, to improve antioxidant capacity and balance the OS in asthmatic children [66-68]. Understanding the critical signaling pathways might provide insights into giving guidance for new medical treatment strategies for individuals exposed to farming environment experiencing adverse respiratory symptoms.

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Appendix

Nederlandse Samenvatting

Blootstelling aan BioPM van veehouderijen liet een aantal effecten zien die verschillende facetten van allergische aandoeningen aan de luchtwegen bevorderen of remmen. Het hoofddoel van dit proefschrift was om te onderzoeken of micro-organismen of hun componenten aanwezig in de binnenlucht van veehouderijen bijdragen aan het initiëren en/of verergeren van ademhalingssymptomen bij astmapatiënten.

In hoofdstuk 2 hebben we de microbiële profielen van BioPM van veehouderijen beschreven en ook hun effect op receptoren van het aangeboren immuunsysteem en op de cytokineproductie door monocyten. De bacterie- en schimmelgemeenschappen van BioPM in de lucht bleken te zijn gegroepeerd naar boerderij. BioPM van alle boerderijen bevatte voornamelijk liganden voor TLR2 en TLR4, alleen BioPM van de varkensboerderijen activeerde TLR5. BioPM van alle boerderijen veroorzaakte een concentratie-afhankelijke toename van IL-6-productie door monocyten, die voornamelijk afhankelijk was van TLR4-activering.

Hoofdstuk 3 beschrijft door BioPM geïnduceerde oxidatieve stress en ontsteking bij het gebruik van PBMC's van gezonde individuen en astmapatiënten, zowel bij stabiele astma als bij exacerbatie van astma. BioPM van alle boerderijen induceerde een verhoging van de ontstekingsreactie (IL-1β, IL-10, TNF-α en IFN-γ), die werd opgeheven door voorbehandeling met de antioxidant NAC, wat wijst op een mechanisme (gedeeltelijk) gerelateerd aan oxidatieve stress. We vonden echter geen duidelijke verschillen in oxidatieve stress en ontstekingsreactie op BioPM tussen PBMC's van gezonde controles en astmapatiënten.

Hoofdstuk 4 laat met behulp van een OVA-geïnduceerd experimenteel muismodel zien hoe BioPM van kippen- en varkenshouderijen allergische reacties van de luchtwegen zouden kunnen veranderen. Ook zijn de verschillen tussen de boerderijen beschreven. BioPM van alle boerderijen veroorzaakte een neutrofiele influx in de BALF van niet-allergische muizen. BioPM afkomstig van de tweede varkenshouderij induceerde een groter aantal ontstekingscellen in de BALF van allergische muizen dan BioPM van de andere boerderijen. BioPM van alle boerderijen wekte de productie op van Th17-cytokine (IL-23), behalve BioPM van de tweede kippenboerderij, die een toename van Th2-cytokine (IL-5) liet zien. Deze resultaten geven aan dat blootstelling aan BioPM van kippen- en varkensboerderijen door inademing kan leiden tot een verhoging van de allergische reactie van de luchtwegen bij muizen na blootstelling aan OVA die een verband heeft met Th17- en Th2-reacties. De verschillende effecten tussen de

boerderijen kunnen in verband worden gebracht met de verschillen in de samenstelling van in de lucht verspreide microbiomen zoals beschreven in hoofdstuk 2.

We hebben het effect van BioPM van geitenboerderijen op de luchtweg allergische reactie verder bestudeerd met behulp van het luchtweg allergische muizenmodel en de mogelijke onderliggende mechanismen beschreven in hoofdstuk 5. Neutrofiele en eosinofiele reacties zijn verhoogd bij niet-allergische en allergische muizen na blootstelling aan BioPM van geitenboerderijen BioPM, wat een aanwijzing is voor een toegenomen luchtweg allergische ontsteking, zowel in muizen gechallenged met fysiologisch zout als in muizen gechallenged met OVA. RNAseq-analyse van longen van muizen blootgesteld aan BioPM geeft aan dat neutrofiele chemotaxis en oxidatie/reductie processen de duidelijkste genomische routes waren bij respectievelijk niet-allergische en allergische muizen.

About the Author

Dingyu Liu was born 8th December 1990 in Ji'an, China. At the age of 16, she moved with her family to Jiaxing, China. In 2008, she studied Environmental Science at Shaoxing University. After graduation in 2012, she started her master program (Heavy metals and cytotoxicity of size-resolved particles emitted by C1 coal of Xuanwei and the deposition character in human body), under supervision of Prof. Senlin Lu, and graduated with a specialization in Environmental Technology of Shanghai University. In 2015, she moved to the Netherlands to start as a PhD candidate on the MARS (Microorganisms and their components present in livestock ambient air in relation to respiratory symptoms) project, a joint project of the Dutch National Institute for Public Health and the Environment (RIVM), Amsterdam Academic Medical Center (AMC), Michigan State University (MSU) and Utrecht University (UU). The major results presented in this thesis.

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List of Publications

This thesis

Liu D., Mariman R., Gerlofs-Nijland M. E., Boere J. F., Folkerts G., Cassee F. R., Pinelli E *. Microbiome composition of airborne particulate matter from livestock farms and their effect on innate immune receptors and cells. Science of the Total Environment, 2019. 688: p. 1298-1307

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