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Integrating farm and air pollution studies in search for immunoregulatory mechanisms operating in protective and high-risk environments

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Abstract

Background: Studies conducted in farm environments suggest that diverse microbial exposure promotes children's lung health. The underlying mechanisms are unclear, and the development of asthma-preventive strategies has been delayed. More comprehensive investigation of the environment-induced immunoregulation is required for better understanding of asthma pathogenesis and prevention. Exposure to air pollution, including particulate matter (PM), is a risk factor for asthma, thus providing an excellent counterpoint for the farm-effect research. Lack of comparable data, however, complicates interpretation of the existing information. We aimed to explore the immunoregulatory effects of cattle farm dust (protective, Finland) and urban air PM (high-risk, China) for the first time using identical research methods.

Methods: We stimulated PBMCs of 4-year-old children (N = 18) with farm dust and size-segregated PM and assessed the expression of immune receptors CD80 and ILT4 on dendritic cells and monocytes as well as cytokine production of PBMCs. Environmental samples were analysed for their composition.

Results: Farm dust increased the percentage of cells expressing CD80 and the cytokine production of children's immune cells, whereas PM inhibited the expression of important receptors and the production of soluble mediators. Although PM samples induced parallel immune reactions, the size-fraction determined the strength of the effects.

Conclusions: Our study demonstrates the significance of using the same research framework when disentangling shared and distinctive immune pathways operating in different environments. Observed stimulatory effects of farm dust and inhibitory effects of PM could shape responses towards respiratory pathogens and allergens, and partly explain differences in asthma prevalence between studied environments.

KEYWORDS
air pollution, asthma, environment, farming, immune cells

1 | INTRODUCTION

The impact of the environment on immune health is being investigated to an increasing extent, since changes in the environmental exposures may have driven the epidemic increase in asthma and allergies in urbanized environments. Interestingly, children who have grown up in farm environment have significantly reduced the risk of developing allergies and asthma compared to general population. Several studies have recognized that early-life exposure to farm dust and to microbial diversity contribute to the healthy immunoregulation.[1-3] Underlying immune mechanisms that could be utilized for preventive interventions remain unidentified. On the other hand, exposure to air pollution is the world's largest single environmental health risk. In 2014, 92% of the world population were living in places where WHO air quality guidelines were not met.[4] Exposure to particulate matter (PM), one important component of air pollution, has been associated with cardiovascular diseases,[5] metabolic disorders[6] and respiratory health outcomes, such as chronic obstructive pulmonary disease and asthma exacerbations[7] but also with the onset of asthma in adults[8] and children.[9] Experimental studies have associated PM exposure with asthma-like airway remodelling and hyperresponsiveness.[7] The exposure to air pollutants might be even more harmful during childhood than in adulthood, since the adverse effects of air pollutants on lung function could be permanent.[10] Studies investigating how PM or its specific constituents may disrupt human immunoregulatory mechanisms and thus predispose exposed individuals to respiratory diseases are, however, rare.

We propose that simultaneous identification of immunoregulatory mechanisms operating in asthma-protective and in asthma-risk environments could accelerate the detection of underlying immune mechanisms and the development of preventive strategies. In this experimental study, we investigated the effects of a proposed protective environment (cattle farm dust) and high-risk environment (urban air PM) on immune responses for the first time by using the same research methods. Peripheral blood mononuclear cells (PBMCs) of 4-year-old children were stimulated with farm dust particles and size-segregated urban air PM samples. To assess the effects of these environmental exposures on the immunoregulatory mechanisms of the innate immunity, we determined the expression of two important immune receptors, cluster of differentiation 80 (CD80) and immunoglobulin-like transcript 4 (ILT4), which are expressed in circulating antigen-presenting cells (myeloid dendritic cells (mDCs), plasmacytoid DCs (pDCs) and monocytes), and represent stimulatory and inhibitory responses. General immune responses were investigated by measuring cytokine secretion of PBMCs. The composition of farm dust and PM samples was analysed in detail.

2 | METHODS

2.1 | Study population

The study population consisted of 4-year-old Finnish children (N = 18, boys N = 11). The study population is a subpopulation from Finnish LUKAS2 cohort study, which is an extended cohort of PASTURE/EFRAIM birth cohort study. LUKAS2 cohort consists of a general population sample of children living in rural and suburban areas in Northern Savonia region, excluding children living in apartment buildings.[11] PBMCs at age 4.5 years were collected from a subsample of LUKAS2 (N = 20) without any preselection. N = 18 had a sufficient number of PBMCs available. Children in this subsample had not been born or lived in farming environment nor had been exposed to significant levels of air pollution. Two out of 18 children (11%) had a doctor-diagnosed asthma at age 4, and 6 of 18 were atopic (specific IgE against any studied allergen >3.5 IU/mL). The study was approved by the Research Ethics Committee, Hospital District of Northern Savo, Kuopio, Finland, and written informed consent was obtained from parents.

2.2 | PBMC isolation

Peripheral blood mononuclear cells were isolated from EDTA blood (Vacutainer, BD) using density gradient centrifugation (Ficoll-Paque, Healthcare Bio-Sciences AB) and cryopreserved in liquid nitrogen as described in Martikainen et al[12]

2.3 | Processing and exposure of blood immune cells

Peripheral blood mononuclear cells were thawed and processed as described earlier.[12] The mean cell viability was 88% (SD ± 3.5). We suspended cells in 10% human AB serum (Innovative Research)

in RPMI 1640, supplemented with 1% glutamine (Invitrogen) and 1% antibiotic/antimycotic (Gibco, Thermo Fisher) to the concentration of 1 x 10^6 cells/mL. One blood sample was taken from each child and then split into five stimulations (control, FD, PM0.2, PM1-0.2 and PM2.5-1). Cells were exposed to farm dust extract (40 μg/mL) or PM samples (75 μg/mL, $PM_{2.5-1}$, $PM_{1-0.2}$ or $PM_{0.2}$) for 18 hours at 37°C in 5% CO_2 on Ultra-Low Attachment surface plates (Corning, Costar). The concentrations were chosen on the basis of dose-response experiments. We also confirmed that the selected doses did not affect cell viability (data not shown). To control the effects of sample collection materials and DMSO on cells, we stimulated PBMCs (N = 6) with a blank filter sample in a similar manner to PM samples. The final concentration of DMSO in cell cultures was 0.15%. To control whether the effect of farm dust was mediated through endotoxin or Gram-negative bacteria, we stimulated PBMCs (N = 2) with farm dust or lipopolysaccharides (LPS) together with polymyxin B (0.01 mg/mL, Sigma-Aldrich).

2.4 | Immunophenotyping of blood immune cells and cytokine measurements

The phenotypes of monocytes and main peripheral blood DC subsets were identified by FACSCantoII cytometer and FACSDiva software v. 8.0.1 (BD Biosciences). Cytokine productions of PBMCs were analysed using Meso Scale Discovery (MSD) Sector Imager™ 2400A with Discovery Workbench® 3.0.18 software. Detailed information of analyses is in Supplementary (Supporting information p. 3).

2.5 | Environmental samples and sample composition

Size-segregated PM samples were collected at Nanjing, China, and airborne farm dust samples were collected from the cattle farm in Northern Savonia, Finland. Detailed information of collection and processing of the samples are in Supplementary (Supporting informationp. 3) and in Jalava et al.[13]

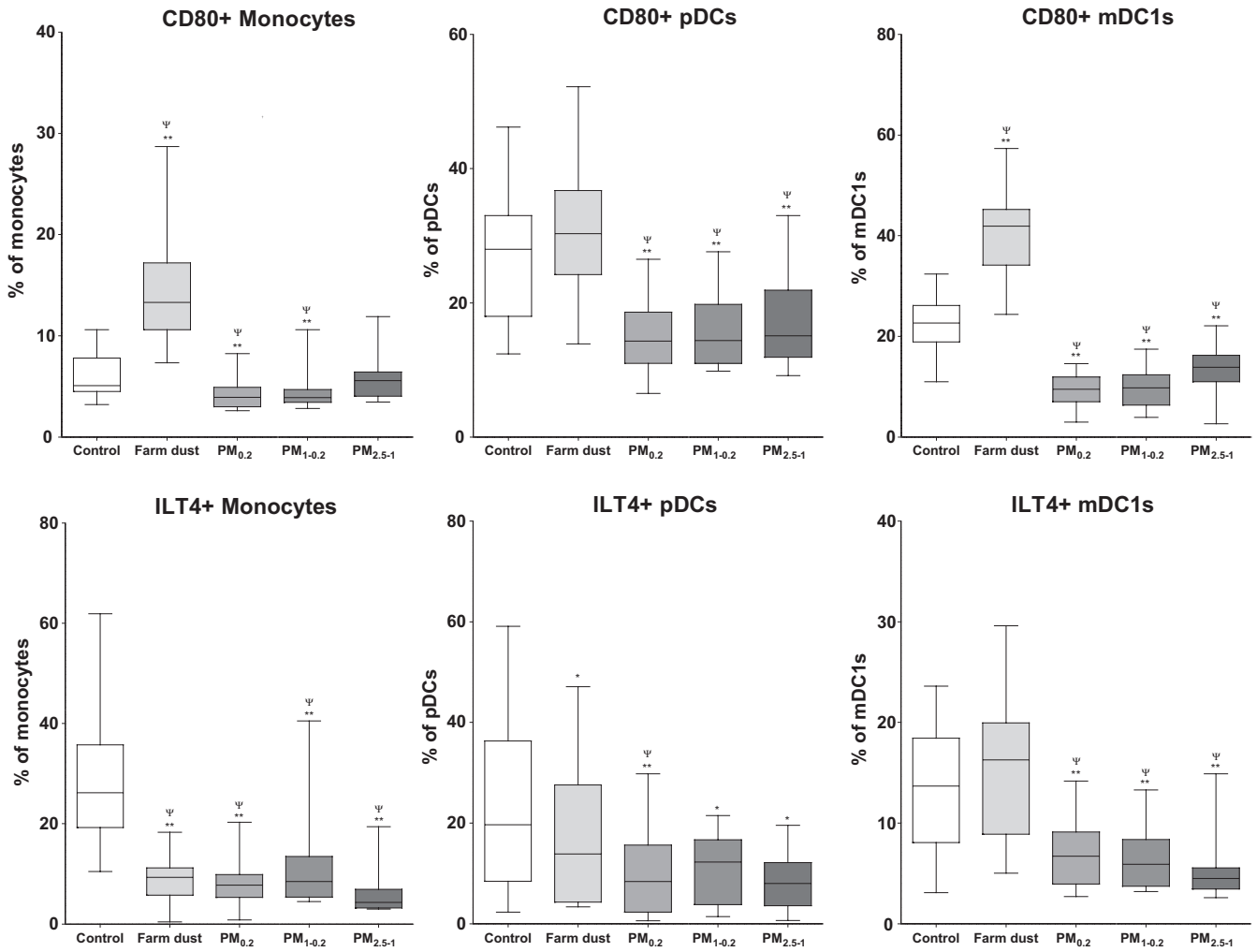

FIGURE 1 The effect of farm dust particles and size-segregated particulate matter (PM) stimulations (18 h) on the percentages of cells positive for CD80 and ILT4 (N = 18). Figures show boxplots with 5%-95% whiskers, and horizontal line indicates the median. Significances were calculated using nonparametric Wilcoxon signed rank test. **P*-value <0.05, compared to control, ***P*-value <0.01, compared to control. $^{\Psi}$*P*-value <0.05 after adjustment for multiple comparisons with Bonferroni correction

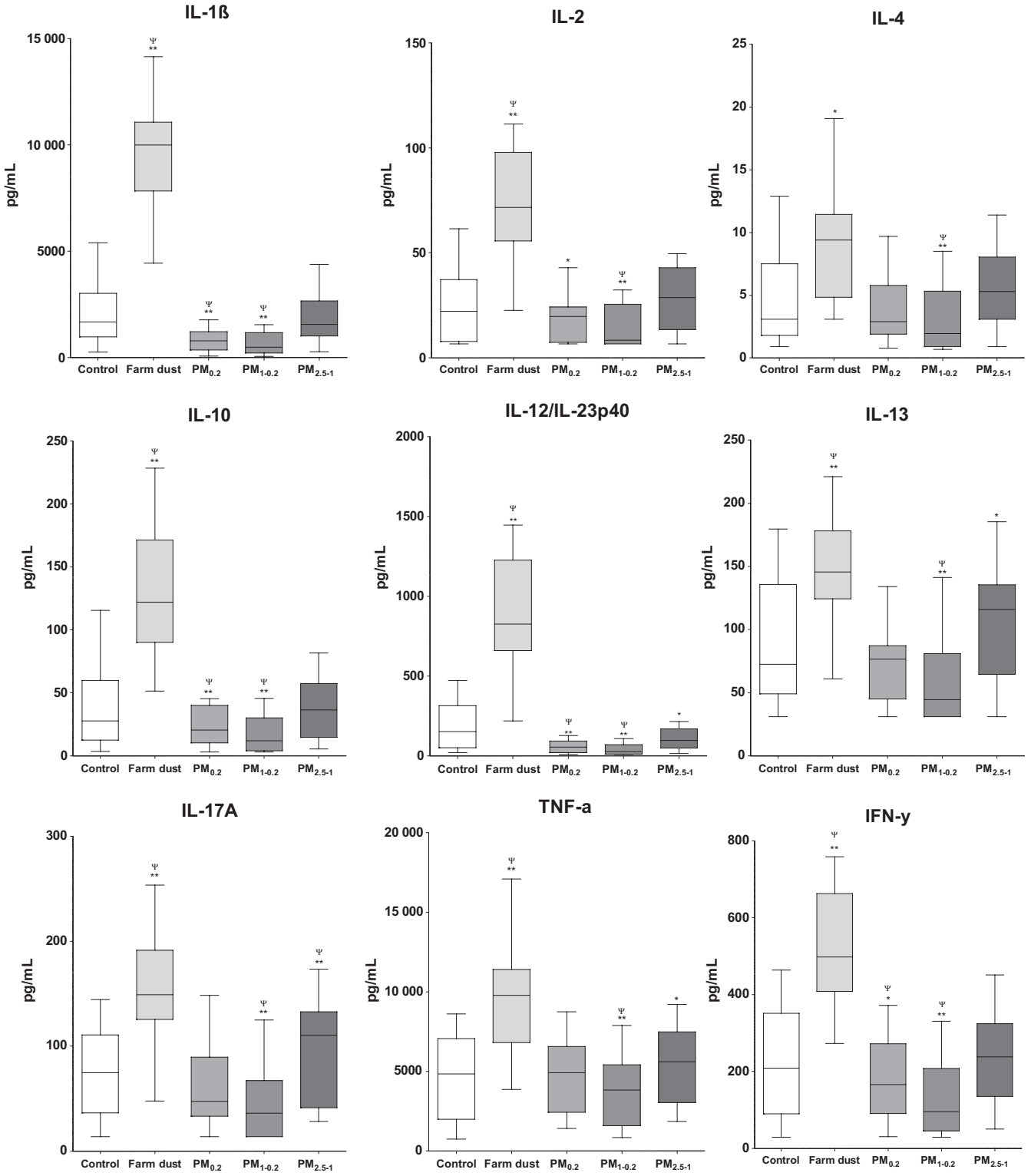

FIGURE 2 The effect of farm dust and size-segregated particulate matter (PM) stimulations (18 h) on the expression of the cytokines. Figures show boxplots with 5%-95% whiskers, and horizontal line indicates the median. Significances were calculated using nonparametric Wilcoxon signed rank test. **P*-value <0.05, compared to control, ***P* value <0.01, compared to control. $^{\Psi}$*P*-value <0.05 after adjustment for multiple comparisons with Bonferroni correction

Particulate matter and farm dust samples were analysed for inorganic ions and elements using inductively coupled plasma mass spectrometry (PM samples) and NexION 350D ICP-MS spectrometer (farm dust). Samples were analysed for polycyclic aromatic hydrocarbon (PAH) compounds using a gas chromatograph mass spectrometer. We also analysed farm dust bacterial microbiota using 16S rRNA gene amplicon sequencing. The detailed information of analyses is in Supplementary (Supporting informationp. 4).

2.6 | Statistical analyses

The data from immunophenotyping (cell variables (DC and monocyte)) were expressed as percentages of cells positive for specific markers and data from cytokine measurements as concentrations of cytokines (pg/mL). Data on PAH contents were expressed as ng/mg of mass and data on water-soluble ionic and elemental compositions as μg/mg of mass. We compared the effects of different stimulations on immune variables using nonparametric Wilcoxon signed rank test and corrected the significances with Bonferroni correction. Correlations between cytokines were calculated using Spearman's two-tailed rank correlation. All statistical analyses were performed using SPSS statistics 21 software (IBM Corporation, USA). Values of $P < 0.05$ were considered statistically significant.

3 | RESULTS

3.1 | Environmental exposures altered the properties of immune cells

Farm dust particles significantly increased the percentages of monocytes and mDC1s expressing immunostimulatory receptor CD80 compared to control (Figure 1). In contrast, urban air PM stimulation induced a statistically significant decrease in the percentage of monocytes, pDCs and mDC1s expressing CD80 (except for $PM_{2.5\text{-}1}$ stimulated monocytes).

All PM size-fractions decreased the percentages of monocytes and mDC1s expressing immune inhibitory receptor ILT4 when compared to control. In pDCs, only decrease induced by $PM_{0.2}$ was statistically significant after adjustment for multiple comparisons. Farm dust particles reduced the proportion of monocytes expressing ILT4, whereas the percentage of DCs expressing ILT4 remained at the control level after farm dust stimulation. Expression of CD80 and ILT4 following exposure to blank filter samples was slightly different when compared to those induced by control samples (Figure S1).

3.2 | Farm dust elevated and PM decreased cytokine production

Farm dust particles induced a statistically significant increase in the production of all studied cytokines, except for IL-4, which did not remain significant after multiple comparisons (Figure 2.). Urban $PM_{1\text{-}0.2}$ had the most pronounced negative effect on the production of all cytokines. $PM_{0.2}$ decreased the levels of IL-1β, IL-10, IL-12 and IFN-γ. $PM_{2.5}$ had opposing effect on the production of IL-13, IL-17 and TNF-α, but only IL-17 remained significant after adjustment for multiple comparisons. Expression of cytokines following exposure to blank filter samples was slightly different when compared to those induced by control samples (Figure S2). In general, correlations between measured cytokines were strong (defined as correlation over $r > 0.9$). When we stratified the results by exposure, the strongest correlations were observed after $PM_{1\text{-}0.2}$ stimulation and weakest after farm dust stimulation (Table S1).

3.3 | Size-fraction determined the strength of the immunological effects

Although PM samples induced parallel immune reactions, the PM size-fraction determined the strength of the effects. Both $PM_{1\text{-}0.2}$ and $PM_{0.2}$ induced significantly different expression of receptors and production of cytokines compared to $PM_{2.5\text{-}1}$, while effects induced by $PM_{0.2}$ did not differ very much from those induced by $PM_{1\text{-}0.2}$ (Table 1).

TABLE 1 Cross-associations between immune responses induced by different particulate matter (PM) size-fractions

Cross-associations	$PM_{0.2}$ vs $PM_{1\text{-}0.2}$ *P*-value	$PM_{0.2}$ vs $PM_{2.5\text{-}1}$ *P*-value	$PM_{1\text{-}0.2}$ vs $PM_{2.5\text{-}1}$ *P*-value
Cell subsets			
CD80+ monocytes (%)	0.756	0.002*	0.001*
CD80+ pDCs (%)	0.346	0.224	0.087
CD80+ mDCs (%)	0.433	0.015	0.009
ILT4+ monocytes (%)	0.005	0.041	0.001*
ILT4+ pDCs (%)	0.53	0.48	0.033
ILT4+ mDCs (%)	0.814	0.433	0.221
Cytokines			
IL-1β	0.069	0.001*	0.000*
IL-2	0.019	0.005	0.001*
IL-4	0.003*	0.003*	0.001*
IL-10	0.004	0.001*	0.000*
IL-12/IL-23p40	0.001*	0.001*	0.000*
IL-13	0.009	0.002*	0.001*
IL-17A	0.022	0.005	0.000*
IFN-γ	0.016	0.008	0.000*
TNF-α	0.001*	0.006	0.000*

Significances were calculated using Wilcoxon signed rank test; PM stimulations were compared to each other.
**P*-value <0.05 after adjustment for multiple comparisons with Bonferroni correction.

3.4 | Composition of the samples

There were some variations in the ionic and elemental compositions of the environmental samples (Table S2). Secondary inorganic ions NO_3^- and SO_4^{2-} dominated the composition of PM samples. Al, Ca, Fe, K and Zn were the most abundant metals in PM samples. The elemental composition of farm dust sample differed from PM samples considerably. PAH contents of PM size-fractions were quite similar, and the highest PAH and genotoxic PAH concentrations were seen in the $PM_{0.2}$ (Table S3). PAH compounds were not detected in farm dust (<0.1 ng/mg). Amplicon sequencing of the bacterial 16S rDNA revealed a dominance of Proteobacteria (83%) and Firmicutes (15%) sequences in the farm dust. (Figure S3).

4 | DISCUSSION

Urbanization, together with industrialization, has led to a lifestyle where the main exposures that an individual encounters have shifted from microbe-rich exposures to exposures with less diverse microbiota and more air pollution. The changes in the environment are paralleled by the increase in the prevalence of allergies and asthma. In this experimental study, we introduced a new research concept for the investigation of the effects of two extremely different environmental exposures on children's immune responses in vitro. Bringing these two areas into one research framework, and by inventing new scenarios and methods, we could gain comparable and valuable data for risk assessment and for the development of preventive strategies.

Cells of the innate immunity, specifically DCs and monocytes, are among the first ones to react to airway exposures. After encountering inhaled antigens, lung DCs, in collaboration with macrophages and epithelial cells, determine whether to direct immune response towards tolerogenic or immunogenic pathways. DCs have a crucial role in determination of subsequent immune responses, as they are responsible for linking innate and adaptive immunity.[14] The importance of DCs has also been recognized in farm exposure studies, as farming has been shown to protect children by modifying the communication between epithelial cells and DCs.[15] In our previous studies, we observed that children living on a farm had less subtype 2 mDCs[12] and that LPS stimulation decreased the percentage of mDC1s in farm children.[16] The ability of immune cells to react to the environment and to different stimuli depends on their functional properties, that is expression of stimulatory and inhibitory receptors and secretion of cytokines.

One of the receptors determining the response of immune cells to foreign antigens is CD80. It is an immune stimulatory receptor triggering the proliferation and activation of effector T cells.[17] In our study, farm dust stimulation increased the percentage of CD80 + cells, associated with the classic activation of immune system. In our earlier study, pDCs positive for CD86, immunostimulatory receptor cooperating with CD80, was associated with lower prevalence of asthma in non-farm-living children.[12] Our results are also in line with the earlier study, in which cowshed dust extract-treated cells exhibited an activated phenotype with high expression of CD86.[18] In contrast, PM stimulation decreased the percentage of cells expressing CD80. This finding is contrary to previous studies suggesting that exposure to PM constituents enhances the expression of CD80/CD86 in mouse bone marrow-derived DCs (BMDCs).[19,20] The use of size-segregated urban air PM and human DCs in this study, together with high spatiotemporal variability of urban PM composition, may explain the differences in the results.

While CD80 receptors act as immune stimulatory receptors, ILT4 receptors have inhibitory effects. ILT4 receptors are involved in immune regulation as they shape T-cell responses towards tolerogenicity, for example by causing CD4+ T helper (Th) cell unresponsiveness.[21] In our study, the proportion of cells expressing ILT4 was decreased after stimulation with PM. This could potentially disrupt immune homoeostasis and partly contribute to the immune-related health outcomes associated with the air pollution exposure. Farm dust could be hypothesized to induce overexpression of ILT4; however, we observed lower expression of this molecule in monocytes, but not in dendritic cells, after farm dust stimulation. In our previous study, ILT4+ mDC1s associated with higher prevalence of asthma in nonfarming children,[12] suggesting that conclusions about cell function cannot be based solely on markers as immune responses, are multilayered.

Farm dust particles increased the cytokine production of PBMCs. In previous studies, PBMCs from farm-living children produced more Th1-associated cytokines and immunoregulatory cytokines compared to nonfarm children.[22] Cowshed dust extract-treated BMDCs cells produced high amounts of cytokines such as IL-10, IL-12p70 and TNF-α.[18] Treatment of murine DCs with grass arabinogalactan resulted in IL-10 production, and interestingly, these DCs were not able to induce an allergic immune response.[23] Urban air PM decreased the cytokine production of PBMCs, whereas previous studies have reported the enhancement of cytokine production.[19,24] Loading of human mDCs with urban air PM has been shown to stimulate memory T cells to secrete cytokines and differentiate into a mixed population of Th cells with high inflammatory potential.[25] Other studies, however, have also reported immunosuppressive effects of PM stimulation. Jalava et al[26] showed that tracers of incomplete biomass and coal combustion, and PAHs in urban air had negative correlations with the inflammatory activity. In mouse studies, biomass combustion samples containing high concentrations of PAHs were linked with overall lower inflammatory responses in mouse lungs.[27] In another study, combustion-derived PM exposure during early life induced an immunosuppressive environment in the mouse lungs, concurrent with increases in tolerogenic DCs and Tregs, resulting in suppression of Th2 responses. However, despite the early-life immunosuppression, adult mice developed severe allergic inflammation when challenged with allergen.[28] These differences in results may be due to different physical and chemical compositions of the PM samples or different capacities of cells to induce immune reactions or the use of reference materials instead of authentic samples.[29-31] We can speculate that our urban air PM samples represent an environment with relatively high concentrations of immunosuppressive agents due to local conditions in Nanjing. Unfortunately, we could

not reliably correlate immunologic parameters with the composition data because of small number of environmental samples.

While PM samples induced parallel immune reactions, the strength of the effects was determined by the PM size-fraction. Smaller size-fractions induced significantly different expression of receptors and production of cytokines compared to $PM_{2.5\text{-}1}$. This is likely due to the differences in chemical composition between the size-fractions, and by the possibly different modes of interactions between cells and particles of different size-fractions. $PM_{0.2}$ consists mainly of primary emission particles, whereas $PM_{1\text{-}0.2}$ contains fresh combustion particles and aged, secondary emission particles, and particles formed via photochemical reactions in the atmosphere. Particles in smallest PM fractions usually share similar chemical properties, whereas larger PM fractions may have different properties due to soil-derived and other mechanically generated dusts. A thorough chemical examination of the urban air PM samples studied here will be reported elsewhere.[32]

Environmental samples were analysed for inorganic ions, elements and PAHs in our study. We also analysed the bacterial microbiota of the farm dust. As expected, compositions of PM and farm dust differed considerably. Although farm dust has been used in studies as an immune stimulatory agent, only few papers have investigated composition of the dust apart from its endotoxin content. Interestingly, farm dust extract was dominated by Gram-negative bacterial DNA, to a large extent being attributable to likely plant-associated taxa, with Gram-negative *Erwinia* and a *Bradyrhizobiaceae* genus comprising together more than 50% of all sequences. Plant-dominated microbiome links with the earlier observations concerning the link between biodiversity and asthma protection.[33] We also analysed whether endotoxin influenced the effects of farm dust by adding LPS-neutralizing polymyxin B to the cell cultures. The immunostimulatory effects of farm dust were only slightly dependent on endotoxin content (data not shown), suggesting that the effect was mediated also by other compounds as also reported by earlier studies.[34,35] Although authentic PM samples may also contain bacteria, fungal spores, pollen and viruses, the role of biological fractions in regard to health effects is still unclear and understudied. Overall, we confirmed that the studied environmental samples represented asthma-protective and asthma-risk environments. We also wanted to highlight the importance of the detailed characterization of environmental samples used in immunological and toxicological studies.

In future studies, the immunoregulatory effects of environmental exposures should be studied using larger study population and broader range of immunological markers. Inclusion of environmental samples collected from various urban and rural locations could support the identification of causative components or their combinations. Our study population was a random sample, which included both healthy and atopic children. A qualitative comparison of immune parameters showed that the direction of responses was similar in both groups (data not shown). Quantitative differences, however, should be studied in a larger number of children. This could uncover shared and distinctive mechanisms operating in healthy children and in those who have already developed allergic conditions, potentially leading to new preventive and perhaps also intervention strategies.

As a conclusion, our study shows the value of investigating different environmental exposures in the same conceptual and methodological framework. Farm dust particles activated children's immune cells, whereas PM seemed to inhibit the expression of important receptors and the production of soluble mediators. This is interesting as the risks of immune diseases are also opposed in these environments. Observed stimulatory effects of farm dust and inhibitory effects of PM could shape responses towards respiratory pathogens and allergens and partly explain differences in asthma prevalence between studied environments. This study offers a new perspective, which could be utilized when studying environment-related immune diseases and their mechanisms. Acquiring comparable data from both environments could lead to the discovery of new immunological pathways and provide new tools for the risk assessment and for the development of preventive strategies.

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CONFLICT OF INTEREST

Authors do not have any actual or potential conflict of interests including any financial, personal or other relationships that could inappropriately influence, or be perceived to influence, their work.

AUTHOR CONTRIBUTIONS

All authors approved the submitted version. Martikainen was responsible for the immunological analysis, statistical analysis and the interpretation of results, completion of the background literature search, drafting and revising the manuscript, and collation of comments from the other authors. Rönkkö and Täubel contributed to data collection, interpretation of the results and to the manuscript. Schaub, Pekkanen, Gu, Wong, Li, Komppula, Hirvonen and Jalava contributed to the data collection and to the manuscript. Roponen obtained funds, designed the study, had responsibility for data collection, interpretation of results and management of the study, and contributed to the manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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